Journal for ImmunoTherapy of Cancer

PLS-α-GalCer: a novel targeted glycolipid therapy for solid tumors

Julian Burks ,[1] Shweta Tiwary,[1] David M Stevens,[2] Sarah L Skoczen,[2] Ruvanthi N Kularatne,[2] Stephan T Stern,[2] Jay A Berzofsky [1]

To cite: Burks J, Tiwary S, Stevens DM, *et al*. PLS-α-GalCer: a novel targeted glycolipid therapy for solid tumors. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e009539. doi:10.1136/jitc-2024-009539

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-009539).

Accepted 16 February 2025

[1]Vaccine Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA
[2]Nanotechnology Characterization Laboratory, Cancer Research Technology Program, Leidos Biomedical Research, Inc, Frederick National Laboratory for Cancer Research Sponsored by the National Cancer Institute, Frederick, Maryland, USA

Correspondence to
Dr Julian Burks;
julian.burks@nih.gov;
burksj394@gmail.com

ABSTRACT

Background The prototypical type I natural killer T (NKT) cell agonist, α-galactosylceramide (α-GalCer), has shown only minimal effects against solid tumors in the clinic. The most promising clinical application of α-GalCer currently entails ex vivo priming of patient-derived dendritic cells; however, this technology suffers from cost, logistical concerns, and safety issues. As a parenteral dendritic cell-targeted alternative, we demonstrate that poly(L-lysine succinylated) (PLS)-α-GalCer, a novel scavenger receptor-A1 targeted α-GalCer prodrug has enhanced antitumor activity compared with α-GalCer.

Methods To compare the antitumor activity of PLS-α-GalCer and α-GalCer, we used mouse syngeneic subcutaneous pancreatic and cervical tumor models using Panc02 and TC-1 cells, respectively. Intratumoral immune cell infiltration was evaluated using flow cytometry and immunohistochemistry whole-slide scan analysis. Serum cytokine levels were examined by ELISA and LEGENDplex analysis. Type I NKT cell intracellular interferon-gamma (IFN-γ) levels were determined by flow cytometry. Immunofluorescence was used to test the uptake and processing of PLS-α-GalCer and α-GalCer in antigen-presenting cells (APCs).

Results The scavenger receptor A1 (SR-A1)-mediated targeting of α-GalCer to APCs by PLS-α-GalCer significantly improves the antitumor function against solid tumors compared with α-GalCer. The Panc02 and TC-1 tumor models demonstrated that PLS-α-GalCer increases intratumoral antigen-specific T, NKT and T cells, and increases the M1/M2 macrophage ratio. In the TC-1 tumor model, we demonstrated that PLS-α-GalCer synergizes with an E7 tumor vaccine to significantly suppress tumor growth and increase the survival of mice. Furthermore, the antitumor function of PLS-α-GalCer is dependent on type I NKT cells and requires SR-A1 targeting. In addition, using SR-A1 knockout RAW cells, a murine macrophage cell line, we showed that PLS-α-GalCer uptake and processing in APCs are more efficient compared with α-GalCer. PLS-α-GalCer also induces significantly less serum Th2 and Th17 cytokines while stimulating significantly more IFN-γ for a longer period and increases Th1:Th2 cytokine ratios compared with α-GalCer.

Conclusions PLS-α-GalCer is a promising immunotherapy for the treatment of solid tumors that has superior antitumor activity compared with α-GalCer and could be combined with tumor vaccines and potentially other immunotherapies such as immune checkpoint inhibitors.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ In clinical trials, α-galactosylceramide (α-GalCer)-based therapies have proven to be safe but display inconsistent results regarding efficacy.
⇒ Currently, the most promising approach is a cell-based therapy using α-GalCer-pulsed dendritic cells. This approach is limited because of the cost and requirement for specialized facilities.

WHAT THIS STUDY ADDS

⇒ This study shows that targeting α-GalCer to antigen-presenting cells (APCs) via scavenger receptor A1 using poly(L-lysine succinylated) (PLS)-α-GalCer is more efficacious in suppressing tumor growth compared with α-GalCer.
⇒ PLS-α-GalCer can turn "cold tumors" that do not respond to vaccines into "hot tumors" which demonstrate high response rates.
⇒ PLS-α-GalCer ability to modulate the tumor microenvironment (TME) to promote antitumor responses by increasing infiltrating immune cells with antitumor activities while decreasing immune cells with protumor functions suggests PLS-α-GalCer can be effective in treating several types of tumors.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In totality, this study provides evidence that PLS-α-GalCer has superior antitumor functions compared with α-GalCer. PLS-α-GalCer's enhanced ability to modulate the TME may unleash the full potential of the antitumor activity of type I natural killer T cells and lead to more consistent clinical trial results.
⇒ PLS-α-GalCer offers to turn current cell-based α-GalCer therapy into a cheap parenteral medicine with greater efficacy with the potential to be used as a monotherapy or combined with checkpoint inhibitors or tumor vaccines.

INTRODUCTION

Although the 5-year relative survival rate for all cancers combined has increased considerably since the early 1960s, in the USA, cancer is the leading cause of death in midlife and may soon become the leading cause of death overall as the number of people diagnosed with and dying from cancer continues to increase.[1] Furthermore, due to the growth

and aging of the population it is predicted by the year 2050 that there will be a 50% increase in the total incident cases.[1] As cancer researchers, we need to continue to innovate, building on what we have learned from the past to create novel therapeutics and treatment strategies to combat the predicted rise in patients diagnosed with cancer.

In a CD1d-dependent manner α-galactosylceramide (α-GalCer) potently stimulates a small population of immune cells called type I natural killer T (NKT) cells.[2–4] Type I NKT cells display antitumor functions and play a role in linking the innate and adaptive immune systems.[5–7] The first clinical trials testing the safety of α-GalCer were conducted over 20 years ago and no serious adverse events were reported. The subsequent clinical trials have tested the use of α-GalCer to induce type I NKT cell-mediated antitumor immunity. These trials include the administration of soluble α-GalCer, α-GalCer-pulsed immature dendritic cells, α-GalCer-pulsed mature dendritic cells, α-GalCer-pulsed antigen-presenting cells (APCs), and activated type I NKT cells.[8–13] The α-GalCer trials thus far have included only a few different types of cancers and most of the results have been modest.[8–13] The potential pitfalls of α-GalCer therapy may be inadequate response to the agonist because of NKT cell anergy and anti-α-Gal antibody neutralization of α-linked NKT cell antigens, as well as the variability in frequency of type I NKT cells across the human population.[11 14] Of all the current α-GalCer therapies under clinical investigation, dendritic cell priming ex vivo shows the most promise, but suffers from cost, logistical challenges, and safety issues.[15] Ideally, parenteral targeting of type I NKT cell agonists to dendritic cells could overcome these hurdles and harness the full potential of NKT cell-mediated antitumor immunity.

In the current study, we demonstrate that a novel scavenger receptor-A1 (SR-A1) targeted α-GalCer prodrug called poly(L-lysine succinylated) (PLS)-α-GalCer or PLS-α-GalCer provides superior antitumor activity against solid tumors in murine models compared with soluble α-GalCer. PLS is a macromolecular structure with a high affinity for SR-A1.[16] SR-A1 is a receptor highly expressed on myeloid and endothelial cells.[17 18] The SR-A1-targeted macromolecular platform, PLS, was developed to deliver drugs to lymphatic tissues.[16] A biodistribution study of Cyanine7.5 (Cy7.5) labeled PLS conducted in mice demonstrated similar distribution of the PLS platform for both the intraperitoneal and intravenous routes of administration, with distribution mainly to organs of the lymphatic system, including liver, spleen and lymph node (online supplemental figure 1). By contrast, subcutaneously administered PLS-Cy7.5 primarily stayed at the site of injection, with some accumulation in liver and muscle. This platform was based on succinylated human serum albumin, which was shown to undergo lymphatic distribution via SR-A1-mediated endothelial transcytosis.[19] SR-A1 is also found on myeloid cells within the lymphatic system,[20] allowing for systemic delivery of PLS-α-GalCer to SR-A1-expressing dendritic cells.[21] As a result, the PLS platform can efficiently deliver therapeutic cargo such as small molecule drugs, peptides, and vaccines to tumor-associated macrophages, the lymphatic system, and myeloid/APC subpopulations.[16] Furthermore, the bioactivity of α-GalCer in vivo is highly dependent on SR-A1, since α-GalCer uptake by SR-A1 plays a major role in CD1d loading and presentation by APCs.[22]

PLS-α-GalCer significantly increased intratumoral type I NKT cell, CD4 and CD8 T cell, and M1 macrophage numbers, while decreasing M2 macrophage numbers, and increasing serum cytokine Th1:Th2 ratios compared with α-GalCer. In addition, PLS-α-GalCer can synergize with cancer vaccines to suppress tumor growth and improve the survival rate in mice. Together, these data suggest that PLS-α-GalCer may provide a new immunotherapy strategy to suppress tumor growth and increase the survival of patients with cancer.

