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Formulation of peptide-based cancer vaccines

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CHAPTER 6

Efficient tumor control by vaccination with MHC class I and class II neoepitopes formulated in cationic liposomes

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ABSTRACT

The efficacy of synthetic peptide-based vaccines can be enhanced by formulating them in cationic liposomes. In this study we developed a multi-epitope vaccine consisting of both MHC class I and MHC class II neoepitopes. A total of seven different synthetic peptides encoding neoepitopes derived from seven point- mutated genes of the mouse colorectal cancer model (MC-38) were individually loaded in cationic liposomes. The peptide-loaded liposomes had comparable size distributions (*Z*-average diameter 125 – 175 nm, polydispersity index 0.05 – 0.2) and were positively charged (zeta-potential: 20 – 35 mV). The liposomally formulated long peptides of MHC-I class neoepitopes present in the mutated Adpgk and Rpl-18 genes efficiently activated neoepitope-specific $CD8^+$ T-cells *in vitro* and *in vivo.* Mice vaccinated intradermally with a mixture of liposomal MHC-I neoepitopes and poly(I:C) developed robust neoepitope-specific $CD8^+$ T-cell responses. Vaccination with a liposomal vaccine of two MHC-I neoepitopes (Adpgk & Rpl-18) combined with three recently identified MHC-II neoepitopes (Ddr2, Zmiz1 & Pcdh18) significantly improved neoepitope-specific $CD8^+$ T-cell induction. Furthermore, mice vaccinated with the liposomal combination vaccine of MHC-I and MHC-II neoepitopes were able to control outgrowth of MC-38 tumors. Surviving mice developed long-term tumor immunity, as they were able to reject a lethal rechallenge of MC-38 cells. In conclusion, cationic liposomes are a powerful delivery system for synthetic peptide-based neoepitope vaccines suited for further clinical development.

1. INTRODUCTION

Cancer vaccines are able to induce anti-tumor immune responses resulting in activated T-cells which specifically recognize and subsequently destroy malignant cells (1-5). T-cell mediated tumor killing operates via the recognition of tumor-specific antigens on the cell surface of tumor cells. An important class of highly-specific tumor antigens which can be utilized for personalized cancer vaccines are neoantigens (3-5). These antigens arise from somatic DNA mutations that result in the expression of tumor-exclusive peptide epitopes, the so called neoepitopes, in MHC class I and MHC class II molecules (2, 5, 6). These mutations are induced randomly and are mostly passenger mutations, therefore they are tumor and patient-specific (1, 2).

Processing-dependent, long synthetic peptides (SPs) containing cytotoxic ($CD8^+$) and helper ($CD4^+$) T-cell epitopes have been used as vaccines to induce polyfunctional, antigen-specific T-cells in multiple preclinical and clinical studies (7-13). However, SPs are by themselves poorly immunogenic due to limited dendritic cell (DC) uptake, lack of maturation signals and consequently poor antigen-specific T-cell priming capacity (3, 7, 14). Therefore SPs should be administered with a potent adjuvant and/or adequate delivery system. In our lab, a cationic SP-loaded liposome vaccination platform have been developed to efficiently induce antigen-specific and polyfunctional $CD8^+$ and $CD4^+$ T-cells (7, 15). Liposomes containing an antigenic human papilloma virus (HPV) E7-derived SP that were adjuvanted with the TLR3 ligand poly(I:C) (polyinosinic:polycytidylic acid) were able to induce T-cell responses that fully cleared established HPV-induced tumors in 75% - 100% of the vaccinated mice (7). The SP loaded liposomes were more effective, even at a 65-fold lower dose than the SP emulsified in the clinically used Montanide oil-in-water formulation, indicating the potency of the particulate cationic delivery system (7). Peptide loading has been optimized so that a wide range of physicochemically different antigenic SPs can be formulated rapidly in DOTAP-based liposomes (14). This is an important prerequisite for this formulation procedure, since every patient will have a unique set of neoepitopes from which a personalized vaccine composed of multiple molecularly different SPs can be designed(3, 14).

In this work we describe the application of cationic DOTAP:DOPC liposomes as a neoepitope vaccine delivery system. Both MHC class I and MHC class II neoepitopes of the colorectal mouse tumor MC-38 were individually loaded in cationic liposomes. We made use of the $CD8^+$ T-cell neoepitopes Adpgk and Rpl-18, which have been reported to play an important role in anti-tumor T-cell responses (9, 16, 17). For the MHC-II neoepitopes we made use of three novel neoepitopes Zmiz1, Ddr2 and Pcdh18, which we have recently identified and were shown to induce functional T-helper responses (Hos et al. submitted for publication). All liposomal formulations had comparable physicochemical characteristics, efficiently activated neoepitope-specific $CD8^+$ T-cells *in vitro* and vaccination induced neoepitope-specific $CD8^+$ T-cells *in vivo*. A combined

vaccine of liposomal MHC class I and MHC class II neoepitopes significantly increased tumor-specific $CD8^+$ T-cell priming efficiency and fully protected the majority of the vaccinated mice against a lethal dose of MC-38 tumor cells.

