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Enhancing Adoptive Cell Therapy of Cancer through Targeted Delivery of Small-Molecule Immunomodulators to Internalizing or Non-Internalizing Receptors

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Abstract

Adoptive cell therapy (ACT) has achieved striking efficacy in B-cell leukemias, but less success treating other cancers, in part due to the rapid loss of ACT T-cell effector function *in vivo* due to immunosuppression in solid tumors. Transforming growth factor- β (TGF- β) signaling is an important mechanism of immune suppression in the tumor microenvironment, but systemic inhibition of TGF- β is toxic. Here we evaluated the potential of targeting a small molecule inhibitor of TGF- β to ACT T-cells using PEGylated immunoliposomes. Liposomes were prepared that released TGF- β inhibitor over $\sim$ 3 days *in vitro*. We compared the impact of targeting these drug-loaded vesicles to T-cells *via* an internalizing receptor (CD90) or non-internalizing receptor (CD45). When lymphocytes were pre-loaded with immunoliposomes *in vitro* prior to adoptive therapy, vesicles targeted to both CD45 and CD90 promoted enhanced T-cell expression of granzymes relative to free systemic drug administration, but only targeting to CD45 enhanced accumulation of granzyme-expressing T-cells in tumors, which correlated with the greatest enhancement of T-cell anti-tumor activity. By contrast, when administered *i.v.* to target T-cells *in vivo*, only targeting of a CD90 isoform expressed exclusively by the donor T-cells led to greater tumor regression over equivalent doses of free systemic drug. These results suggest that *in vivo*, targeting of receptors uniquely expressed by donor T-cells is of paramount importance for maximal efficacy. This immunoliposome strategy should be broadly applicable to target exogenous or endogenous T-cells and defines parameters to optimize delivery of supporting (or suppressive) drugs to these important immune effectors.

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Supporting information

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In adoptive cell therapy (ACT), tumor-specific lymphocytes are either isolated from patient tumor biopsies or peripheral blood, activated and expanded *ex vivo* then infused back into patients, or alternatively, polyclonal peripheral T-cells are genetically transduced with a tumor-specific receptor to impart tumor specificity prior to infusion.^{1–3} In early clinical trials, ACT has mediated up to 78% sustained complete remissions in chronic lymphoblastic leukemia.^{4, 5} Despite these promising results in early clinical findings, the objective response rates of ACT in solid tumors have been lower, for example 22% durable complete responses in patients with advanced metastatic melanoma.^{6–9} Poor *in vivo* functional persistence has been one of the limiting factors believed to hinder the overall efficacy of adoptive T-cell therapy.¹⁰ Although ACT T-cells often exhibit robust effector function *ex vivo*, they are usually quickly subjected to immunosuppression within the tumor microenvironment and/or lymphoid organs. Most ACT T-cells become non-functional before tumor eradication¹¹ owing to a variety of immunosuppression mechanisms in tumors, including the recruitment of suppressive host immune cells, activation of negative costimulatory signaling pathways, and intratumoral production of immunosuppressive factors.¹²

TGF- β , a pleiotropic cytokine, is a key immunosuppressive signal produced in the tumor microenvironment promoting tumor evasion from the immune response.^{13, 14} TGF- β suppresses both the activation and proliferation of cytotoxic T lymphocytes (CTLs, CD8⁺ killer T-cells) and decreases CTLs' cytotoxicity. TGF- β signaling upregulates the regulatory gene FoxP1 and inhibits production of the key effector molecules interferon- γ (IFN- γ), perforin, Granzyme A and B, and Fas ligand by CTLs.^{15–20} In addition, TGF- β exerts its negative inhibitory effects by attenuating the potency of natural killer (NK) cells and promoting the generation of regulatory T lymphocytes (T_{regs}).^{21, 22} The relevance of TGF- β in immunosuppression is illustrated by transgenic mice expressing a dominant-negative TGF- β type II receptor specifically in CD4⁺ and CD8⁺ T-cells, which spontaneously reject thymoma and aggressive B16F10 melanoma tumors.²³

Based on these findings, therapeutic strategies targeting TGF- β synthesis, interaction of the cytokine with its receptor, or interfering with downstream signal transduction have been evaluated in preclinical studies. For example, systemic administration of a soluble TGF- β receptor, which neutralizes extracellular TGF- β , suppressed pancreatic tumor growth in mice.²⁴ Adoptive transfer of tumor-specific CD8⁺ T-cells bearing a non-functional TGF- β receptor also eradicated prostate cancer in mice.²⁵ Small molecule TGF- β receptor inhibitors have shown efficacy in preclinical models when used alone^{26–28} or in combination with agonistic antibodies, cytokines, or chemotherapy.^{29–32} Motivated by these promising preclinical results, several small molecule inhibitors and neutralizing antibodies targeting TGF- β ligands or receptors have entered clinical trials.³³ However, the only published first-in-human dose study of TGF- β receptor small molecule inhibitor Galunisertib (LY2157299)

used as a monotherapy in glioma patients revealed modest results.³⁴ For an ongoing study in advanced hepatocellular carcinoma patients, Galunisertib had better efficacy in patients with high alpha-fetoprotein levels, but the overall response rate was still low.^{33, 35}

In parallel to the challenge of achieving therapeutic efficacy, modulation of a highly pleiotropic cytokine/receptor system such as TGF- β faces many potential issues of systemic toxicity. As TGF- β is required to maintain homeostasis of immune cells,²¹ systemic blockade of TGF- β signaling pathway may promote autoimmune pathology. This is predicted by studies of TGF- β knockout mice, which develop multifocal inflammation and autoimmune disease.^{36, 37} Humans deficient in TGF- β have increased risk of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.^{38, 39} Small molecule inhibitors of TGF- β have shown significant cardiac toxicities in animal models;^{40, 41} Eli Lilly reported severe preclinical systemic toxicities from a candidate pan-TGF- β ligand inhibitor, which was not further developed.³⁵ Thus, clinical testing of systemic TGF- β inhibitors has been an exercise in balancing efficacy and safety in dosing.