MATERIALS AND METHODS

Cell lines

Panc02 murine pancreatic cancer cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C in humidified air with 5% CO_2. The Panc02 were a kind gift from Professor Jill P Smith (Georgetown University, Washington, DC). The TC-1 cell line is derived from murine lung epithelial cells transduced with human papillomavirus (HPV-16) E7 and E6 oncogenes.[23] They were grown in Roswell Park Memorial Institute 1640 supplemented with 10% FBS containing 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 100 µg/mL G418 at 37°C in humidified air with 5% CO_2. The RAW 264.7 (RAW WT) cells were purchased from American Type Culture Collection and cultured using DMEM supplemented with 10% FBS containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C in humidified air with 5% CO_2. SR-A1 knockout RAW 264.7 cells (clone 1/2) (RAW SR-A1 KO) cells were grown in the same condition as the RAW wild-type cells.[24]

Mice

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). C57BL/6 Jα18 knockout mice[25] were bred at the Frederick National Laboratory for Cancer Research. Female mice older than 6 weeks of age were used for all experiments. All mice were housed in a specific pathogen-free facility. All experimental protocols were approved by and performed under the guidelines of the National Cancer Institute's animal care and use committee.

Tumor studies

For the Panc02 studies, 1×10^6 cells were injected subcutaneously into the flanks. Treatment with 429 ng of

Table 1 Immunohistochemistry reagents, dilution, incubation time, antigen retrieval, fixation, chromogen, and control tissue information

Antibody	Dilution & time	Antigen retrieval	Proteolytic digestion	2° AB Kit	Fixative	Chromagen	Control tissue	Manufacturer	Catalog #
CD4	1:250, 60'	HIER EDTA, 10'	Not used	Bond Polymer Refine Kit minus the PostPrimary reagent/ Biot Rabbit a/Rat (w/o serum)	10% NBF	DAB	Spleen	eBioscience	13–9766 (Biot Rat IgG1)
CD8a	1:50, 60'	HIER Citrate, 10'	Not used	Bond Polymer Refine Kit minus the PostPrimary reagent/ Biot Rabbit a/Rat (w/o serum)	10% NBF	DAB	Spleen	eBioscience	14-0195-82 (rat IgG2a)
CD206	1:4000, 60'	HIER Citrate, 10'	Not used	Bond Polymer Refine Kit minus the PostPrimary reagent	10% NBF	DAB	Spleen, ms melanoma	Abcam	ab64693 (rabbit polycl gG)
iNOS	1:100, 60'	HIER Citrate, 10'	Not used	Bond Polymer Refine Kit minus the PostPrimary reagent	10% NBF	DAB	Liver, uterus	Abcam	ab64693 (rabbi polycl IgG)

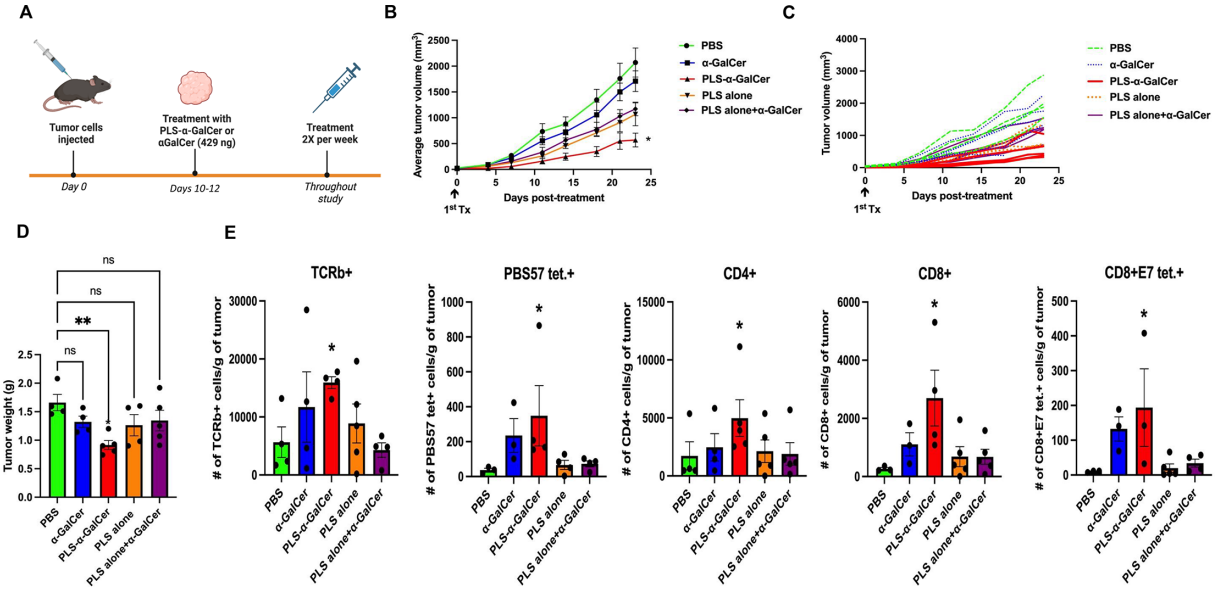

Figure 1 PLS-α-GalCer suppresses TC1 tumor growth and increases tumor-infiltrating antigen-specific CD8 T cells, NKT cells, and CD4 and CD8 T cells. (A) Schematic of tumor study model. (B) Tumor growth curve showing average volume. The formula (½ (Length × Width^2)) was used to calculate the volume. *PLS-α-GalCer versus PBS, p value<0.05, n=5. (C) Tumor growth curve showing tumor volume of individual mice. (D) Final average tumor weights. **PLS-α-GalCer versus PBS, p value<0.05, n=4–5, ns= (not significant). (E) Flow cytometry analysis of tumor-infiltrating T cells and NKT cells. *PLS-α-GalCer versus PBS, p value<0.05, n=3–5. NKT, natural killer T; PBS, phosphate-buffered saline; PLS-α-GalCer, poly(L-lysine succinylated)-α-galactosylceramide.

α-GalCer or α-GalCer equivalents of PLS-α-GalCer, or phosphate-buffered saline (PBS) vehicle control started 7–9 days after tumor cell inoculation and was administered intraperitoneally twice a week throughout the study.

For the TC-1 studies, 0.5×10^5 cells were injected subcutaneously into the flanks. Treatment with 429 ng of α-GalCer or α-GalCer equivalents of PLS-α-GalCer or HPV16 $E7_{43–77}$ peptide (GQAEPDRAHYNIVTFCCKCDSTLR LCVQSTHVDIR) vaccine[26] (160 µg $E7_{43–77}$ peptide+20 µg DOTAP (Roche)+1 µg Granulocyte-macrophage colony-stimulating factor (PeproTech)), or PBS vehicle control started 10–12 days after tumor cell inoculation and was administered intraperitoneally twice a week throughout the study.

The mice were randomized according to their tumor volume size to balance the average tumor volumes for each group before treatment started in all experiments. The tumors were measured using a digital caliper twice a week.

Flow cytometry

At the end of the tumor studies, tumor mononuclear cells were collected using a MACS tumor dissociation kit (Miltenyi Biotec) followed by Ficoll-Paque PLUS and centrifugation following manufacturer's instructions. Next, the cells were washed with PBS and stained with LIVE/DEAD Fixable Blue Dead Cell Stain (Thermo Fisher Scientific) at 4°C for 20 min. Next, the cells were washed with Fluorescence-activated cell sorting (FACS) buffer (PBS+1% Bovine serum albumin (BSA)+0.05% sodium azide) and stained with Fc block (anti-mouse CD16/32 antibody, BioLegend) and tetramers for 30 min at 4°C. The cells were then stained with surface fluorescent protein labeled antibodies against CD4, CD8, TCRβ, and CD45 (BioLegend or eBioscience). HPV16 $E7_{49–57}$ peptide-loaded H-2D^b tetramer and PBS57-loaded CD1d-tetramer were provided by the National Institutes of Health tetramer core facility. Fluorescence was measured using a FACSymphony (BD Bioscience) and analyzed using FlowJo (Becton, Dickinson and Company).

Immunofluorescence staining

First, coverslips were coated with Poly-L-Lysine Solution (Millipore) (0.01%) at 4°C and rinsed with PBS before cell plating. Next, 0.5×10^5 RAW wildtype or RAW SR-A1 knockout cells were plated on the coverslips overnight and incubated overnight at 37°C in humidified air with 5% CO_2. The next day the cells were treated with 1 µg/mL of α-GalCer or α-GalCer equivalents of PLS-α-GalCer in cell culture media and incubated at 37°C in humidified air with 5% CO_2. After 18 hours, the cells were stained for immunofluorescence. Briefly, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 12 min at room temperature. Next, fixative was aspirated, and coverslips were washed three times with PBS for 5 min each. Then the cells were permeabilized and blocked with 0.1% saponin and 1% BSA in PBS for 50 min at room temperature. Next, the permeabilization buffer was aspirated and primary antibodies diluted in (1× PBS/1% BSA/0.1% Saponin) were added and incubated overnight at 4°C. After incubation, the cells were washed with PBS three times for 5 min each. Next, a secondary antibody was added, and cells were incubated in the dark for 1 hour at room temperature. After 1 hour, the cells