2. MATERIALS & METHODS

2.1. Materials

The synthetic peptides (SPs) comprising MHC-I and MHC-II neoepitope sequences as well the 24-mer OVA24 SP, containing the ovalbumin derived $CD8^+$ epitope SIINFEKL, were synthesized and purified at the peptide facility of the Department of Immunology at the Leiden University Medical Center (LUMC). In every SP of 15 to 23 amino acid residues, the minimal epitope was surrounded by the natural flanking regions (table 1) (8, 9) (Hos et al., submitted for publication). Both lipids, 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Chloroform, methanol and acetonitrile were obtained from Biosolve BV (Valkenswaard, the Netherlands). Ammonium hydroxide 25% (w/v) was purchased from Brocacef BV (Maarssen, the Netherlands) and trifluoracetic acid from Sigma Aldrich (Zwijndrecht, the Netherlands). Vivaspin 2 centrifuge membrane concentrators from Sartorius Stedim Biotech GmbH *(*Göttingen, Germany*)*. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza Verviers, Belgium) supplemented with 8% (v/v) fetal calf serum (Greiner Bio-One, Alphen a/d Rijn, the Netherlands), 50 μM β-mercaptoethanol *(*Sigma-Aldrich, Zwijndrecht, the Netherlands*)* and either 2 mM Glutamax (Thermo Fisher, Bleiswijk, the Netherlands), 80 IU/ml sodium-penicillin G (Astellas, the Netherlands) and 30% supernatant derived from murine GM-CSF producing NIH/3T3 cells, for the D1 dendritic cells (18) or 100 IU/ml penicillin/streptomycin and 2 mM glutamine (Thermo Fisher, Bleiswijk, the Netherlands) for mouse derived T-cells. The fluorescent antibodies CD8α-brilliant violet 605 and CD3-brilliant violet 421 (Biolegend) as wells as the viability staining solution 7-Aaminoactinomycin (7-AAD) were obtained from eBiosciences (Landsmeer, the Netherlands) and BD biosciences (Breda, the Netherlands). Both fluorescently labelled MHC-I tetramers, Adpgk-Phycoerythrin (D^b- A S M T N **M** E L M-PE) and Rpl-18-allophycocyanin (K^b- K I L T F D **R** L-APC), were produced and purified at the peptide facility of the LUMC. Phosphate buffer (PB) was composed of 7.7 mM $Na_2HPO_4 \cdot 2\ H_2O$ and 2.3 mM $NaH_2PO_4 \cdot 2\ H_2O$, pH 7.4 in deionized water with a resistivity of 18 MΩ•cm, produced by a Millipore water purification system (MQ water). The PB buffer was filtered through a 0.22 μm Millex GP PES-filter (Millipore, Ireland) prior to use. Phosphate-buffered saline was purchased from Frensius Kabi (Graz, Austria).

Table 1. Amino acid sequences of the SPs containing the MC-38 neoepitopes. Amino acid residue positions are noted in between brackets and the mutated amino acids are noted in red.

Gene name harbouring neoepitope	Amino acid sequence synthetic peptide	Peptide solvent
Adpgk (305 – 319)	E L A S M T N **M** E L M S S I V	0.04% NH_4OH
Rpl-18 (118 - 132)	G K I L T F D **R** L A L E S P K	0.04% NH_4OH
Reps-1 (38 - 52)	L F R A A Q L **A** N D V V L Q I	0.04% NH_4OH
Dpagt (206 – 220)	S I I V F N L **L** E L E G D Y R	0.04% NH_4OH
Ddr2 (308 - 330)	S E A S E W E P **H** A V Y F P L V L D D V N P S	0.04% NH_4OH
Zmiz 1 (271 - 287)	R P P A D F T Q P A A **S** A A A A A	0.04% NH_4OH
Pcdh 18 (480 - 494)	P **W** A Y I T T V T A T D P D L	$CHCl_3$:MeOH:MQ

2.2. Mice

Female C57BL/6 (H-2^b) mice were purchased from Charles River (L'Arbresle, France) and Janvier labs (Le Genest-Saint-Isle, France) and housed in the LUMC animal facility. Experiments were started when mice were 8 – 12 weeks old and all studies were carried out under the guidelines of the animal ethic committee of the Netherlands.