One approach to enhance the efficacy of TGF- β inhibitors is to target these therapeutics to immune effector cells and/or the tumor microenvironment, promoting the full potential of these inhibitors while minimizing systemic side effects. Nanocarriers are often employed to increase delivery of drugs to immune cells in the tumor microenvironment, whereby particle entry occurs *via* the leaky vasculature of tumors. For example, lipid-calcium phosphate nanoparticles carrying siRNA against TGF- β lowered levels of this cytokine in melanoma tumors, promoting the efficacy of a therapeutic cancer vaccine.⁴² Park *et al.* demonstrated that nanoparticles carrying a small molecule TGF- β inhibitor together with interleukin-2 have impressive efficacy in suppressing the growth of solid tumors or lung metastases.³⁰ However, nanoparticle accumulation in tumors following systemic administration remains very inefficient with current particle designs,⁴³ due to slow passage through vascular/ECM barriers in tumors and rapid clearance of particles by the reticuloendothelial system. As an alternative, we have previously explored strategies using T-cells themselves as delivery vehicles of supporting drugs to the tumor microenvironment, by conjugating cytokine- or drug-loaded nanoparticles to the surfaces of T-cells *ex vivo* prior to transfer into tumor-bearing recipients.^{44–46} These studies demonstrated that tumor-specific T cells can efficiently transport nanocarriers into tumors at levels difficult to achieve with free particles, but are constrained by the requirement for *ex vivo* loading of the cells with nanocarriers. To overcome this limitation, we recently explored the potential of targeting anti-tumor lymphocytes directly *in vivo* using liposomes functionalized with cytokines or antibody fragments targeting receptors specifically expressed by ACT T-cells.⁴⁷ An advantage of this strategy is that adoptively transferred T-cells recirculate through the blood, where they can be readily targeted by systemically-administered nanoparticles without the need to overcome the clearance and tissue-entry barriers facing nanoparticles' entry to the tumor microenvironment. Subsequently, these circulating tumor-specific lymphocytes can carry the particle and associated drug into the tumor microenvironment.

Here we report on using this approach for targeted delivery of a potent small molecule inhibitor of TGF- β receptor I (SB525334, hereafter referred to as TGF- β I)⁴⁸ to ACT T-cells. We show that an antibody-targeted PEGylated liposomal form of TGF- β I efficiently

inhibited TGF- β signaling in primary T-cells, maintaining T-cell proliferation and cytotoxicity *in vitro*. Comparing liposomal delivery of TGF- β I targeting an internalizing receptor (CD90, or Thy1) vs. a non-internalizing receptor (CD45), we found that T-cells pre-loaded *ex vivo* with liposomes targeting CD45 infiltrated B16F10 melanoma tumors more efficiently and expressed higher levels of granzyme. However, when we used these same two targeting ligands to direct liposomal TGF- β I to lymphocytes directly *in vivo*, adoptive cell therapy with melanoma-specific CD8⁺ T-cells supported by repeated injections of anti-Thy1-targeted liposomes slowed tumor growth more than anti-CD45-targeted liposomes. These results provide insight into designing and selecting appropriate targeting agents for specific delivery of immunosuppression-reverting drugs to anti-tumor lymphocytes.

RESULTS AND DISCUSSION

Synthesis and characterization of TGF- β inhibitor-loaded liposomes

To encapsulate the hydrophobic TGF- β inhibitor SB525334, which has poor solubility in water but high solubility in ethanol (68 mg/ml), liposomes were formulated *via* ethanol dilution. Lipids and the drug were mixed in ethanol and added to an excess aqueous phase dropwise *via* a syringe pump, followed by removal of ethanol and free drug by centrifugal filtration and washing with buffer. We first evaluated the impact of membrane composition on drug loading in 2.5/30/67.5 mole ratio maleimide-PEG-DPSE/PC/cholesterol liposomes, testing lipids of varying liquid/gel state transition temperatures (T_m s). Liposomes formed using low- T_m DOPC ($T_m = -20$ °C) encapsulated only 8 ± 0.1 μ g drug/mg lipid, while higher T_m DMPC or HSPC lipids with more rigid membranes encapsulated 11 ± 0.1 μ g and 12 ± 0.7 μ g/mg lipid, respectively (Fig. 1A). Based on these results and the expected enhanced stability of high- T_m liposomes *in vivo*, we focused on HSPC-based liposomes for subsequent studies. The molar percentage of HSPC was then varied from 37% to 77% (with PEG-DSPE content fixed at 2.5 mol% and cholesterol amounts adjusted accordingly) to optimize the lipid composition for drug loading. Over this lipid composition range, TGF- β I loading was relatively constant (Fig. 1B). Finally, with lipid composition fixed at 67.5% HSPC, 30% cholesterol and 2.5% maleimide-PEG-DSPE, the concentration of TGF- β I in the initial lipid/drug mixture was also titrated. Drug loading reached a plateau around 12 μ g/mg liposomes as the TGF- β I concentration in the initial solution was raised above 2.4 mg/ml (Fig. 1C). We also used these optimized lipid composition and drug input amounts to form liposomes *via* the lipid film rehydration method (co-drying the drug with the lipids), but this process yielded only 1 ± 0.2 μ g/mg liposomes which was 12-fold less than the ethanol dilution process (Fig. 1D). TGF- β I-loaded liposomes formed under these optimized conditions *via* ethanol dilution had a mean diameter of 83 ± 11 nm (Fig. 1E) and released the drug over ~3 days in the presence of serum *in vitro* (Fig. 1F).

Free and liposomal SB block TGF- β signaling *in vitro*

When activated CD8⁺ T-cells were treated with soluble TGF- β 1, the downstream signaling mediator Smad2²¹ was phosphorylated within an hour; the mean fluorescence intensity (MFI) of phospho-Smad2 (pSmad2) was increased 3-fold in TGF- β -pulsed cells compared to untreated T-cells (Fig 2A, B). However, co-incubation of cells with TGF- β I maintained pSmad2 at basal levels in the presence of TGF- β (Fig. 2A, B). To evaluate the protective

efficacy of TGF- β I at later time points and when it was encapsulated in liposomes, we incubated activated pmel-1 CD8⁺ T-cells (T-cells specific for the melanoma antigen gp100) with liposomal TGF- β I (free liposomes with no targeting ligand) or equivalent doses of free drug in the presence of TGF- β . After 36 hr of incubation with TGF- β , pSmad2 levels remained elevated ~1.5-fold relative to untreated cells (Fig. 2C, D). TGF- β I both in free drug form or encapsulated in liposomes downregulated pSmad2 expression to basal levels, indicating the liposomal formulation did not impair drug function and the amount of drug released from liposomes during the assay time was sufficient to protect T-cells from TGF- β signaling (Fig. 2C, D). Doubling the amount of liposomal TGF- β I (“TGF- β + 2xTGF- β I lipo”) also showed a trend toward further decreases in pSmad2 below the levels in untreated cells, though this did not reach statistical significance (Fig. 2C, D). This would reflect quenching of basal signaling derived from autocrine TGF- β production by activated T-cells.¹⁵