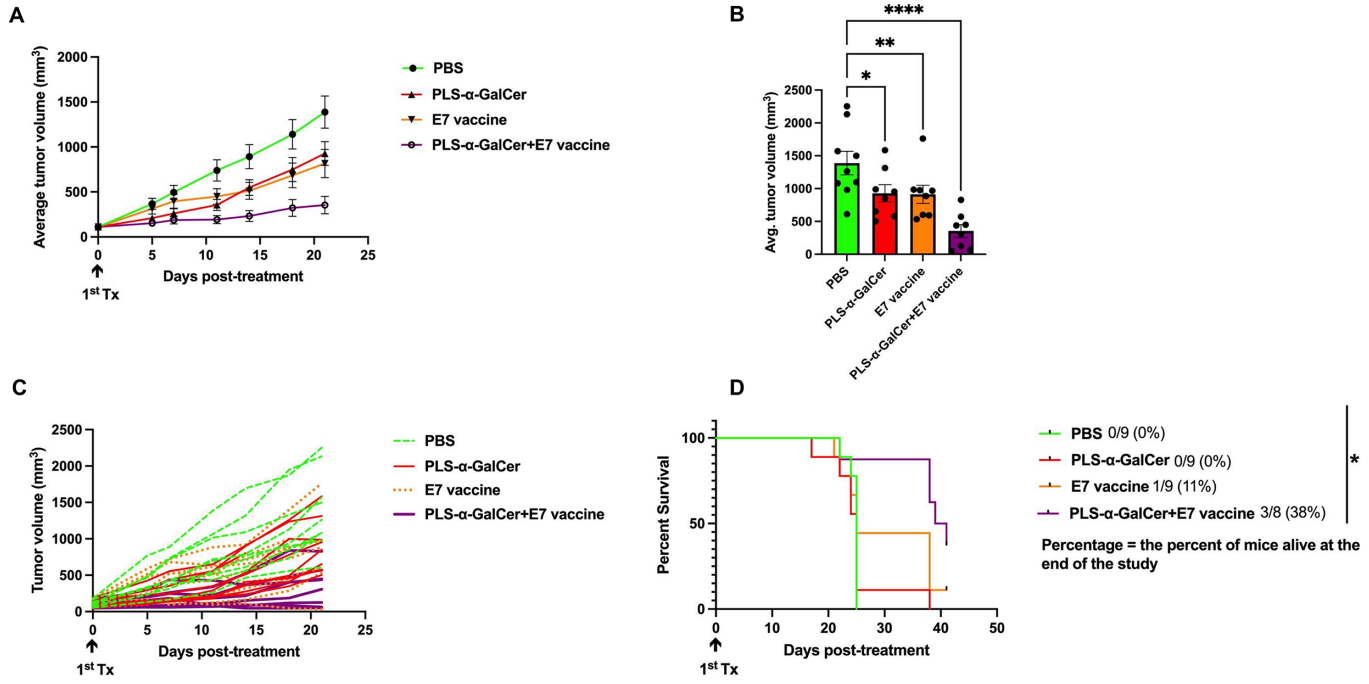

Figure 2 PLS-α-GalCer synergizes with the E7 $_{(43–77)}$ vaccine to suppress TC1 tumor growth and increase the survival rate. (A) Tumor growth curve showing average volume. The formula (½ (Length × Width^2)) was used to calculate the volume. (B) Quantitation of average tumor volume on day 21. *PLS-α-GalCer versus PBS, p value<0.05; **E7 vaccine versus PBS, p value<0.001; ****PLS-α-GalCer+E7 vaccine versus PBS, p value<0.0001. n=8–9. (C) Tumor growth curve showing tumor volume of individual mice. (D) Kaplan-Meier survival analysis of percent survival. *PLS-α-GalCer+E7 vaccine versus all other groups individually, p value<0.05, n=8–9, percentage represents the percent of mice alive at the end of the study. PBS, phosphate-buffered saline; PLS-α-GalCer, poly(L-lysine succinylated)-α-galactosylceramide.

were washed three times with PBS and then coverslips were mounted using Prolong gold antifade with DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes). The primary antibodies used were LAMP1 (Abcam, 1:100) and L363 (Millipore, 1:200). The secondary antibody used was Goat anti-mouse Cy5 (Abcam, 1:5000) and Goat anti-mouse Alexa Fluor 488 (Abcam, 1:500). Images were taken at 40× using a Zeiss LSM880 confocal microscope.

Immunohistochemistry

The staining and whole-slide analysis were performed by the Molecular Histopathology Laboratory at the Frederick National Laboratory for Cancer Research. Formalin-Fixed Paraffin-Embedded (FFPE) tissues were submitted for H&E and chromogenic immunohistochemistry; annotations were made to include tumor tissue for quantification of immunohistochemistry using the reagents listed in table 1 . Negative controls included replacing the primary antibody with non-specific antibody from the same species and of the same isotype. Controls stained appropriately. Whole slide imaging (WSI) was performed with an Aperio ScanScope XT (Leica) at 200× in a single z-plane. Digital pathology for biomarker quantification was performed following WSI. Thresholds for positivity were determined using known positive controls. Cell detection algorithms were run to quantify positive cells, which are expressed as the number of positive cells per mm^2 of tissue.

Serum cytokine analysis

Mice were treated with 429 ng of α-GalCer or α-GalCer equivalents of PLS-α-GalCer, or PBS and serum was collected 4, 8, 24 and 28 hours later. The interferon-gamma (IFN-γ) cytokine levels were measured by ELISA using a R&D Systems DuoSet kit (DY485). The Th2 and Th17 cytokines were measured by LEGENDplex analysis (BioLegend). The time points where cytokine levels are within the assay detection limits are shown.

Statistics

Statistical analyses were performed with GraphPad Prism (V.10.2.0) and data are presented as mean±SEM. The data representing a single time point and multiple time points were analyzed by one-way analysis of variance and two-way analysis of variance, respectively with multiple comparison correction. The survival data were analyzed by the Mantel-Cox test. P values<0.05 were considered statistically significant.

RESULTS

PLS-α-GalCer synthesis and characterization

α-GalCer was conjugated to PLS via a Steglich esterification using 4-dimethylaminopyridine and N,N'-diisopropylcarbodiimide in a solvent mixture containing dimethylformamide and dimethyl sulfoxide (online supplemental figure 2A). Prior to

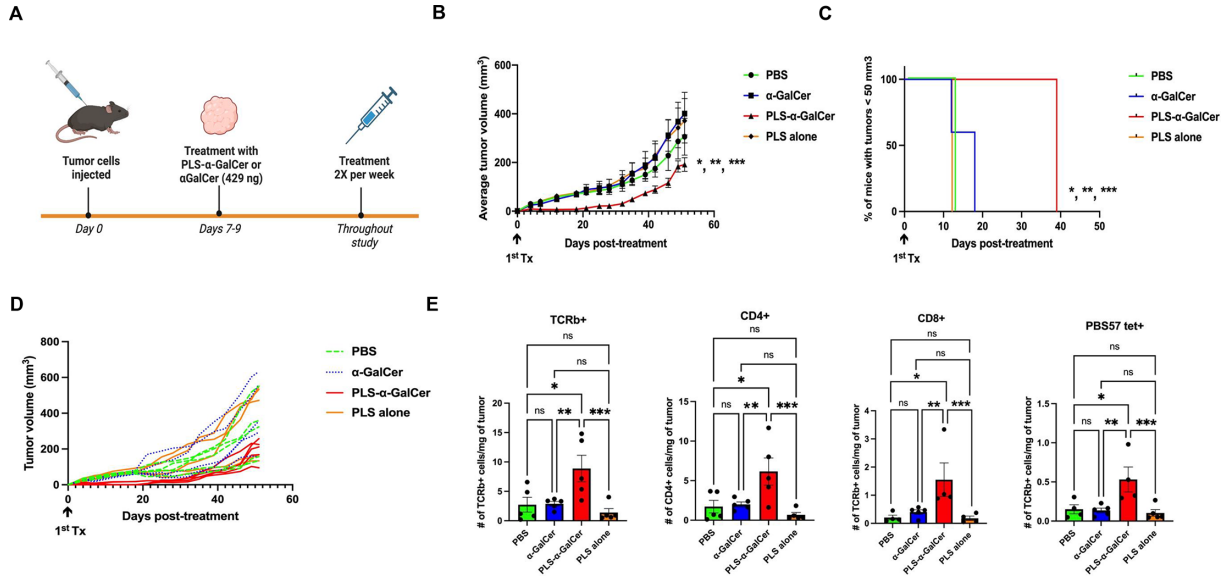

Figure 3 PLS-α-GalCer suppresses Panc02 tumor growth and increases tumor-infiltrating T cells and NKT cells. (A) Schematic of tumor study model. (B) Tumor growth curve showing average volume. The formula (½ (Length × Width^2)) was used to calculate the volume. *PLS-α-GalCer versus PBS, p value<0.05; **PLS-α-GalCer versus α-GalCer, p value<0.05; ***PLS-α-GalCer versus PLS alone, p value<0.05; n=5. (C) Kaplan-Meier analysis of the percent of mice with tumors<50 mm^3. *PLS-α-GalCer versus PBS, p value<0.05; **PLS-α-GalCer versus α-GalCer, p value<0.05; ***PLS-α-GalCer versus PLS alone, p value<0.05; n=5. (D) Tumor growth curve showing tumor volume of individual mice. (E) Flow cytometry analysis of tumor-infiltrating T cells and NKT cells per gram of tumor. *PLS-α-GalCer versus PBS, p value<0.05; **PLS-α-GalCer versus α-GalCer, p value<0.05; ***PLS-α-GalCer versus PLS alone, p value<0.05; n=5, ns= (not significant). NKT, natural killer T; PBS, phosphate-buffered saline; PLS-a-GalCer, poly(L-lysine succinylated)-α-galactosylceramide.

the reaction, pendant carboxylate groups were converted to the corresponding carboxylic acids. α-GalCer contains several hydroxyl groups and thus it is expected the esterification reaction to take place at any of the given sites. Therefore, the resulting polymer-prodrug could have a mixture of products depending on the site of conjugation. To avoid cross-linking the reaction was carried out in very dilute conditions. The resulting polymer, PLS-α-GalCer, was purified via a series of dialysis in acetone, acetate buffers and water. These steps also regenerated the carboxylate groups to render aqueous solubility.