2.3. Formulation of neoepitope loaded liposomes

The SP loaded liposomes were prepared by using the thin film dehydration-rehydration method as described earlier (14, 15). In brief, both lipids (DOTAP:DOPC) were dissolved in chloroform and mixed in a 1:1 molar ratio in a round bottomed flask followed by rotary evaporation to obtain a dry lipid film. The SP was added either to the dry lipid film as a 1 mg/ml solution in 0.04% (w/v) ammonium hydroxide or prior to rotary evaporation as a 1 mg/ml solution in $CHCl_3$:MeOH:MQ (60:36:4, v/v) (table 1) to the, in chloroform, dissolved lipids (14). The dry lipid film was hydrated, with either the 1 mg/ml SP solution in 0.04% (w/v) NH_4OH or MQ, and briefly vortexed. The resulting dispersion was snap frozen in liquid nitrogen, followed by overnight freeze-drying in a Christ alpha 1–2 freeze-dryer (Osterode, Germany). The lipid-SP cake was rehydrated in three consecutive steps with PB to reach the initial volume. Next, the liposomes were down-sized by sequential extrusion through polycarbonate filters (Nucleopore Milipore, Kent, UK) of 400 and 200 nm (four cycles through each filter). Purification and concentration of the SP loaded liposomes was done by centrifugation (931 G) in Vivaspin 2 centrifugation concentrators (molecular weight cut-off: 300 kDa).

2.4. Physicochemical characterization

The hydrodynamic diameter, polydispersity index (PDI) and zeta-potential were determined by making use of dynamic light scattering (DLS) and laser Doppler electrophoresis. All measurements were performed using a Zetasizer Nano (Malvern Instruments, Malvern Panalytical, UK) and samples were diluted 100-fold in PB prior to the measurement.

2.5. Peptide and lipid recovery

The recovery of SP in the final formulation was determined by reversed phase UPLC-UV analysis (Waters Acquity UPLC® combined with an Acquity UV detector and a Waters BEH C18 – 1.7 mm (2.1 ´ 50 mm) column) (Heuts et al. submitted for publication). An ACN/MQ with 0.1% TFA gradient with a flow rate of 0.5 ml/min was used and the SPs were detected by measuring the UV absorbance at λ = 214 nm. Quantification was done by integration of the area under the curve of the calibration lines by using MassLynx software (Waters, software 4.2.). The final peptide recovery was calculated according to equation 1:

(Eq. 1) $Peptide\ recovery\ (\%) = \left(\frac{Encapsulated\ peptide}{Total\ added\ peptide}\right) x\ 100\%$

Lipid recovery was determined by using the same reversed phase UPLC system coupled to an Waters Evaporative Light Scattering Detector (ELSD). Quantification of both lipids was done by integrating the area under the curve of both the DOTAP and DOPC calibration curves.

(Eq. 2) $(\%) = \left(\frac{Lipid\ content\ final\ formulation}{Total\ added\ lipid}\right) x\ 100\%$

2.6. In vitro antigen presentation

D1 dendritic cells were incubated overnight with either free or liposomal SPs containing the Adpgk or Rpl-18 $CD8^+$ T-cell neoepitopes. The following day T-cell bulks specific for the neoepitopes of Adpgk and Rpl-18 were added to the SP loaded D1 cells (8). The cells were co-cultured during 5 hours in the presence of brefeldin A (2 μg/mL), after which an intracellular cytokine staining was performed with fluorescently labeled antibodies against IFNy and TNFα, CD8 and CD3. During the co-culture cells were cultured in supplemented IMDM in an incubator at 37°C and 5% CO_2. Frequencies of cytokine-producing neoepitope-specific T-cells were determined after sample acquisition with the BD LSR-II flow cytometer (Biolegend, San Diego, USA) followed by data processing with Flowjo V10 (Biolegend).

2.7. vaccination of naïve mice

Mice were immunized intradermally (i.d.) at the tail base with a total volume of 30 μL. SP loaded liposomes or the free SP (1 nmol per peptide) were adjuvanted with poly(I:C) (1 μg/mouse) and administered in PBS. The prime vaccination was given on day 0 and the booster vaccine on day 14.

2.8. Ex vivo analysis of neoepitope-specific CD8+ T-cells

Frequencies of neoepitope-specific $CD8^+$ T-cells were determined in the peripheral blood of vaccinated mice. Blood samples of mice were obtained from the tail vein at different time points during the *in vivo* experiments. Next, the red blood cells in the samples were lysed followed MHC class I tetramer staining specific for the $CD8^+$ T-cell neoepitopes Adpgk and Rpl-18. The staining was performed for 30 minutes at room temperature.