Liposomal TGF- β I maintains cytotoxicity and proliferation of T-cells in the presence of immunosuppression *in vitro*

Next we sought to assess the potency of liposomal TGF- β I in maintaining the cytotoxic potential of TGF- β -treated T-cells, by monitoring expression of the key cytotoxic enzyme granzyme B.⁴⁹ Culture of activated pmel CD8⁺ T-cells with TGF- β suppressed granzyme B expression to levels ~40% of that in untreated cells by 36 hr of culture (Fig. 3A–C). Addition of free inhibitor to TGF- β -treated cells partially restored granzyme B expression to 70% of untreated cells, while liposomal TGF- β I maintained 80% of uninhibited granzyme levels (Fig. 3A–C). Granzyme B maintenance due to liposomal TGF- β I was also dose dependent; doubling the concentration of liposomes further restored over 90% of granzyme B levels (Fig. 3B). Both free and liposomal SB were effective in maintaining granzyme B expression over 5 days in the presence of TGF- β (Fig. 3C).

TGF- β signaling is also known to block the division of T-cells.^{15–17} To track effects on T-cell proliferation, we labeled pmel T-cells with CFSE, a dye which is serially diluted during cell division. We found that TGF- β treatment could modestly limit T-cell expansion even in response to strong stimulation by anti-CD3/anti-CD28 beads, as evidenced by CFSE dilution and total cell counts (Fig. 3D–F). By contrast, T-cells activated in the presence of TGF- β together with liposomal or free TGF- β I were stimulated to divide and expand similarly to untreated cells (Fig. 3D–F). Together, these results demonstrate that TGF- β I can be released from liposomes at concentrations relevant to maintaining T-cell proliferation and expression of key effector genes over several days.

Ex vivo attachment of targeted TGF- β I-loaded liposomes to T-cells prior to adoptive transfer

To deliver liposomal TGF- β I to adoptively-transferred T-cells *in vivo*, we tested the use of antibody-mediated targeting to permit pre-loading of T-cells with liposomes prior to infusion or for direct *in vivo* targeting of transferred T-cells. For this purpose, we reasoned that an important issue is the behavior of the target receptor on liposome binding. Classic studies with antibody-targeted liposomes delivering chemotherapy agents to tumor cells demonstrated that liposomes targeting internalizing receptors are more effective than

liposomes targeting non-internalizing receptors,^{50, 51} suggesting that for such drugs, delivery of liposomes to endosomal compartments enhances drug delivery to the cytosol/nucleus. To support adoptively-transferred T-cells, it was unclear if more sustained TGF- β inhibition would be achieved by liposomes bound to the cell surface that continuously release drug over several days, or alternatively through liposomes that are internalized and degraded in the endolysosomal pathway.

To compare these two possibilities, we selected two candidate receptors for targeting, one that internalizes rapidly, and a second which shows very low/slow internalization (Fig. 4A). We previously demonstrated targeting of liposomes to ACT T-cells using antibodies against CD90 (Thy1.1), a congenic internalizing cell surface receptor uniquely expressed by the transferred T-cells.⁴⁷ For comparison, we tested liposomes targeted through an anti-CD45 antibody, which in preliminary experiments staining with antibody alone we found did not appear to exhibit rapid internalization (data not shown). Liposomes covalently conjugated to anti-Thy1 (anti-Thy1-Lip) or anti-CD45 (anti-CD45-Lip) were synthesized, which were linked with 0.55 ± 0.03 nmol and 0.35 ± 0.04 nmol antibody per mg lipid, respectively. We first prepared empty liposomes conjugated with anti-Thy1 or anti-CD45 and assessed their internalization by pmel-1 T-cells. Higher levels of cell surface CD45 expression led to higher maximal loading of T-cells with anti-CD45-Lip compared to anti-Thy1-Lip at saturating liposome concentrations (Fig. 4B). To equalize the amount of vesicles delivered to T-cells *via* these two different targeting ligands, liposome binding to T-cells as a function of vesicle concentration was measured. By using a binding titration curve (Fig. 4C), we could choose concentrations of anti-CD45-Lip and anti-Thy1-Lip needed to achieve equivalent total liposome binding to T-cells. Unlike anti-Thy1 liposomes, which were 70 % internalized by pmel-1 T-cells within 19 hr, anti-CD45 liposomes remained on the cell surface through this time point (Fig. 4D). Control liposomes lacking the targeting antibodies showed negligible uptake by T-cells (Supplementary Fig. 1).

To compare the efficacy of Thy1 vs. CD45 targeting *in vivo*, we tested the effect of liposome-mediated drug delivery on pmel-1 melanoma-specific CD8⁺ T-cells in mice bearing B16F10 melanomas. To focus on the question of whether binding to an internalizing vs. non-internalizing receptor was more effective for TGF- β I delivery, we first tested an experimental setting where T-cells were conjugated with TGF- β I-loaded liposomes *in vitro* prior to adoptive transfer, so that the liposomes could be compared under conditions where the same dose of liposomes/drug were coupled to the donor cells, and only the ACT cells were modified with liposomes. Activated pmel-1 CD8⁺ T-cells were prepared and either left unmodified or conjugated with anti-Thy1- or anti-CD45-targeted TGF- β I-loaded liposomes *in vitro* prior to adoptive transfer into lymphodepleted B16F10 tumor-bearing mice (Fig. 5A). Control animals received unmodified T-cells followed by an equivalent total dose of systemic TGF- β I. Four days after adoptive transfer, all groups received a second injection with the same combination of T-cells/liposomes/TGF- β I. Animals were sacrificed three days after the second T-cell infusion and tumors, spleen, and blood were processed and analyzed by flow cytometry (Fig. 5A). Examining tumor size, only T-cells “backpacked” with CD45-binding liposomes elicited a statistically significant reduction in tumor area (Fig. 5B). Flow cytometry analysis revealed that pmel-1 T-cells infiltrating tumors upregulated granzyme compared to transferred cells in the spleen, and T-cells conjugated with either Thy1-