Liquid chromatography–mass spectrometry (LC-MS) was used to determine the amount of conjugated α-GalCer as a percent weight of the total construct. This was carried out by base hydrolysis of the ester linkages. Polymer samples were incubated with Sodium hydroxide (NaOH) at room temperature for 1 hour to facilitate complete hydrolysis, after which the samples were analyzed by LC-orbitrap. Non-hydrolyzed PLS-α-GalCer samples were used to determine free α-GalCer and the NaOH hydrolyzed samples were used to determine the total α-GalCer in the sample. The total α-GalCer was 3.4% (wt/wt) with 0.5% (wt/wt) being free α-GalCer (online supplemental figure 2B). In online supplemental figure 2C and D, PLS-α-GalCer release in mouse plasma was slow and not concentration-dependent, with a release half-life of 10.3–11.1 days for PLS-α-GalCer concentrations ranging from 0.1 to 1 µg α-GalCer equivalents/mL. Purified polymer was characterized using ^{1}H nuclear magnetic resonance (NMR) (online supplemental figure 3) for structure confirmation. Due to the low amount of α-GalCer conjugated to the polymer backbone (~3 units from 250 succinylated lysine repeat units, based on drug loading), the only visible peak of α-GalCer corresponds to the ceramide chain at 1.25 ppm. Further, due to peak overlap determination of the number of α-GalCer units through integration is difficult.

PLS-α-GalCer stimulates type I NKT cells more efficiently than α-GalCer in vitro

To test whether PLS-α-GalCer is more effective in activating type I NKT cells than α-GalCer, we titrated the concentration of both using IFN-γ (online supplemental figure 4A) and tumor necrosis factor alpha (online supplemental figure 4B) production as the readout for type I NKT cell activation. At the concentrations of 50 nM and 100 nM of α-GalCer or α-GalCer equivalents of PLS-α-GalCer used in online supplemental figure 4A and B, the NKT cell T-cell receptor (TCR) is markedly downregulated, indicated by the reduced ability to detect type I NKT cells using the PBS57 tetramers (online supplemental figure 4C). Therefore, it is difficult to compare PLS-α-GalCer and α-GalCer ability to stimulate type I NKT cells. To address this problem, we titrated the concentration of PLS-α-GalCer and α-GalCer further to find a concentration where the type I NKT cell TCR is not downregulated while still being able to detect IFN-γ

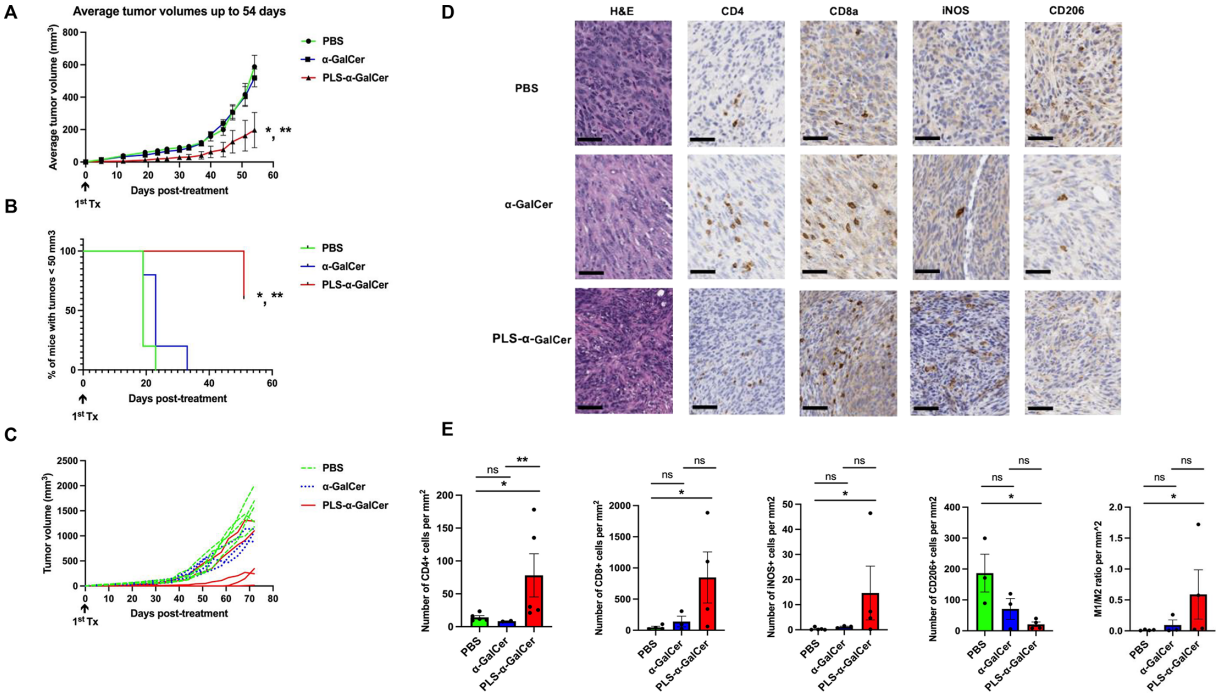

Figure 4 PLS-α-GalCer suppresses Panc02 tumor growth and increases M1/M2 macrophage tumor ratio**.** (A) Tumor growth curve showing average volume. The formula (½ (Length × Width^2)) was used to calculate the volume. *PLS-α-GalCer versus PBS, p value<0.05; **PLS-α-GalCer versus α-GalCer, p value<0.05 n=5. (B) Kaplan-Meier analysis of the percent of mice with tumors<50 mm^3. *PLS-α-GalCer versus PBS, p value<0.05; **PLS-α-GalCer versus α-GalCer, p value<0.05 n=5. (C) Tumor growth curve showing tumor volume of individual mice. (D) Immunohistochemistry staining of CD4 and CD8 T cells, and iNOS and CD206 positive cells scale bar=50 µm. (E) Whole-slide scan analysis showing the number of immune cells per mm^2 of tumor. *PLS-α-GalCer versus PBS, p value<0.05; **PLS-α-GalCer versus α-GalCer, p value<0.05 n=3–5, ns= (not significant). PBS, phosphate-buffered saline; PLS-α-GalCer, poly(L-lysine succinylated)-α-galactosylceramide.

levels (online supplemental figure 4D and E). Although not statistically significant, in online supplemental figure 3D, it is shown that at the lowest two concentrations at which tetramer staining is not reduced based on online supplemental figure 4E, PLS-α-GalCer activates substantially more type I NKT cells than α-GalCer. In online supplemental figure 4F, we show the confirmatory result with more replicates that PLS-α-GalCer more efficiently stimulates type I NKT cells compared with α-GalCer at a concentration where the TCR is not downregulated, as shown in online supplemental figure 4G.

PLS-α-GalCer suppresses TC-1 tumor growth and increases tumor-infiltrating antigen-specific CD8 T cells, NKT cells, and CD4 and CD8 T cells

To test the ability of PLS-α-GalCer to induce tumor-antigen specific CD8 T cells and suppress tumor growth we used the TC-1 cell line that expresses (HPV-16) E7 and E6 oncogenes, and forms non-immunogenic "cold" tumors.[27 28] The schematic of the tumor study is shown in figure 1A in which 10–12 days after TC-1 tumor cell inoculation, treatment twice a week with 429 ng of α-GalCer or α-GalCer equivalents of PLS-α-GalCer was started. The mice treated with PLS-α-GalCer grew significantly smaller tumors compared with PBS-treated control mice (figure 1B, C and D). Figure 1B shows means of groups while figure 1C shows individual mice and each group of individual mice is shown in online supplemental figure 5. In contrast, no significant difference was detected in the mice treated with α-GalCer, PLS alone, or PLS alone+α-GalCer compared with PBS group (figure 1B and D). Thus, the PLS-α-GalCer conjugate was more effective than the mix of each component not linked. Just the initial two doses of PLS-α-GalCer were not sufficient to significantly suppress tumor growth, so repeated treatment was necessary (data not shown). Furthermore, PLS-α-GalCer significantly increased tumor-infiltrating antigen-specific CD8 T cells (E7-tetramer positive), NKT cells, and CD4 and CD8 T cells compared with the PBS group (figure 1E). The group with α-GalCer alone also showed some ability to increase NKT cells and E7-specific CD8 T cells in the tumor microenvironment (TME), but it was not significant. In addition, there was a substantial percentage of CD4/CD8 double negative cells from the gated TCRb+population in all experimental groups (online supplemental figure 6). These cells may be immunosuppressive cells such as type II NKT cells[29–31] or regulatory T cells,[32 33] conventional T cells that have robust antitumor activity,[34 35] or a combination of cell types.