Subsequently, the cells were stained with fluorescently-labeled antibodies specific for CD8 and CD3 for 30 minutes on ice. The live and dead cells were distinguished by making use of the 7-AAD viability staining solution. Data were acquired with the BD LSR-II flow cytometer and analyzed by making use of the Flowjo software.

2.9. Prophylactic and therapeutic vaccination

In the prophylactic vaccination setting mice were vaccinated according to the prime-boost regimen (2.7). For the tumor experiments a dose of 10 nmol per neoepitope was used based on recent literature (16). On day 23 the mice were subcutaneously injected in the flank with $3.5x10^5$ MC-38 tumor cells in 200 mL PBS. In the therapeutic tumor setting mice were injected $3.5x10^5$ MC-38 tumor cells in the flank. When tumors were palpable (day 5 – 7), mice were given a prime vaccination and one week thereafter a booster vaccination. Tumors were measured in three dimensions 2 to 3 times a week by making use of a caliper. When tumor size exceeded 1000 mm^3, the mice were sacrificed for ethical reasons. Throughout the complete experiment the animal wellbeing was monitored.

2.10. Statistical analysis

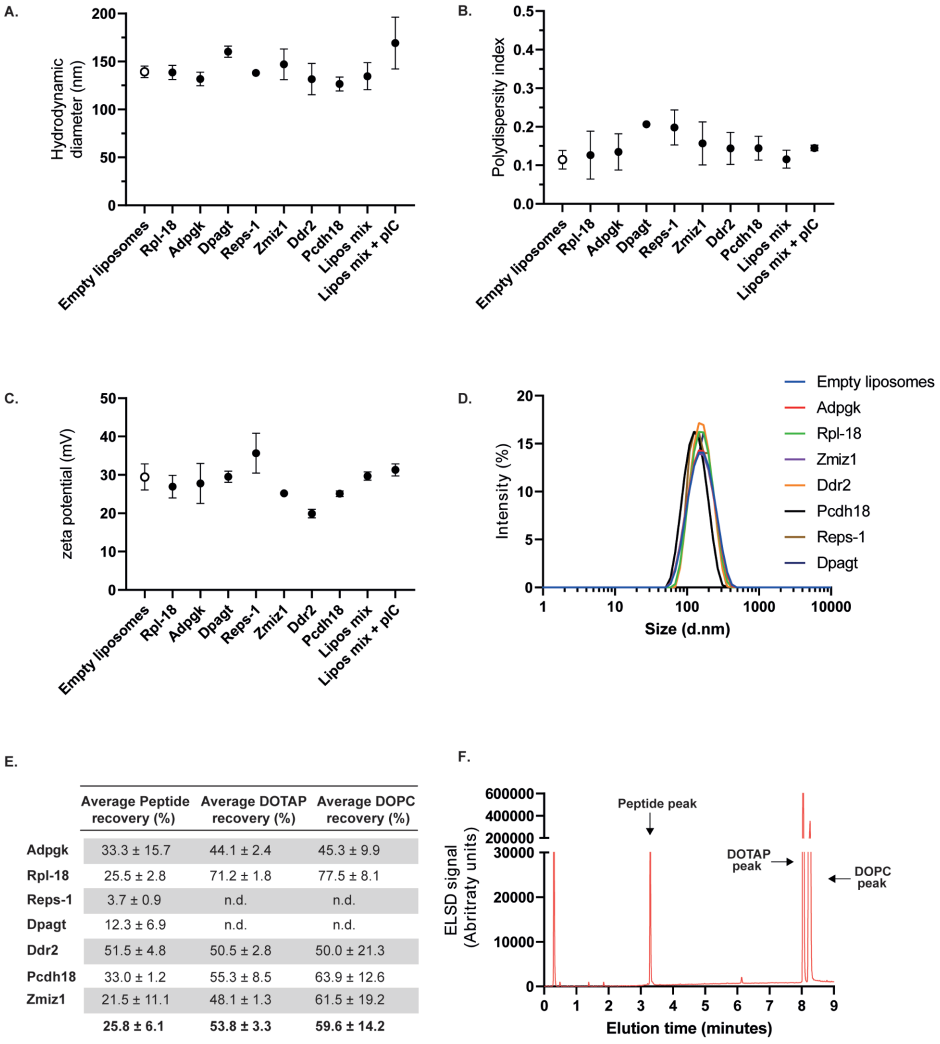
Statistical significance was calculated by making use of the GraphPad Prism 8 software (San Diego, USA). Depending on the experiment the unpaired T-test, analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test or the log-rank (Mandel-Cox) test was used.