targeting or CD45-targeting liposomes exhibited higher granzyme levels than T-cells alone. By contrast, systemic TGF- β I administration did not increase T-cell granzyme expression above the T-cells alone group (Fig. 5C). Increased granzyme expression was observed for liposome-treated T-cells in both the blood and tumors using either targeting ligand (Fig. 5D–E). However, anti-CD45-targeted liposomes led to a greater infiltration of granzyme B⁺ ACT T-cells into tumors compared to the other treatment groups (Fig. 5F). This higher number of granzyme B⁺ ACT T-cells was not due to activation caused by binding to CD45 or Thy1.1 (Supplementary Fig.2). Thus, in the setting of pre-loading T-cells with liposomes *in vitro*, binding to T-cells through the non-internalizing receptor CD45 elicited greater granzyme-expressing donor T-cell infiltration of tumors, which correlated with greater therapeutic efficacy.

Internalizing liposomes encapsulating TGF- β I slow tumor growth and outperform anti-CD45 liposomes for *in vivo* targeting

Although anti-CD45-Lip were more effective than anti-Thy1-Lip for enhancing adoptive cell therapy in the setting of *ex vivo* loading of T-cells with liposomes, we expected *in vivo* targeting of ACT T-cells using anti-CD45 would be complicated by the fact that this receptor is expressed on the surface of all nucleated hematopoietic cells and their precursors.^{52, 53} Anti-Thy1-Liposomes on the other hand target a receptor uniquely expressed by the donor T-cells. To compare the therapeutic efficacy of these liposomes for direct *in vivo* targeting, B16F10 tumor cells were injected subcutaneously into Thy1.2⁺ C57Bl/6 mice on day 0 and allowed to establish tumors for 6 days. Animals were then sublethally lymphodepleted by irradiation on day 5 and received i.v. adoptive transfer of activated pmel-1 Thy1.1⁺ CD8⁺ T-cells pre-conjugated with anti-CD45-Lip or anti-Thy1.1-Lip encapsulating TGF- β I, T-cells alone, or pmel-1 T-cells together with systemic injection of an equivalent dose of free drug. On days 8 and 10, mice were boosted by i.v. injections of either anti-Thy1.1-Lip or anti-CD45-Lip loaded with TGF- β I or an equivalent dose of free drug (Fig. 6A). In addition, a control group of animals received T-cells together with non-targeted liposomes carrying the same dose of TGF- β I. In this setting of repeated *in vivo* targeting, anti-CD45-Liposomes were equivalent to untargeted liposomes, and did not slow tumor growth to a statistically significant degree compared to T-cells combined with free drug. However, anti-Thy1.1-Liposomes slowed tumor growth and significantly increased overall survival compared to all other groups (Fig. 6B, C). We hypothesize the low efficacy of anti-CD45-Liposomes is due to the uptake of these liposomes by endogenous T-cells, peripheral B-cells, dendritic cells and macrophages, which all express CD45.⁵⁴ Anti-CD45-Liposomes bound to only 52% of ACT T-cells in blood and 25% of these cells in lymph nodes; a similar percentage of endogenous T-cells took up these liposomes (Fig. 6D, E). By contrast, we previously showed that anti-Thy1.1-Liposomes specifically target ACT T-cells *in vivo*.⁴⁷ Non-targeting liposomes also did not provide additional therapeutic efficacy compared to free drug, indicating that passive tumor targeting *via* the enhanced permeability and retention (EPR) effect was ineffective in this setting (Fig. 6B, C). Though the enhancement in survival seen in this challenging tumor model was modest, this was achieved with only 2 “booster” injections of TGF- β I, suggesting promise of this approach for allowing repeated dosing to enhance responses to ACT.

Comparing the two strategies tested here—"pre-loading" of drug-loaded liposomes vs. direct injection of liposomes for *in vivo* targeting—the pre-loaded strategy provides transferred T-cells with pseudo-autocrine drug delivery with essentially perfect association of the drug carriers with target cells, maximizing the effective dose of drug delivered to the anti-tumor donor cells and thereby achieving greater initial anti-tumor activity. However, the one-time nature of this intervention means that for repeated drug dosing, repeated adoptive transfers must be performed (as done in the studies summarized in Fig. 5). By contrast, direct i.v. injection of targeted liposomes (e.g. Anti-Thy1.1-Lip) cannot match the efficiency of drug delivery to the donor cells (as some liposomes inevitably are scavenged by macrophages or otherwise cleared prior to reaching donor T-cells). However, targeted liposomes can be repeatedly administered at will for as long as anti-liposome antibody responses do not develop in the recipient, and for this reason, direct targeting may be practically more attractive in the clinic, where repeated T-cell infusions are limited by donor cell availability, cost, and clinical complexity.

CONCLUSIONS

Strategies to boost anti-tumor immunity can rely on boosting endogenous T-cell responses, or employ adoptively transferred T-cells prepared and expanded *ex vivo*. While boosting of the endogenous response through methods like therapeutic vaccination are of great interest for their potential simplicity in clinical implementation, to date no therapeutic vaccines or other immunotherapy interventions have been devised that can expand T-cell populations to the scale reached by adoptive cell therapy. Thus, approaches to augment this approach are of significant translational interest. Here we synthesized and characterized targeting liposomes encapsulating a potent small molecule TGF- β receptor inhibitor, SB525334. To elucidate if more sustained TGF- β inhibition would be achieved by liposomes bound to the ACT T-cells surface that continuously release drug over several days, or alternatively through liposomes that are internalized and degraded in the endolysosomal pathway, we synthesized liposomes targeting to two different receptors to represent the two mechanisms respectively. In the setting of pre-loading T-cells with liposomes *in vitro*, binding to T-cells through the non-internalizing receptor CD45 elicited greater granzyme expression in ACT T-cells systemically and particularly led to greater donor T-cell infiltration of tumors, which correlated with greater therapeutic efficacy. By contrast, when antibody-functionalized liposomes were used to target TGF- β I directly to T-cells *in vivo*, internalizing anti-Thy1.1 liposomes elicited enhanced tumor regression and survival. This result may reflect the superior donor cell specificity of Thy1 vs. CD45, and emphasizes the importance of concentrating the TGF- β I on the effector T-cells as opposed to other immune cell populations. ACT T-cells can be engineered to express unique receptors mimicking the unique Thy1 isoform used in this small animal model, and our results suggest that optimal targeting might be achieved by targeting a receptor unique to the transferred T-cells which is also engineered for low/minimal internalization.