PLS-α-GalCer synergizes with the E7 $_{(43–77)}$ vaccine to suppress TC-1 tumor growth and increase the survival rate

Since PLS-α-GalCer can increase the number of tumor-infiltrating antigen-specific CD8 T cells, we tested whether the E7 $_{(43–77)}$ vaccine could synergize with PLS-α-GalCer in suppressing TC-1 tumor growth. In figure 2A, B and C

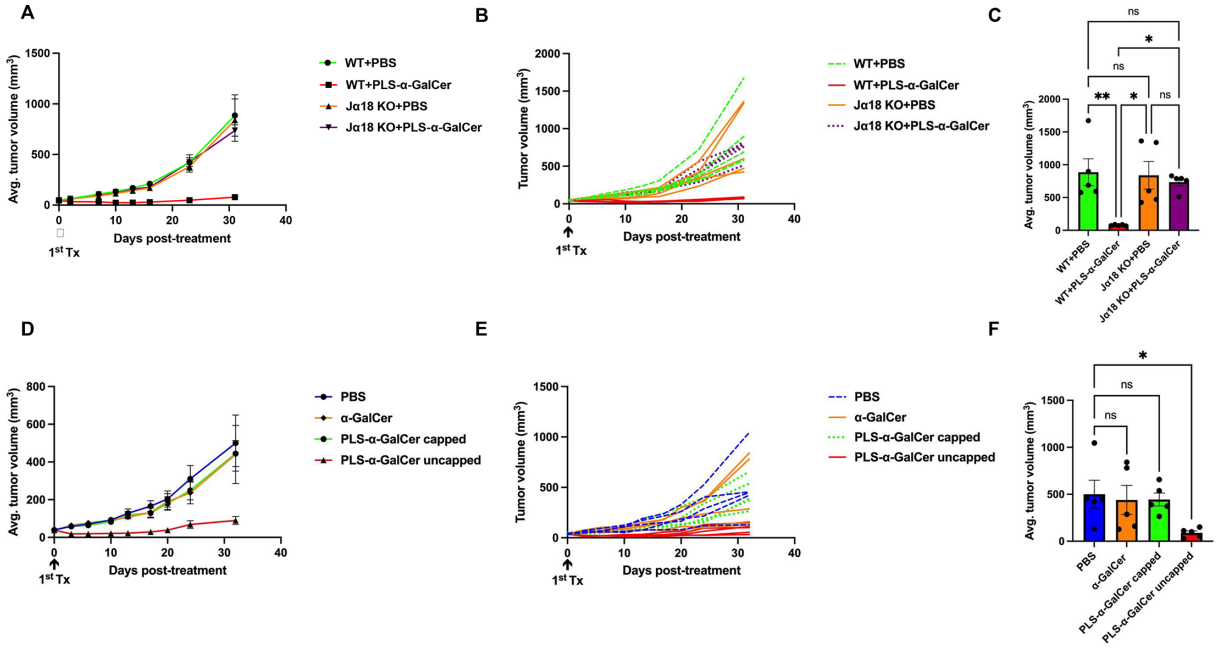

Figure 5 PLS-α-GalCer antitumor function is type I NKT cell dependent and requires SR-A1 targeting. (A) Tumor growth curve showing average volume. The formula (½ (Length × Width^2)) was used to calculate the volume. (B) Tumor growth curve showing tumor volume of individual mice. (C) Quantitation of final average tumor volume *p value<0.05, **p value<0.01, n=5, ns= (not significant). (D) Tumor growth curve showing average tumor volume. The formula (½ (Length × Width^2)) was used to calculate the volume. (E) Tumor growth curve showing average volume of individual mice. (F) Quantitation of final average tumor volume. *p value<0.05, **p value<0.01, n=5, ns=(not significant). NKT, natural killer T; PBS, phosphate-buffered saline; PLS-α-GalCer, poly(L-lysine succinylated)-α-galactosylceramide; SR-A1, scavenger receptor A1.

we show that both PLS-α-GalCer and the E7 $_{(43–77)}$ vaccine could significantly suppress tumor growth when used as a monotherapy but when combined they led to a synergistic antitumor effect. The tumor volumes of individual mice of each group are shown in online supplemental figure 7. In addition, the combination of PLS-α-GalCer and E7 $_{(43–77)}$ vaccine significantly increases the survival time compared with PBS, PLS-α-GalCer alone, and E7 $_{(43–77)}$ vaccine alone treated mice (figure 2D). Collectively, the data from figures 1 and 2 demonstrate that PLS-α-GalCer can significantly increase tumor-infiltrating CD4 and CD8 T cells, NKT cells, antigen-specific T cells, and suppress TC-1 tumor growth as a monotherapy. But when combined with the E7 $_{(43–77)}$ vaccine the antitumor response is synergistically enhanced, leading to a significant improvement in survival time.

PLS-α-GalCer suppresses Panc02 tumor growth and increases tumor-infiltrating T cells and NKT cells

To test the effect of PLS-α-GalCer in suppressing pancreatic tumor growth we used the Panc02 cell line. We chose to test PLS-α-GalCer effect on pancreatic tumor growth for three reasons: (1) It has been demonstrated that NKT cells play a critical role in pancreatic cancer progression,[36] (2) Pancreatic cancer is considered a cold tumor with few infiltrating T cells,[37 38] and we wanted to test whether PLS-α-GalCer could turn a cold tumor into a hot tumor with more infiltrating T cells, and (3) Pancreatic cancer typically does not respond to immunotherapy,[37 39] and we wanted to show the potential of PLS-α-GalCer to be an effective alternative treatment for tumors that do not respond to immunotherapy. The tumor study design is shown by the schematic in figure 3A in which 7–9 days after Panc02 tumor cell inoculation, treatment twice a week with 429 ng of α-GalCer or α-GalCer equivalents of PLS-α-GalCer started. The mice treated with PLS-α-GalCer grew significantly smaller tumors compared with PBS, α-GalCer, or PLS alone (figure 3B, C and D). The tumor volumes of individual mice of each group are shown in online supplemental figure 8. The analysis of tumor-infiltrating immune cells showed a significant increase in T cells and NKT cells in the PLS-α-GalCer group compared with PBS, α-GalCer, or PLS alone treated mice (figure 3E).

PLS-α-GalCer increases the M1/M2 macrophage tumor ratio and suppresses Panc02 tumor growth

To confirm the tumor-infiltrating T cell data in figure 3F and to test the effect of PLS-α-GalCer on tumor-associated macrophage polarization, we repeated the Panc02 tumor study. It is known that M2-macrophage polarization is associated with worse overall survival in pancreatic cancer and long-term pancreatic cancer survivors have significantly reduced levels of M2 macrophages.[40 41] Furthermore, it has been shown that α-GalCer-induced type I NKT cell activation increases M1 macrophage polarization and iNOS$^+$CD206$^-$ macrophage frequency in the TME.[42] In figure 4A, B and C, we demonstrate that PLS-α-GalCer significantly suppresses pancreatic tumor growth compared with PBS or α-GalCer treated mice. The tumor

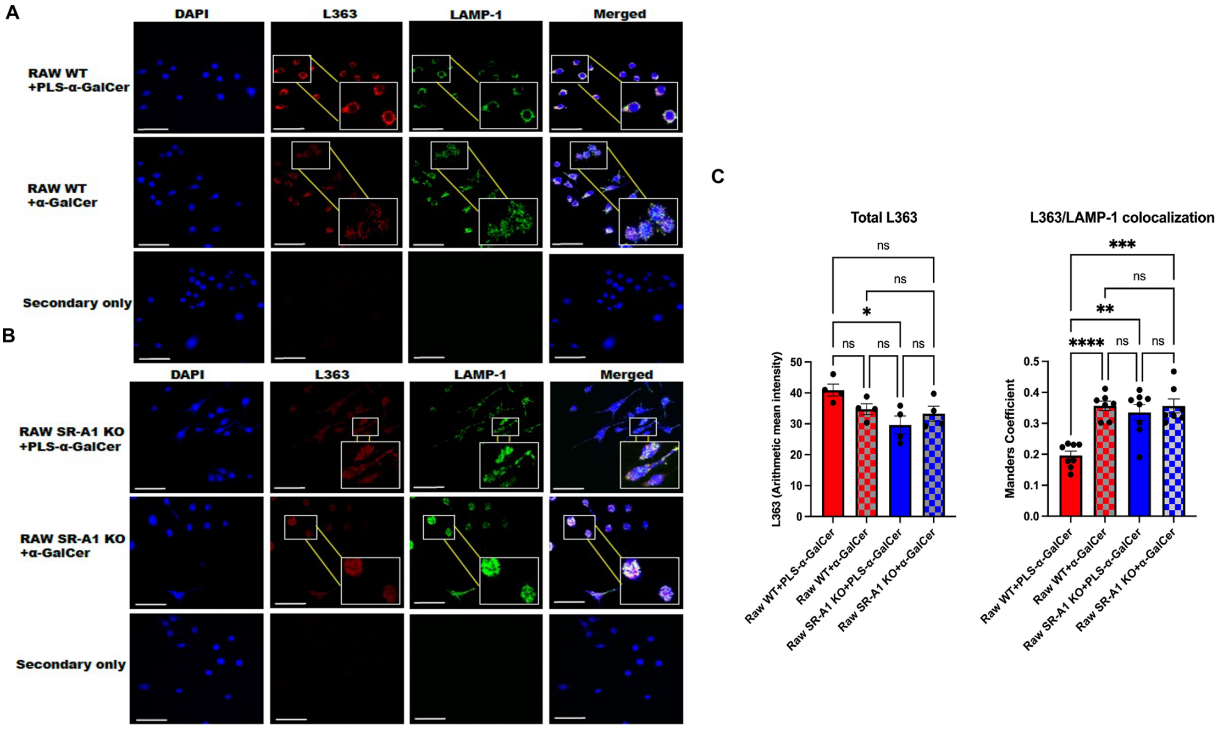

Figure 6 PLS-α-GalCer is more efficiently processed by RAW WT cells compared with α-GalCer in an SR-A1 dependent manner. Immunofluorescence staining of α-GalCer/CD1d complex using L363 antibody and lysosomes using LAMP-1 antibody in RAW WT cells in A and RAW SR-A1 KO cells in B. Image magnification is 40×, inset is a 200% magnification image. (**C**) left panel. Quantitation of total α-GalCer/CD1d complex, *p value<0.05; n=4, right panel. Quantitation of α-GalCer/CD1d complex colocalized with lysosomes, **p value<0.01, ***p value<0.001, ****p value<0.0001; n=7–8. ns=(not significant). PLS-α-GalCer, poly(L-lysine succinylated)-α-galactosylceramide; SR-A1, scavenger receptor A1.