3. RESULTS

3.1 Characterization of the synthetic peptide loaded liposomes

Seven different SPs comprising neoepitopes with naturally-flanking sequences from the murine colon carcinoma MC-38 model (see Table 1) were individually loaded in cationic liposomes (DOTAP:DOPC, 1:1 molar ratio). The seven different SPs were individually encapsulated and the corresponding liposomal dispersions had comparable hydrodynamic diameters (Z-average: 125 – 175 nm) and polydispersity (PDI: 0.05 – 0.2), and were positively charged (zeta potential 20 – 35 mV) (Fig. 1A–D). The five different liposomal formulations could be mixed without notable changes in the physicochemical characteristics (Fig. 1 A-C). The physicochemical characteristics of the liposomes did not notably change upon storage during 8 weeks at 4 °C (Supplementary Fig. 1). Peptides and lipids were separated by UPLC, the SPs eluted between 2 and 4 minutes (the exact elution time was SP dependent), DOTAP at 8 minutes and DOPC at 8.25 minutes (Fig. 1F). Allowing efficient separation followed by the quantification of both lipids and the SPs in one UPLC run. The average peptide recovery for the formulated SPs was 25.8 ± 6.1 %, indicating efficient loading of the SPs. Peptide recovery appeared to be dependent on the physicochemical characteristics of the SPs, which is in line with our previous findings with DOTAP:DOPC liposomes (Fig. 1E) (14). Both lipids, DOTAP and DOPC, showed

comparable recoveries of 53.8 ± 3.3 % and 14.2 ± 59.6 %, respectively. Altogether, all seven SPs were encapsulated with reproducible efficiencies.



	Average Peptide recovery (%)	Average DOTAP recovery (%)	Average DOPC recovery (%)
Adpgk	33.3 ± 15.7	44.1 ± 2.4	45.3 ± 9.9
Rpl-18	25.5 ± 2.8	71.2 ± 1.8	77.5 ± 8.1
Reps-1	3.7 ± 0.9	n.d.	n.d.
Dpagt	12.3 ± 6.9	n.d.	n.d.
Ddr2	51.5 ± 4.8	50.5 ± 2.8	50.0 ± 21.3
Pcdh18	33.0 ± 1.2	55.3 ± 8.5	63.9 ± 12.6
Zmiz1	21.5 ± 11.1	48.1 ± 1.3	61.5 ± 19.2
	25.8 ± 6.1	**53.8 ± 3.3**	**59.6 ± 14.2**

Figure 1. Physicochemical characteristics of neoepitope loaded liposomes. **(A)** Z-average **(B)** average polydispersity index and **(C)** average zeta potential of empty liposomes, liposomes loaded with 7 different neoepitopes (four CD8 & three CD4 neoepitopes), the mix of the liposomal formulations and the mix adjuvanted with poly(I:C) (pIC). Z-average and PDI were determined by dynamic light scattering (DLS) and the zeta-potential by laser doppler velocimetry. Data shown as mean *± SD, n=3* **(D)** Representative size distribution plots as determined with DLS. **(E)** Average recovery of peptide, DOTAP and DOPC after formulation, quantified by UPLC-UV and UPLC-ELSD. Data represent mean ± SD, n=3 **(E)** UPLC chromatogram of liposomal Ddr2 with ELSD detection, illustrating the separation of peptide and both lipids. n.d. = not determined.

3.2 Efficient CD8⁺ T-cell activation by liposomally formulated CD8 neoepitopes

The liposomal formulated MHC-I neoepitopes Adpgk and Rpl-18 were first evaluated *in vitro*. Dendritic cells loaded with liposomal SPs efficiently activated two neoepitope-specific CD8⁺ T-cell lines for Adpgk and Rpl-18 respectively, to produce TNF and IFNy (Fig. 2). This shows that the SPs, when encapsulated in cationic liposomes, were efficiently processed and presented by DCs. When the free SPs were admixed with empty liposomes (instead of encapsulated), only low frequencies of neoepitope-specific CD8⁺ T-cell activation were observed (data not shown), which is in line with previous our previous studies with other antigenic peptides (15). DCs incubated with empty liposomes or liposomes loaded with the OVA24 peptide, containing an ovalbumin derived CD8⁺ T-cell epitope, as a negative control for this study, did not activate the neoepitope-specific CD8⁺ T-cells (Fig. 2)(7).