METHODS AND EXPERIMENTAL

Materials

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(poly ethylene glycol)-2000 (maleimide-PEG-DSPE), cholesterol, hydrogenated Soy L- α -phosphatidylcholine (HSPC), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were from Avanti Polar Lipids (Alabaster, AL) and dissolved in ethanol before use. Anti-Thy1.1 (clone 19E12) and Anti-CD45 (clone HB220) were purchased from BioXCell (West Lebanon, NH). Ethanol was from VWR (Radnor, PA). F(Ab')₂ Preparation Kits, BCA Protein Assay Kits, and 7k MWCO Zeba spin desalting columns were from Pierce Thermo Scientific (Rockford, IL). Protein A agarose columns and Amicon Ultra-15 30kDa MWCO Centrifugal Filter Units were from Millipore (Billerica, MA). ACK lysis buffer and 1,1'-Diocetadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD) were obtained from Invitrogen Life Technologies (Grand Island, NY). Dithiothreitol (DTT), Concanavalin A Type VI (ConA), and Triton X-100 were from Sigma-Aldrich (St. Louis, MO) and used as received. Recombinant interleukin-2 (IL-2) and interleukin-7 (IL-7) were obtained from PeproTech (Rocky Hill, NJ). Ficoll-Paque Plus was from GE Health Care (Waukesha, WI). EasySep™ Mouse CD8⁺ T Cell Enrichment Kit was from Stemcell (Vancouver, BC, Canada). SB525334 (TGF- β I) was from Selleckchem (Houston, TX). Recombinant mouse TGF- β 1 was bought from R&D systems (Minneapolis, MN). Hank's Balanced Salt Solution (HBSS) were purchased from (Gibco-Invitrogen, Carlsbad, CA). B16F10 melanoma cells were from American Type Culture Collection (Manassas, VA). Anti-mouse CD16/32 (clone 93), anti-mouse Thy1.1-PE (clone HIS51), anti-mouse CD8a-FITC (clone 53-6.7), anti-mouse CD8a-APC-Cy7 (clone 53-6.7), anti-mouse CD4-FITC (clone GK1.5), anti-mouse CD4-Percp-Cy5.5 (clone GK1.5), anti-mouse NK1.1-FITC (clone PK136), Foxp3-PE-Cy7 (FJK-16s), and anti-perforin-PE (clone dG9) were purchased from eBioscience (San Diego, CA). Anti-pSmad2-APC (clone 072-670), cytoperm/cytofix buffer kit and Perm Buffer III were bought from BD Biosciences (San Jose, CA). Anti-human/mouse Granzyme B-APC (clone GB11) was obtained from BioLegend (San Diego, CA). Live/Dead fixable Aqua dead cell stain kit and mouse anti-CD3/CD28 dynabeads were obtained from Life Technologies (Grand Island, NY). AccuCount rainbow fluorescent count beads (10.1 μ m) was bought from Spherotech (Lake forest, IL).

Preparation of antibody fragments

Monoclonal antibodies (Abs) against Thy1.1 and CD45 were digested with pepsin to generate F(Ab')₂ using a F(Ab')₂ Preparation Kit following the manufacturer's instructions, and characterized by gel electrophoresis. Antibodies or F(Ab')₂ used for liposome coupling were concentrated by using centrifugal filter units (Amicon Ultra-15 30kDa MWCO) and their concentrations were measured by infrared spectroscopy (Direct Detect, Millipore, Billerica, MA).

Liposome synthesis

Liposomes were prepared by ethanol dilution. Briefly, a lipid solution (0.8 mg in 150 μ l ethanol) composed of maleimide-PEG-DSPE/cholesterol/HSPC in a 2.5/30/67.5 molar ratio

was combined with TGF- β I (500 μ g in 100 μ l ethanol). The drug/lipid solution was added dropwise at 200 μ l/min *via* a syringe pump (New Era Pump Systems, NE-1000) to a 5 ml deionized water reservoir in a glass vial with vigorous stirring at 1100 rpm (RT 10 power stir plate, IKA). After lipid/drug addition was complete, the bulk aqueous solution was further stirred at a lower speed for 2 min (200 rpm, power 4 on VWR 371 hot plate/stirrer). Solvent and free drug were removed using centrifugal filter units (Amicon Ultra-15 30kDa MWCO) by concentrating the preparation to ~200 μ L followed by washing 1 $\times$ with 5 ml PBS. For low- T_m and medium- T_m liposomes, equivalent moles of DOPC or DMPC, respectively, were used to replace HSPC. For experiments with fluorescently-labeled liposomes, 0.1 mol % of a fluorescent lipophilic tracer dye DiD was added to the initial lipid mixture.

For the rehydration synthesis method, vacuum-dried lipid films composed of maleimide-PEG-DSPE/cholesterol/HSPC in a molar ratio of 2.5/27.5/69 together with 1% of DiD were rehydrated in 250 μ l of 50 mM HEPES/150 mM NaCl-buffer (pH6.5). Lipids were vortexed every 10 min for 1 hr at 62°C to form vesicles and size extruded through a polycarbonate membrane (0.1 μ m) 21 times. After washing in excess phosphate buffered saline (PBS) pH7.4 and spinning down by ultracentrifugation at 110,000 $\times$ g for 4 hr, liposomes were re-suspended in 100 μ l PBS per 1.4 mg of lipids and stored at 4°C until use.