volumes of individual mice of each group are shown in online supplemental figure 9. Furthermore, the representative whole-slide immunohistochemistry images for CD4 and CD8 T cells, and M1 and M2 macrophages are shown in figure 4D with the quantitation of the whole-slide analysis in figure 4E. iNOS expression is used as a marker of M1 macrophages, CD206 a marker of M2 macrophages, and a higher M1/M2 ratio is associated with a negative impact on tumor growth.[42–45] The result of the quantitation reveals that PLS-α-GalCer significantly increases tumor-infiltrating T cells and M1/M2 macrophage ratio compared with PBS or α-GalCer treated mice (figure 4E). Collectively, the data from figures 3 and 4 reveal that PLS-α-GalCer can significantly increase pancreatic tumor NKT, CD4 and CD8 T cell numbers, while also increasing the M1/M2 macrophage ratio resulting in a significant reduction in tumor growth.

PLS-α-GalCer antitumor function is type I NKT cell dependent and requires SR-A1 targeting

Because α-GalCer is a type I NKT cell agonist, we asked whether the antitumor effect of PLS-α-GalCer is type I NKT cell-dependent. For these experiments, we used the Panc02 tumor model and followed the same treatment regimen shown in figure 3A. As expected, PLS-α-GalCer treatment significantly reduced tumor growth in WT mice but was not effective in Jα18 KO mice, which completely lack type I NKT cells because they cannot form without this receptor (figure 5A). The growth curves of the individual mice are presented in figure 5B, and the quantitation of the final tumor volume is shown in figure 5C.

Since SR-A1 plays a critical role in the processing of α-GalCer for antigen presentation and PLS targets SR-A1, we tested whether the antitumor function of PLS-α-GalCer requires SR-A1 targeting. The antitumor activity of PLS-α-GalCer was compared with a version of PLS-α-GalCer in which all carboxylate groups are capped with 2-(2-methoxyethoxy)ethanamine which prevents the interaction of the negative charges of PLS with SR-A1. In figure 5D and E, we show that the antitumor function of PLS-α-GalCer is lost when mice are treated with the capped version. The quantitation of the final average tumor volume shows that uncapped PLS-α-GalCer significantly decreased tumor growth compared with PBS control while no significant difference was found in the mice treated with the capped version (figure 5F). This result confirms our hypothesis that the enhanced activity of PLS-α-GalCer is due to its ability to bind to SR-A1.

PLS-α-GalCer is more efficiently processed by RAW WT cells compared with α-GalCer in an SR-A1 dependent manner

α-GalCer is rapidly taken up by APCs and processed with α-GalCer/CD1d complexes detected at plasma membrane in 18–24 hours.[46 47] Moreover, it is known that SR-A1 plays a vital role in the antigen processing of α-GalCer by the endosome–lysosome pathway.[22] To

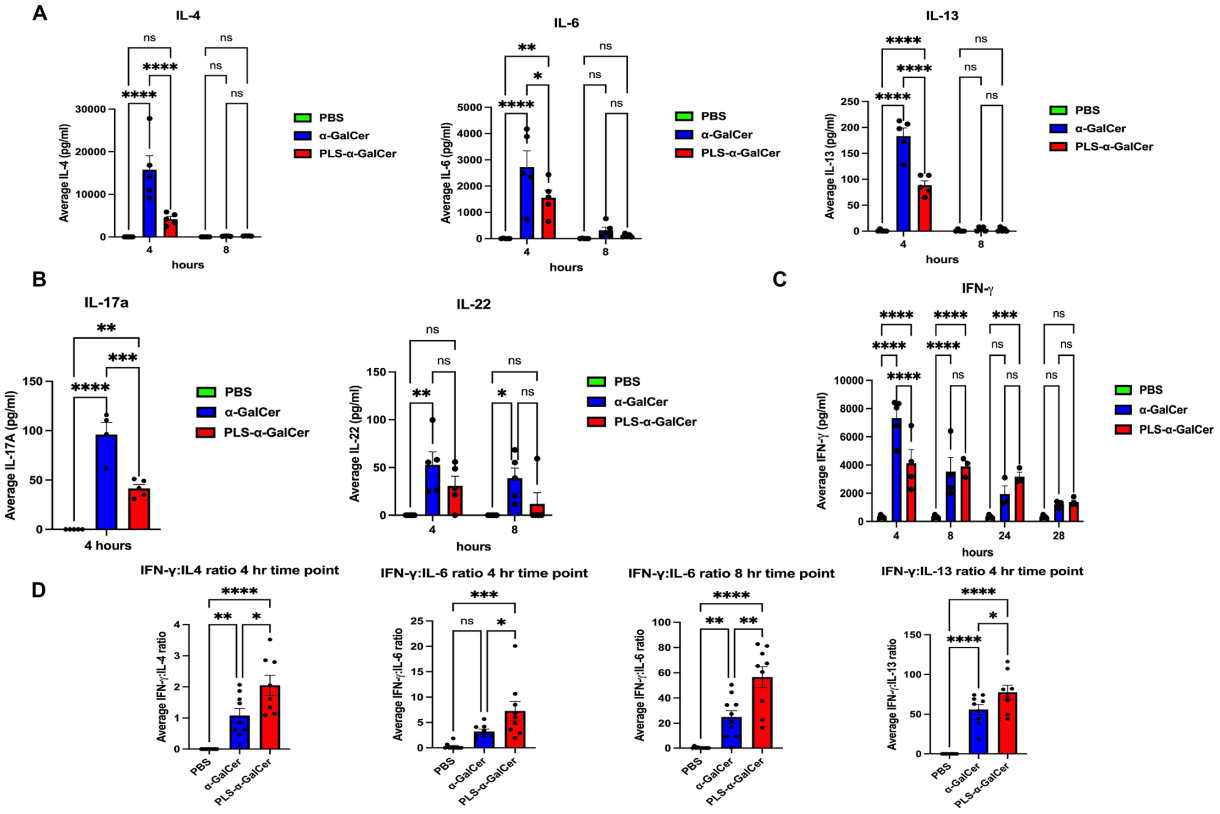

Figure 7 PLS-α-GalCer induces significantly less serum Th2 and Th17 cytokines while stimulating significantly more IFN-γ for a longer period and increases Th1:Th2 cytokine ratios compared with α-GalCer. (A) LEGENDplex analysis of serum Th2 cytokine levels after treating mice with 429 ng of PLS-α-GalCer or α-GalCer; *p value<0.05, **p value<0.01, ****p value<0.0001; n=5. (B) LEGENDplex analysis of serum Th17 cytokine levels after treating mice with 429 ng of PLS-α-GalCer or α-GalCer; *p value<0.05, **p value<0.01, ***p value<0.001, ****p value<0.0001; n=5. (C) ELISA analysis of serum IFN-γ levels after treating mice with 429 ng of PLS-α-GalCer or α-GalCer; ***p value<0.001, ****p value<0.0001; n=3–5 per group. (D) Th1:Th2 cytokine ratios; *p value<0.05, **p value<0.01, ****p value<0.0001; n=8 per group. PBS was used as the control and ns=(not significant). IFN-γ, interferon-gamma; PBS, phosphate-buffered saline; PLS-α-GalCer, poly(L-lysine succinylated)-α-galactosylceramide.

test the ability of PLS-α-GalCer to be internalized and processed by APCs compared with α-GalCer, we used the RAW macrophage cell line, which abundantly expresses SR-A1, and RAW SR-A1 KO cells.[24] The L363 antibody was used to detect α-GalCer/CD1d complexes and LAMP-1 was used to detect lysosomal compartments. Although not statistically significant, the RAW WT cells that were pulsed with PLS-α-GalCer showed more total α-GalCer/CD1d complexes than α-GalCer pulsed cells (figure 6A and C left panel). Interestingly, the RAW WT cells pulsed with PLS-α-GalCer had the majority of α-GalCer/CD1d complexes located at the plasma membrane while the α-GalCer pulsed cells had significantly fewer α-GalCer/CD1d complexes located at the plasma membrane with a diffuse L363 staining pattern located primarily in the cytoplasm (figure 6A). The colocalization quantitation data in figure 6C right panel shows that significantly more α-GalCer/CD1d complexes colocalized with lysosomes in RAW WT cells pulsed with α-GalCer compared with PLS-α-GalCer-pulsed cells. Moreover, the RAW SR-A1 KO cells pulsed with PLS-α-GalCer displayed a diffused L363 staining pattern like α-GalCer pulsed RAW SR-A1 KO cells (figure 6B) with significantly more α-GalCer/CD1d complexes colocalized with lysosomes compared with RAW WT cells pulsed with PLS-α-GalCer (figure 6C right panel). The total level of α-GalCer/CD1d complexes was significantly lower in SR-A1 KO cells pulsed with PLS-α-GalCer than in RAW WT cells pulsed with PLS-α-GalCer (figure 6C left panel). Collectively, the data suggest that PLS-α-GalCer is more efficiently internalized and processed by APCs in an SR-A1-dependent manner than α-GalCer.