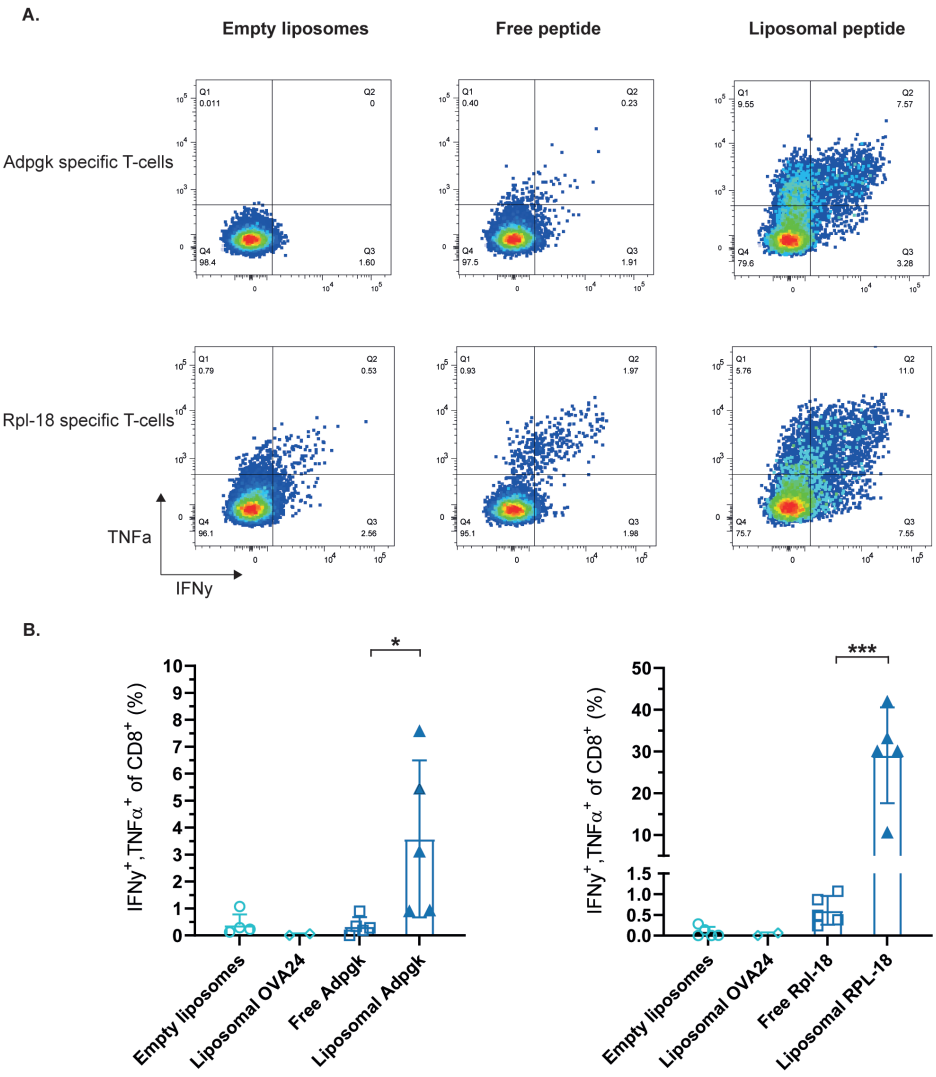


Figure 2. *In vitro* activation of neoepitope specific CD8⁺ T-cells. Murine dendritic cells were loaded with either free or liposomal Adpgk or Rpl-18 peptide, control liposomes loaded with the OVA24 peptide or empty liposomes (2.5 μM per peptide). DCs were co-cultured with Adpgk or Rpl-18 specific CD8⁺ T-cell lines followed by intracellular cytokine staining. CD8⁺ T-cells positive for both INFγ and TNFα were quantified by flow cytometry. **(A)** Representative dot plots of (top) Adpgk and (bottom) Rpl-18 specific CD8⁺ T-cells and **(B)** average of five independent T-cell bulk lines experiments. Data shown as mean ± SD, *p < 0.05, ** p<0.01, one-way Anova with Bonferroni's multiple comparisons test.

To analyze the formulated SPs *in vivo*, naïve mice received a prime vaccination (day 0) followed by a booster vaccination (day 7) with either the free SP or the liposomal SP containing a MHC-I the neoepitope, Adpgk or Rpl-18, (1 nmol) adjuvanted with the TLR-3 ligand poly(I:C) (1 μg/mouse). Induction of neoepitope-specific CD8⁺ T-cells in blood was

3 to 5 fold higher in blood when the SPs were formulated in cationic liposomes compared to free SPs admixed with poly(I:C) (Fig. 3). Robust T-cell priming *ex vivo* was observed for all four neoepitopes and was not influenced by the physicochemical characteristics (e.g., solubility, iso-electric point) of the SP (Fig. 3). The administration of a vaccine composed of a mixture of all four liposomal SPs was comparable to T-cell priming with three out of four single liposomal SP vaccines (Fig. 3). This indicates the feasibility of mixing multiple liposomal formulations for these neoepitopes without compromising T-cell priming capacity. The *in vivo* experiments show that encapsulation of the SPs is essential, as SPs admixed with empty liposomes did not induce high levels of neoepitope specific CD8⁺ T-cells (Fig. 3).