Coupling of ligands to liposome surface

Antibodies/F(Ab')₂ (2–5 mg/ml) were 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 using Zeba desalting columns before mixing with maleimide-bearing liposomes (1 mg protein/1 mg lipid) in PBS pH 7.4. After incubation for 9 hr at 25°C on a rotator, antibody-conjugated liposomes were washed in excess PBS pH7.4 and pelleted by ultracentrifugation at 110,000 $\times$ g for 2.5 hr. Conjugated vesicles were then re-suspended in 500 μ l PBS per 1.6 mg of lipids before spinning at 800 $\times$ g for 3.5 minutes using a bench top centrifuge (Eppendorf centrifuge 5424) to remove a small fraction of aggregates/drug precipitates that formed during overnight incubation with the antibodies. Liposomes in the supernatants were used for *in vitro* experiments or T-cell conjugation.

To quantify antibody functionalization, anti-Thy1.1-FITC or anti-CD45-FITC were concentrated to 3–5 mg/ml using Ultra-15 Centrifugal Filters before coupling to liposomes as described above. 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 490/520nm 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 or anti-CD45-FITC stock solutions.

Characterization of liposomes and drug loading quantification

Liposome sizes were characterized by dynamic light scattering (90Plus Particle Size Analyzer, Brookhaven, Holtsville, NY). To quantify drug loading, liposomes were dissolved in a lipid-dissolving buffer (LDB, aqueous 38 vol% ethanol containing 1.5 vol% Triton X) followed by measurement of TGF- β I absorbance at 338 nm by spectrometer (Thermo scientific MULTISKAN GO) for drug quantification.

TGF- β I release kinetics

Liposomes (0.8 mg) were resuspended in 1 ml PBS supplemented with 10% fetal bovine serum in Eppendorf tubes at 37°C on a rotator in triplicate. At each time point, 100 μ l of the solution was taken for measuring the total amount of drug while 200 μ l of each tube was centrifuged (Airfuge® Air-Driven, Beckman Coulter) at 160,000 $\times$ g for 45 minutes, and 100 μ l of supernatant was taken for quantification of released drug. Both the initial solution and supernatant samples were added to 400 μ l LDB (total 500 μ l) for absorbance measurements at 338 nm.

Activation of Pmel-1 Thy1.1⁺ CD8⁺ T cells

All animal care and use was performed in the USDA-inspected MIT Animal Facility under federal, state, local and NIH guidelines under an institute-approved animal protocol. Female Thy1.2⁺ C57Bl/6 mice (6–8 weeks of age) and pmel-1 Thy1.1⁺ mice were purchased from Jackson Laboratories. Spleens from pmel-1 Thy1.1⁺ mice were ground through a 70 μ m cell strainer and red blood cells were removed by incubating with ACK lysis buffer (1 ml per spleen) for 4 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 μ g/ml and IL-7 at 1 ng/ml were added to activate and expand pmel-1 T-cells. After two days, dead cells were removed by Ficoll-Paque 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 0.75 $\times$ 10⁶ per ml RPMI containing 10 ng/ml recombinant murine IL-2. After 24 hr, cells were washed 3 times in PBS and re-suspended in 100 $\times$ 10⁶ per ml of complete RPMI for liposome conjugation or adoptive transfer.

In vitro liposome binding to T cells

Anti-Thy1.1 liposomes (1 mg) were incubated with 120 $\times$ 10⁶ activated pmel-1 Thy1.1⁺ T-cells in 10 ml complete RPMI supplemented with 10% FCS for 20 min at 37°C with gentle agitation every 10 min. After incubation, T-cells were spun down and supernatants were collected for conjugation efficiency measurements. After 1 wash in ice cold PBS, T-cells with conjugated liposomes were re-suspended at 100 $\times$ 10⁶ /ml in PBS for adoptive transfer.

Titration of liposome concentration for *in vitro* conjugation

Varying concentrations of DiD-labeled anti-Thy1.1-Lip and anti-CD45-Lip were added to 5 $\times$ 10⁶ activated pmel-1 Thy1.1⁺ T-cells. The total volume for all groups was topped up with RPMI with 10% FCS to 280 μ l and incubated at 37°C for 20 min. After two washes in ice cold PBS to remove unbound liposomes, cells were resuspended in FACS buffer and analyzed by flow cytometry on a BD FACS Canto.

Evaluation of TGF- β signaling and granzyme B expression of T-cells

For short incubations with TGF- β 1 (1 hr), activated Pmel-1 CD8⁺ T-cells (1 $\times$ 10⁶) were treated with serum-free RPMI, TGF- β 1 alone (1.5 nM), TGF- β I (2 μ M) alone or both for 1 hr at 37°C in 220 μ l serum-free RPMI. For extended incubations with TGF- β 1 (12 hr, 36 hr, 60 hr), activated pmel-1 CD8⁺ T-cells were expanded in IL-2 (10 ng/ml) for one day, and

then T-cells (0.5×10^6) were treated with complete RPMI, TGF- β 1 alone (1.5 nM), TGF- β I alone (2 μ M), TGF- β 1 and TGF- β I together, equivalent doses of liposomal TGF- β I, or 2 $\times$ equivalent doses of TGF- β I in liposomes in the presence of TGF- β 1 at 37°C. All T-cells were incubated in complete RPMI supplemented with 5 ng/ml IL-2. After incubation, T-cells were spun down and washed 1 $\times$ with ice cold PBS followed by Aqua live dead staining and intracellular staining for phosphorylated Smad2 and granzyme B. T-cells were analyzed on a FACS Canto flow cytometer after staining.

***In vitro* T-cell proliferation assay**

Naïve CD8⁺ T cells were isolated from Pmel-1 splenocytes *via* magnetic negative selection using an EasySep™ Mouse CD8⁺ T Cell Enrichment Kit, and re-suspended at 10×10^6 /ml in pre-warmed serum-free RPMI. Carboxyfluorescein succinimidyl ester (CFSE) was added to the cells at a final concentration of 2 μ M, which were incubated at 37°C for 15 minutes. Staining was quenched by adding a 1:1 volume ratio of cold RPMI with 10% FBS and cells were spun down followed by two more washes in cold RPMI with FBS. Anti-CD3/CD28 beads were added to CFSE-stained naïve CD8⁺ T-cells (0.15×10^6) at a 1:1 beads:T-cells ratio before treatment with TGF- β 1 alone (1.2 nM), SB alone (1.6 μ M), both drugs, or equivalent doses of liposomal TGF- β I in the presence of TGF- β 1 in 280 μ l complete RPMI media with 10% FBS at 37°C. Two days after activation, T-cells were mixed with counting beads, washed once with FACS buffer before analysis by flow cytometry on a BD FACS Canto.