PLS-α-GalCer induces significantly less serum Th2 and Th17 cytokines while stimulating significantly more IFN-γ for a longer period and increases Th1:Th2 cytokine ratios compared with α-GalCer

To gain additional insight into the mechanism of the antitumor activity of PLS-α-GalCer, we compared the cytokine profile with α-GalCer analyzing Th2 and Th17 cytokines, and IFN-γ, the primary Th1 cytokine induced by α-GalCer. It has been shown that Th2 cytokines, such as IL-4, IL-6, and IL-13, and Th17 cytokines, such as IL-17a and IL-22, play a role in suppressing tumor immunity.[48–53] In figure 7A and B, the mice treated with PLS-α-GalCer induced

significantly less serum Th2 and Th17 cytokines compared with α-GalCer. Moreover, PLS-α-GalCer treated mice maintained significantly elevated serum IFN-γ levels over a 24-hour period compared with control while α-GalCer did not (figure 7C). Note that these levels are below the level to raise any concern about cytokine storm or other toxicity. In addition, we analyzed the serum Th1:Th2 cytokine ratios because a higher ratio is known to be associated with good prognosis in patients with cancer.[44 45] The mice treated with PLS-α-GalCer displayed a significantly higher Th1:Th2 cytokine profile compared with α-GalCer (figure 7D).

DISCUSSION

The potent antitumor effect of type I NKT cells induced by α-GalCer was discovered more than a decade ago.[3 54 55] This discovery led to several clinical trials testing the antitumor effect of soluble α-GalCer, α-GalCer-pulsed dendritic cells, α-GalCer-activated NKT cells, and α-GalCer-pulsed APCs from the peripheral blood.[8–11 56] Although some antitumor effects were seen, mostly suboptimal results were achieved in the clinical trials.[11] Regarding patients, the variability in the number of NKT cells prior to treatment plays a role in the inconsistent results observed. Furthermore, the long-term anergic state of type I NKT cells induced by α-GalCer prevents effective repeated administration of the drug.[57–59] Thus far, the most promising method is treating patients with α-GalCer-pulsed antigen-presenting or dendritic cells. Although this method has been shown to prevent NKT cell anergy,[60] the results from the clinical trials suggest there is a need for improvement as the clinical responses have not been consistent and predictable.[11] Moreover, the cell-based α-GalCer therapies are very expensive, logistically demanding, and risky. The good news is that α-GalCer is well tolerated, and no severe adverse effects have been reported.[11] Thus, there is potential to unlock the potent antitumor effect of α-GalCer to treat patients with cancer by targeting α-GalCer to APC/dendritic cells in vivo.

In this study, we introduced "PLS-α-GalCer"; a novel technology that delivers α-GalCer to APCs in the TME and lymphatic system.[16] The targeting mechanism involves the high affinity and specificity of PLS interacting with SR-A1 on APCs.[16] Our studies demonstrated that SR-A1-mediated uptake of PLS-α-GalCer results in more efficient processing of α-GalCer than free α-GalCer in APCs. In addition, the tumor studies showed that PLS-α-GalCer significantly decreases tumor growth, increases tumor-infiltrating antigen-specific CD8 T cells, NKT cells, and CD4 and CD8 T cells, and increases M1/M2 macrophage ratio compared with α-GalCer. Moreover, studies using RAW SR-A1 KO cells and untargeted capped PLS-α-GalCer revealed the importance of SR-A1 in processing PLS-α-GalCer and mediating antitumor effects. Our findings are supported by previous studies that showed SR-A1-mediated uptake of α-GalCer plays a major role in CD1d loading and presentation by APCs.[22] Also, the bioactivity of α-GalCer in vivo is highly dependent on SR-A1 uptake.[22] Therefore, the ability of PLS-α-GalCer to target SR-A1 is an ideal strategy to reveal the full potential of α-GalCer in suppressing tumor growth.

The targeting mechanism of PLS-α-GalCer delivering α-GalCer to APCs in the primary tumor and lymphatic system suggests it may be effective in suppressing tumor metastasis. In support, PLS-α-GalCer induces significantly less serum Th2 and Th17 (tumor-promoting) cytokines and significantly more serum IFN-γ for a longer period with a significantly higher Th1:Th2 ratio compared with α-GalCer. The effect of PLS-α-GalCer on metastasis was not addressed in this study but is an important aspect to test in follow-up studies.

The current study demonstrated that PLS-α-GalCer can synergize with the $E7_{(43–77)}$ vaccine to significantly suppress the growth of E7 oncogene-expressing TC-1 tumors and increase the overall survival. This suggests that combination therapies with PLS-α-GalCer might provide a strategy to effectively treat patients with cancer. In fact, it is known that anti-programmed cell death protein-1 (PD-1) therapy prevents α-GalCer-mediated induction of type I NKT cell anergy.[57] The conclusions from the restimulation experiments testing whether PLS-α-GalCer causes type I NKT cell anergy were not clear, because after a second dose of PLS-α-GalCer a month later, NKT cell intracellular IFN-γ was detected but no secreted form was detected in vitro or in vivo (online supplemental figure 10). Therefore, combining PLS-α-GalCer with anti-PD-1 treatment could provide a more robust antitumor response than PLS-α-GalCer monotherapy. Several clinical trials have also shown the benefit of treating patients with neoantigen vaccines.[61 62] Since PLS-α-GalCer is targeted to APCs, another approach to explore is to attach neoantigens, tumor-associated antigens, or patient-derived tumor antigens to the PLS-α-GalCer prodrug to induce a highly specific antitumor response. In this manner, the PLS-α-GalCer prodrug would serve as a cancer vaccine, vaccine vehicle, and adjuvant all in one treatment. Overall, this study provides evidence that PLS-α-GalCer is effective in suppressing tumor growth as a monotherapy by inducing an antitumor microenvironment and the antitumor response synergizes with tumor vaccine treatment. Therefore, strategies to optimize PLS-α-GalCer therapy for patients with cancer are warranted.

Acknowledgments We thank the NCI/CCR/LRBGE Optical Microscopy Core for their expertise in confocal microscopy, Frederick National Laboratory for Cancer Research (FNLCR) Molecular Histopathology Laboratory for their expertise in immunohistochemistry, NCI CCR Vaccine Branch Flow Cytometry Core staff for their expertise in flow cytometry, FNLCR Small Animal Imaging Program for assistance with performing the PLS-Cy7.5 biodistribution study, and the staff of the NCI Animal Facility for their assistance with the maintenance of our mice.

Contributors JB is the first author and contributed significantly to the conception of the project, experimental designs, performing experiments, analyzing data, wrote the manuscript and is a guarantor. ST helped process samples. DMS contributed to synthesizing poly(L-lysine succinylated) (PLS)-α-galactosylceramide (GalCer) constructs. RNK contributed to synthesizing PLS-α-GalCer constructs and wrote the methods for the synthesis and characterization of PLS-α-GalCer. SLS performed PLS-α-GalCer drug loading and half-life determination assays and wrote those method sections. STS contributed to the conception of the project, synthesizing PLS-α-GalCer constructs, and edited the manuscript. JAB contributed to experimental designs, provided technical advice, edited the manuscript and is a guarantor.

Funding This study was supported by the National Cancer Institute intramural research program at the National Institute of Health, Common account number (8365532) and in part (STS, DMS, and RNK) from the National Cancer Institute, National Institute of Health, under contract 75N91019D00024.

Competing interests No, there are no competing interests.

Patient consent for publication Not applicable.

Ethics approval Experiments involving mice were conducted in accordance with the protocol approved by the National Cancer Institute Animal Care and Use Committee. Reference number: METB-033-2.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iDs
Julian Burks http://orcid.org/0000-0002-8760-766X
Jay A Berzofsky http://orcid.org/0000-0002-0342-3194