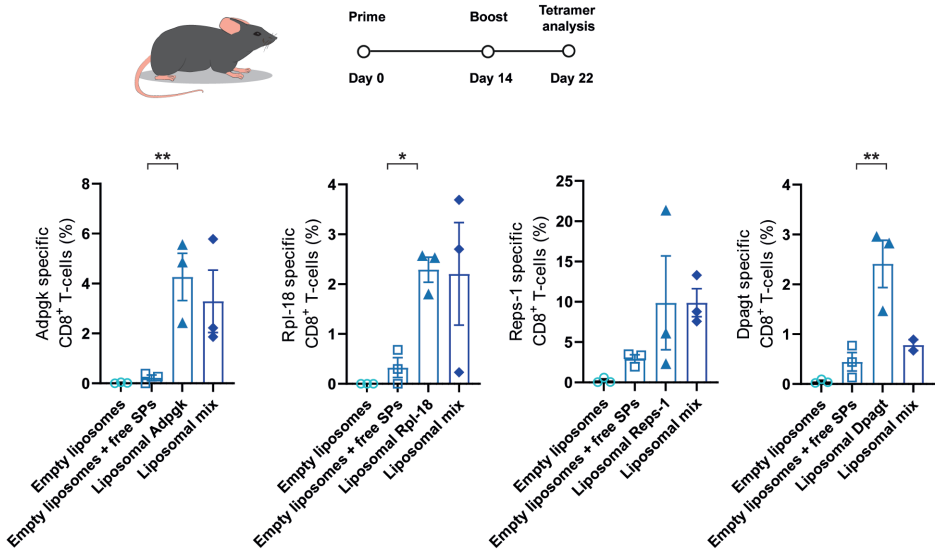


Figure 3. *In vivo* CD8⁺ T cell priming of liposomal neoepitope cocktail. Naïve C57BL/6 received a prime and boost vaccination with either a single MC-38 neoepitope in liposomes (from left to right: Adpgk, Rpl-18, Reps-1 and Dpagt), the liposomal cocktail of all 4 neoepitopes or the free SPs admixed with empty liposomes. Empty liposomes alone served as the negative control. Mice were vaccinated on day 0 and 14 with a dose of 1 nmol per SP adjuvanted and all vaccines were adjuvanted with 1 μg poly(I:C) per mouse. Levels of neoepitope specific CD8⁺ T-cells in C57BL/6 mice were determined in blood on day 22 by flow cytometry. Neoepitope-specific CD8⁺ T-cells were fluorescently stained with in-house developed MHC class I-peptide tetramers. Data represented as mean ± SD, n=3, *p < 0.05, **p<0.01, one-way anova with Bonferroni's multiple comparisons test.

3.3 Liposomal MHC class I and MHC class II neoepitopes administered as a cocktail efficiently enhance neoepitope-specific CD8⁺ T-cell priming

Next, we studied neoepitope-specific T-cell priming by a liposomal vaccine containing CD8⁺ as well as CD4⁺ T-cell neoepitopes. The neoepitopes were administered *in vivo* as a liposomal mixture in one cocktail. In both naïve (Fig 4B) and tumor-bearing mice (Fig 4A,C) which were vaccinated with liposomal CD8⁺ and CD4⁺ T-cell neoepitopes, at least twofold higher frequencies of both Adpgk and Rpl-18 neoepitope-specific CD8⁺

T-cells in blood were observed compared to mice vaccinated with only liposomal CD8⁺ T-cell neoepitopes. Thus, the addition of liposomal CD4⁺ T-cell neoepitopes to CD8⁺ T-cell neoepitopes significantly improved priming of tumor specific CD8⁺ T-cells. The encapsulated CD8⁺ and CD4⁺ SPs outperformed the mixture of free SPs with empty liposomes (Fig. 4B,C), indicating that liposomal encapsulation improved CD4⁺ T-help supported CD8⁺ T-cell priming for these neoepitopes. Based on these results, we analyzed our liposomally formulated SPs in a therapeutic setting in tumor-bearing mice.