***In vivo* targeting by anti-CD45-Lip**

Animals were sublethally lymphodepleted by total body irradiation (5 Gy) and received i.v. adoptive transfer of 15×10^6 activated pmel-1 Thy1.1⁺ CD8⁺ T-cells the next day. Mice were either received liposomes without targeting ligands or anti-CD45-Lips (0.5 mg) two days after adoptive transfer. One day after injection of ACT T-cells, mice were sacrificed for flow cytometric analysis.

Tumor therapy using *ex vivo* liposome-loaded T-cells

B16F10 melanoma cells were suspended at 0.5×10^6 cells in 100 μ l HBSS and inoculated subcutaneously (*s.c.*) to induce tumors in C57Bl/6 mice (6–8 weeks old, Jackson Laboratory, Bar Harbor, ME) (day 0). Animals were then sublethally lymphodepleted by total body irradiation (5 Gy) 5 days post tumor inoculation (day 5) and received i.v. adoptive transfer of 8×10^6 activated pmel-1 Thy1.1⁺ CD8⁺ T-cells the next day. For T-pharmacocyte groups, T cells were either conjugated with anti-CD45 liposomes or anti-Thy1.1 liposomes encapsulating TGF- β I before adoptive transfer. Other groups of mice either receive equivalent doses of systemic free TGF- β I (1 μ g) in addition to T-cells or T-cells alone. After four days, mice in respective groups were boosted with 12×10^6 activated T-cells and 1.5 μ g drugs either in liposomes or free form same as the first dose. Mice were sacrificed three days after boost for flow cytometric analysis.

Tumor therapy with *in vivo* targeting of lymphocytes

B16F10 tumor cells (0.5×10^6) were injected subcutaneously into Thy1.2⁺ C57Bl/6 mice on day 0 and allowed to establish tumor for 6 days. Animals were then sublethally lymphodepleted by irradiation (5 Gy) on day 5 and received i.v. adoptive transfer of 12×10^6 activated pmel-1 Thy1.1⁺ CD8⁺ T-cells the next day. For ex vivo-conjugated groups, T cells were either conjugated with anti-CD45-Lip or anti-Thy1.1-Lip encapsulating TGF- β I before adoptive transfer. Other groups of mice either received equivalent doses of systemic free TGF- β I (1.5 μ g) in addition to T-cells or T-cells alone. On day 8 and 10, mice were boosted with either 0.6 mg of anti-Thy1.1-Lip or anti-CD45-Lip loaded with SB or equivalent dose of systemic drug (5 μ g). Tumor size were monitored every two days afterwards. Tumor area was calculated as the product of 2 measured orthogonal diameters ($D_1 \times D_2$). Body mass of treated mice was measured daily as an indicator of overall body condition and systemic toxicity. Mice were euthanized when tumor size reached 150 mm².

Necropsy and sample preparation for flow cytometry analysis

Inguinal lymph nodes and spleens were ground through a 70 μ m cell strainer and washed once with ice cold PBS. Splenocytes were treated with ACK lysis buffer (1 ml per spleen) for 4 min at 25°C to remove red blood cells before washing in ice cold FACS buffer (PBS with 1% BSA). Blood samples were lysed with 2 $\times$ 1ml ACK lysis buffer for 5 min at 25°C and then washed 1 $\times$ with ice cold PBS. Tumors were weighed and ground through a 70 μ m cell strainer and washed once with ice cold FACS buffer. All cells were added with counting beads and washed in ice cold PBS once before Aqua live/dead staining. After Aqua staining, cells were washed 1 $\times$ in FACS buffer followed by surface-staining with Ab. Cells were then surface-stained for Thy1.1, CD8, CD4 and NK 1.1, washed 2 $\times$ in FACS buffer and fixed before splitting into two halves for intracellular staining of p-Smad2 and granzyme B. Samples for p-Smad2 study were permeabilized in BD PERM Buffer III while others were treated with BD Cytofix/cytoperm buffer. After intracellular staining, cells were washed once in FACS buffer and re-suspended in FACS buffer before analyzing on a BD LSR Fortessa flow cytometer. All data was processed using FlowJo software.

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 $\pm$ SEM of sample groups. Graphs show the mean $\pm$ SEM of sample groups. Tumor areas were compared using two-way ANOVA followed by Sidak's multiple comparison test (against T-only group) or two-way ANOVA followed by Tukey's multiple comparison test, and Kaplan-Meier survival curves were compared by log rank test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of TGF- β I-loaded liposomes

(A) Liposomes with a 2.5/30/67.5 mol ratio of maleimide-PEG-DSPE/cholesterol/PC were prepared using DOPC, DMPC, or HSPC lipids and evaluated for encapsulation of SB by UV spectroscopy. *, $p < 0.05$, by one-way ANOVA followed by Tukey's multiple comparison test (B) Liposomes containing a fixed 2.5 mol% maleimide PEG-DSPE were prepared with varying HSPC/cholesterol ratios and TGF- β I encapsulation was measured. (C) Drug loading as a function of TGF- β I concentration in the initial lipid/drug solution. (D) Comparison of lipid rehydration vs. ethanol dilution methods for TGF- β I encapsulation in maleimide-PEG-DSPE/cholesterol/HSPC 2.5/30/67.5 liposomes. (E) Typical mean particle size distributions for liposomes before antibody conjugation determined by dynamic light scattering. (F) Kinetics of TGF- β I release from liposomes at 37 °C in 10% FBS. Data shown are means $\pm$ SEM ($n=3$). ***, $p < 0.001$, by two-tailed unpaired student t-test.