REFERENCES

1 Weir HK, Thompson TD, Stewart SL, *et al*. Cancer Incidence Projections in the United States Between 2015 and 2050. *Prev Chronic Dis* 2021;18:E59.
2 Hayakawa Y, Godfrey DI, Smyth MJ. Alpha-galactosylceramide: potential immunomodulatory activity and future application. *Curr Med Chem* 2004;11:241–52.
3 Kawano T, Cui J, Koezuka Y, *et al*. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997;278:1626–9.
4 Bendelac A, *et al*. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 1995;268:863–5.
5 Smyth MJ, Godfrey DI. NKT cells and tumor immunity—a double-edged sword. *Nat Immunol* 2000;1:459–60.
6 Berzofsky JA, Terabe M. NKT Cells in Tumor Immunity: Opposing Subsets Define a New Immunoregulatory Axis. *J Immunol* 2008;180:3627–35.
7 Terabe M, Berzofsky JA. The role of NKT cells in tumor immunity. *Adv Cancer Res* 2008;101:277–348.
8 Ishikawa A, Motohashi S, Ishikawa E, *et al*. A Phase I Study of α-Galactosylceramide (KRN7000)–Pulsed Dendritic Cells in Patients with Advanced and Recurrent Non–Small Cell Lung Cancer. *Clin Cancer Res* 2005;11:1910–7.
9 Giaccone G, *et al*. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002;8:3702–9.
10 Uchida T, Horiguchi S, Tanaka Y, *et al*. Phase I study of α-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. *Cancer Immunol Immunother* 2008;57:337–45.
11 Waldowska M, Bojarska-Junak A, Roliński J. A brief review of clinical trials involving manipulation of invariant NKT cells as a promising approach in future cancer therapies. *Cent Eur J Immunol* 2017;42:181–95.
12 Toyoda T, Kamata T, Tanaka K, *et al*. Phase II study of α-galactosylceramide-pulsed antigen-presenting cells in patients with advanced or recurrent non-small cell lung cancer. *J Immunother Cancer* 2020;8:e000316.
13 Ishibashi F, Sakairi Y, Iwata T, *et al*. A phase I study of loco-regional immunotherapy by transbronchial injection of α-galactosylceramide-pulsed antigen presenting cells in patients with lung cancer. *Clin Immunol* 2020;215:108457.
14 Sullivan BA, Kronenberg M. Activation or anergy: NKT cells are stunned by -galactosylceramide. *Journal of Clinical Investigation* 2005;115:2328–9.
15 Zhang Y, Springfield R, Chen S, *et al*. α-GalCer and iNKT Cell-Based Cancer Immunotherapy: Realizing the Therapeutic Potentials. *Front Immunol* 2019;10:1126.
16 Stevens DM, Adiseshaiah P, Dasa SSK, *et al*. Application of a Scavenger Receptor A1-Targeted Polymeric Prodrug Platform for Lymphatic Drug Delivery in HIV. *Mol Pharm* 2020;17:3794–812.
17 Draude G, *et al*. Distinct scavenger receptor expression and function in the human CD14(+)/CD16(+) monocyte subset. *Am J Physiol* 1999;276:H1144–9.
18 Apostolov EO, *et al*. *Scavenger Receptors of Endothelial Cells Mediate the Uptake and Cellular Proatherogenic Effects of Carbamylated LDL*. 29. Arterioscler Thromb Vasc Biol, 2009:1622–30.
19 Swart PJ, *et al*. Homing of negatively charged albumins to the lymphatic system: general implications for drug targeting to peripheral tissues and viral reservoirs. *Biochem Pharmacol* 1999;58:1425–35.
20 Zani IA, Stephen SL, Mughal NA, *et al*. Scavenger receptor structure and function in health and disease. *Cells* 2015;4:178–201.
21 Ingersoll MA, Spanbroek R, Lottaz C, *et al*. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 2010;115:e10–9.
22 Freigang S, Landais E, Zadorozhny V, *et al*. Scavenger receptors target glycolipids for natural killer T cell activation. *J Clin Invest* 2012;122:3943–54.
23 Lin KY, *et al*. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;56:21–6.
24 França A, Aggarwal P, Barsov EV, *et al*. Macrophage scavenger receptor A mediates the uptake of gold colloids by macrophages in vitro. *Nanomedicine (Lond)* 2011;6:1175–88.
25 Chandra S, Zhao M, Budelsky A, *et al*. A new mouse strain for the analysis of invariant NKT cell function. *Nat Immunol* 2015;16:799–800.
26 Zwaveling S, Ferreira Mota SC, Nouta J, *et al*. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol* 2002;169:350–8.
27 Becker W, Olkhanud PB, Seishima N, *et al*. Second-generation checkpoint inhibitors and Treg depletion synergize with a mouse cancer vaccine in accordance with tumor microenvironment characterization. *J Immunother Cancer* 2024;12:e008970.
28 Das K, Belnoue E, Rossi M, *et al*. A modular self-adjuvanting cancer vaccine combined with an oncolytic vaccine induces potent antitumor immunity. *Nat Commun* 2021;12:5195.
29 Kaer LV. Natural killer T cells in health and disease. *Front Biosci* 2011;S3:236–51.
30 Liu J, Hill BJ, Darko S, *et al*. The peripheral differentiation of human natural killer T cells. *Immunol Cell Biol* 2019;97:586–96.
31 Ambrosino E, Terabe M, Halder RC, *et al*. Cross-Regulation between Type I and Type II NKT Cells in Regulating Tumor Immunity: A New Immunoregulatory Axis. *J Immunol* 2007;179:5126–36.
32 Wu Z, *et al*. CD3(+)CD4(-)CD8(-) (Double-Negative) T Cells in Inflammation, Immune Disorders and Cancer. *Front Immunol* 2022;13:816005.
33 Bafor EE, Valencia JC, Young HA. Double Negative T Regulatory Cells: An Emerging Paradigm Shift in Reproductive Immune Tolerance? *Front Immunol* 2022;13:886645.
34 Velikkakam T, Gollob KJ, Dutra WO. Double-negative T cells: Setting the stage for disease control or progression. *Immunology* 2022;165:371–85.
35 Li Y, Dong K, Fan X, *et al*. DNT Cell-based Immunotherapy: Progress and Applications. *J Cancer* 2020;11:3717–24.
36 Janakiram NB, Mohammed A, Bryant T, *et al*. Loss of natural killer T cells promotes pancreatic cancer in LSL-Kras$^{G12D/+}$ mice. *Immunology* 2017;152:36–51.
37 Ajina R, Weiner LM. T-Cell Immunity in Pancreatic Cancer. *Pancreas* 2020;49:1014–23.
38 Hartupee C, Nagalo BM, Chabu CY, *et al*. Pancreatic cancer tumor microenvironment is a major therapeutic barrier and target. *Front Immunol* 2024;15:1287459.
39 Yoon JH, Jung YJ, Moon SH. Immunotherapy for pancreatic cancer. *World J Clin Cases* 2021;9:2969–82.
40 Lin H-J, Liu Y, Caroland K, *et al*. Polarization of Cancer-Associated Macrophages Maneuver Neoplastic Attributes of Pancreatic Ductal Adenocarcinoma. *Cancers (Basel)* 2023;15:3507:13:.

41 Cui R, Yue W, Lattime EC, *et al*. Targeting tumor-associated macrophages to combat pancreatic cancer. *Oncotarget* 2016;7:50735–54.
42 Paul S, Chhatar S, Mishra A, *et al*. Natural killer T cell activation increases iNOS+CD206- M1 macrophage and controls the growth of solid tumor. *j immunotherapy cancer* 2019;7:208.
4 Martinez FO, Helming L, Gordon S. Alternative Activation of Macrophages: An Immunologic Functional Perspective. *Annu Rev Immunol* 2009;27:451–83.
44 Jayasingam SD, *et al*. Evaluating the Polarization of Tumor-Associated Macrophages Into M1 and M2 Phenotypes in Human Cancer Tissue: Technicalities and Challenges in Routine Clinical Practice. *Front Oncol* 2019;9:1512.
45 Zhang M, He Y, Sun X, *et al*. A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients. *J Ovarian Res* 2014;7:19.
46 Bai L, Sagiv Y, Liu Y, *et al*. Lysosomal recycling terminates CD1d-mediated presentation of short and polyunsaturated variants of the NKT cell lipid antigen alphaGalCer. *Proc Natl Acad Sci U S A* 2009;106:10254–9.
47 Kain L, Webb B, Anderson BL, *et al*. The identification of the endogenous ligands of natural killer T cells reveals the presence of mammalian α-linked glycosylceramides. *Immunity* 2014;41:543–54.
48 Budhu A, Forgues M, Ye Q-H, *et al*. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* 2006;10:99–111.
49 Mantovani A, Allavena P, Sica A, *et al*. Cancer-related inflammation. *Nature New Biol* 2008;454:436–44.
50 Greten TF, Zhao F, Gamrekelashvili J, *et al*. Human Th17 cells in patients with cancer: Friends or foe? *Oncoimmunology* 2012;1:1438–9.
51 Zhang J-P, Yan J, Xu J, *et al*. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *J Hepatol* 2009;50:980–9.
52 De Monte L, Reni M, Tassi E, *et al*. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *J Exp Med* 2011;208:469–78.
53 Burkholder B, Huang R-Y, Burgess R, *et al*. Tumor-induced perturbations of cytokines and immune cell networks. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2014;1845:182–201.
54 Morita M, Motoki K, Akimoto K, *et al*. Structure-Activity Relationship of .alpha.-Galactosylceramides against B16-Bearing Mice. *J Med Chem* 1995;38:2176–87.
55 Kobayashi E, *et al*. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* 1995;7:529–34.
56 Motohashi S, Ishikawa A, Ishikawa E, *et al*. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 2006;12:6079–86.
57 Parekh VV, Lalani S, Kim S, *et al*. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* 2009;182:2816–26.
58 Parekh VV, Wilson MT, Olivares-Villagómez D, *et al*. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 2005;115:2572–83.
59 O'Konek JJ, Kato S, Takao S, *et al*. β-mannosylceramide activates type I natural killer t cells to induce tumor immunity without inducing long-term functional anergy. *Clin Cancer Res* 2013;19:4404–11.
60 Fujii S, Shimizu K, Kronenberg M, *et al*. Prolonged IFN-γ–producing NKT response induced with α-galactosylceramide–loaded DCs. *Nat Immunol* 2002;3:867–74.
61 Ott PA, Hu Z, Keskin DB, *et al*. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature New Biol* 2017;547:217–21.
62 Keskin DB, Anandappa AJ, Sun J, *et al*. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature New Biol* 2019;565:234–9.