Figure 2. Free and liposomal TGF- β I block TGF- β signaling in activated T-cells

(A–B) Activated pmel-1 CD8⁺ T-cells were treated with PBS, TGF- β alone (1.5 nM), TGF- β I (2 μ M) alone or both drugs for 1 hr at 37°C, and pSmad2 levels were evaluated by intracellular staining and flow cytometry. (A) Overlay of representative histograms of pSmad2 expression. (B) Quantification of mean fluorescence intensity of pSmad2 in T-cells under different treatments ($n = 3$ samples/group). (C–D) Activated pmel-1 CD8⁺ T-cells expanded for 1 day in IL-2 were incubated with TGF- β alone (1.5 nM), TGF- β I (2 μ M) alone, TGF- β with TGF- β I, or TGF- β combined with an equivalent dose of liposomal TGF- β I (or a 2-fold greater dose of TGF- β I) at 37°C for 36 hrs, followed by intracellular staining for pSmad2. Shown are histograms of pSmad2 expression (C) and quantification of mean fluorescence intensity of pSmad2 ($n = 4$ samples/group) (D) *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 3. Liposomal SB maintains granzyme expression in activated T-cell and proliferates T-cells in the presence of immune suppression *in vitro*

(A–C) Activated pmel-1 CD8⁺ T-cells expanded for 1 day in IL-2 were replated in 5 ng/ml IL-2 for 12 to 60 hr with or without TGF- β 1 alone (1.5 nM), TGF- β 1 (2 μ M) alone, TGF- β 1 and free TGF- β 1 together, or TGF- β together with equivalent doses of liposomal TGF- β 1 at 37°C, followed by intracellular staining for granzyme B expression. Shown are histograms of granzyme B expression in T-cells (A) and quantification of MFI ($n = 3$ samples/group) after 36 hr (B), and relative granzyme expression levels over time (C). (D–F) Naïve Pmel-1 CD8⁺ T-cells (0.15×10^6) were labeled with CFSE and activated by culture with anti-CD3/CD28 beads at a 1:1 beads:T-cells ratio. in the presence of TGF- β alone (1.2 nM), TGF- β 1 alone (1.6 μ M), the cytokine and inhibitor together, or TGF- β and equivalent doses of liposomal TGF- β 1 for 48 hr. Shown are histograms of CFSE dilution patterns of T-cells (D), quantification of CFSE MFI ($n = 4$ samples/group) (E), and total cell counts under different treatments after two days (F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 4. Targeted binding of liposomes to T-cells through an internalizing or non-internalizing receptor

(A) Schematic of two different mechanisms of delivery resulting from binding of internalizing- vs. non-internalizing liposomes. (B–C) DiD-labeled anti-Thy1.1-Lip and anti-CD45-Lip were incubated with 5×10^6 activated pmel-1 Thy1.1⁺ T-cells at 37°C for 20 min, washed, and analyzed by flow cytometry. Shown are sample histograms of T-cell liposome fluorescence after incubation with 1 mg/ml anti-CD45-Lip and 0.4 mg/ml anti-Thy1.1-Lip (B) and mean fluorescence of cells after conjugation with different concentrations of anti-CD45-Lip or anti-Thy1.1-Lip (C). (D) Pmel-1 T-cells were incubated with biotinylated anti-Thy1.1-Lip or anti-CD45-Lip for 20 min at 37°C, washed into fresh medium, and then stained with fluorescent streptavidin (SAv) to detect cell surface-accessible particles immediately or after 19 hr in culture before analysis by flow cytometry.

Figure 5. Pre-loading T-cells with SB liposomes targeting non-internalizing CD45 receptor leads to greater tumor infiltration by donor T-cells and enhanced therapeutic efficacy of ACT
Thy1.2⁺ C57Bl/6 mice were injected with B16F10 tumor cells (0.5×10^6) subcutaneously day 0, sublethally lymphodepleted by irradiation on day 5, and received i.v. adoptive transfer of 8×10^6 activated pmel-1 Thy1.1⁺ CD8⁺ T-cells on day 6, when tumors had a mean size of 17 ± 4 mm². T-cells were either conjugated with anti-CD45 liposomes or anti-Thy1.1 liposomes encapsulating TGF- β I before adoptive transfer. Other groups of mice either receive equivalent dose of systemic free TGF- β I (1 μ g) in addition to T-cells or T-cells alone. After four days, animals in respective groups were boosted with 12×10^6 activated T-cells

and 1.5 μg TGF- βI either in liposomes or free form, and sacrificed for analysis by flow cytometry on day 13. **(A)** Experimental timeline. **(B)** Relative average tumor growth normalized to day 6 tumor areas. *, $p<0.05$, by two-way ANOVA followed by Sidak's multiple comparison test (against T-only group) on tumor size on day 13. **(C)** Sample histograms of granzyme B expression of tumor infiltrating adoptively transferred T-cells. **(D, E)** Mean fluorescence intensities of granzyme B expression for ACT T-cells in tumors **(D)** and blood **(E)**. **(F)** Quantification of number of granzyme B⁺ CD8⁺ Thy1.1⁺ T-cells per gram of tumor. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$, by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 6. Anti-Thy1.1 liposomes encapsulating TGF- β inhibitor slow tumor growth and outperform anti-CD45 liposomes for *in vivo* targeting

(A–C) Thy1.2⁺ C57Bl/6 mice were injected with B16F10 tumor cells (0.5×10^6) subcutaneously on day 0, sublethally lymphodepleted by irradiation on day 5, and received i.v. adoptive transfer of 12×10^6 activated pmel-1 Thy1.1⁺ CD8⁺ T-cells on day 6. T-cells were either conjugated with anti-CD45-Lip or anti-Thy1.1-Lip encapsulating TGF- β I before adoptive transfer. Other groups of mice received equivalent doses of systemic free TGF- β I (1.5 μ g) in addition to T-cells or T-cells alone. On days 8 and 10, mice were boosted with either anti-Thy1.1-Lip or anti-CD45-Lip loaded with TGF- β I or equivalent doses of free systemic drug (5 μ g). (A) Timeline of injections. (B) Average tumor area vs. time; shown are means $\pm$ SEM (n=4–6 animals/group). **, p<0.01, ***, p<0.001, ****, p<0.001, by two-way ANOVA followed by Tukey's multiple comparison test for tumor areas on day 21. (C) Kaplan-Meier survival curves. (D–F) Activated pmel-1 Thy1.1⁺ CD8⁺ T-cells (15×10^6) were adoptively transferred 1 day after lymphodepletion. Fluorescently labeled Liposomes without antibody coupling and anti-CD45-Lip (0.5 mg) were injected i.v. two days after adoptive transfer. Mice were sacrificed one day after injection of liposomes, and blood and LNs were analyzed by flow cytometry. Shown are percentage of respective cells types labeled with liposome fluorescence in blood (D), LNs (E) and spleens (F).