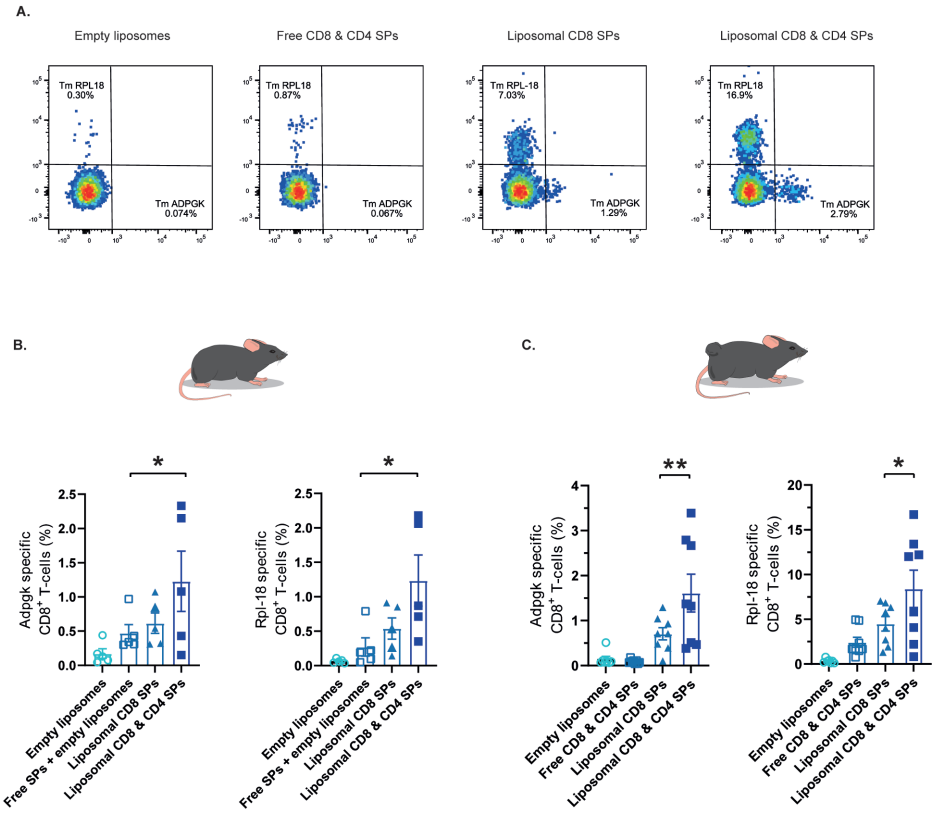


Figure 4. ***In vivo*** **CD8⁺ T-cell priming by liposomal vaccine containing MHC class I (Adpgk & Rpl-18) and MHC class II neoepitopes (Ddr2, Zmiz1 & Pcdh18). (A**) Representative dot plots of neoepitope-specific CD8⁺ T-cell responses for Adpgk or Rpl-18 in blood of vaccinated tumor-bearing mice. Mice were vaccinated with 1 nmol of SP per neoepitope adjuvanted with 1 μg poly(I:C) per mouse. Vaccination with the liposomal cocktail induced the highest levels of Adpgk and Rpl-18 specific CD8⁺ T-cells in **(B)** naïve mice and **(C)** tumor-bearing mice. Tetramer positive CD8⁺ T cells were measured in blood by flow cytometry on day 21. Data shown as mean ± SEM, n= 5 (B) and n=8 (C). *p<0.05, **p<0.01, determined by one-way anova with Bonferroni's multiple comparisons test.

3.4 Liposomal vaccine containing multiple neoepitopes protects mice against MC-38 tumor outgrowth

The functionality of the induced neoepitope-specific T-cells by the liposomal vaccine was evaluated by a MC-38 tumor challenge in vaccinated mice. The animals received the liposomal vaccine composed of five neoepitopes, two MHC class I (Adpgk and Rpl-18) and three MHC class II (Ddr 2, PcdH18, and Zmiz1) neoepitopes, after which the mice received a lethal dose of MC-38 tumor cells. In both the free SP group and the empty liposome group no detectable induction of tumor-specific T-cells in blood was observed after vaccination (Supplementary Fig. 2). The MC-38 challenge resulted in a rapid outgrowth of tumors in these control groups and all mice were sacrificed within 25 days after the challenge (Fig. 5 A-C). Tumor-specific $CD8^+$ T-cells were, as previously observed, effectively induced after vaccination with the liposomal combination vaccine containing both MHC class I and MHC class II neoepitopes (Supplementary Fig. 2). In this group only three out of eight mice developed a tumor with a delayed tumor growth (Fig. 5A). The remaining five mice fully controlled tumor outgrowth and survived the tumor challenge. The surviving mice received a second challenge with MC-38 cells 100 days after the first challenge. No tumor outgrowth was observed and all mice survived the lethal dose of MC-38 cells up until at least 116 days after the booster vaccination (Fig. 5D). This indicates that the mice developed a functional memory response against MC-38 cells**.** Finally, the liposomal vaccine was tested in a therapeutic setting in which tumor-bearing mice were vaccinated. Mice received a booster vaccine 7 days after the first therapeutic vaccination, without additional treatment. The liposomal multi-neoepitope was able to significantly delay the outgrowth of the MC-38 tumors in the vaccinated mice (Fig. 5 E).

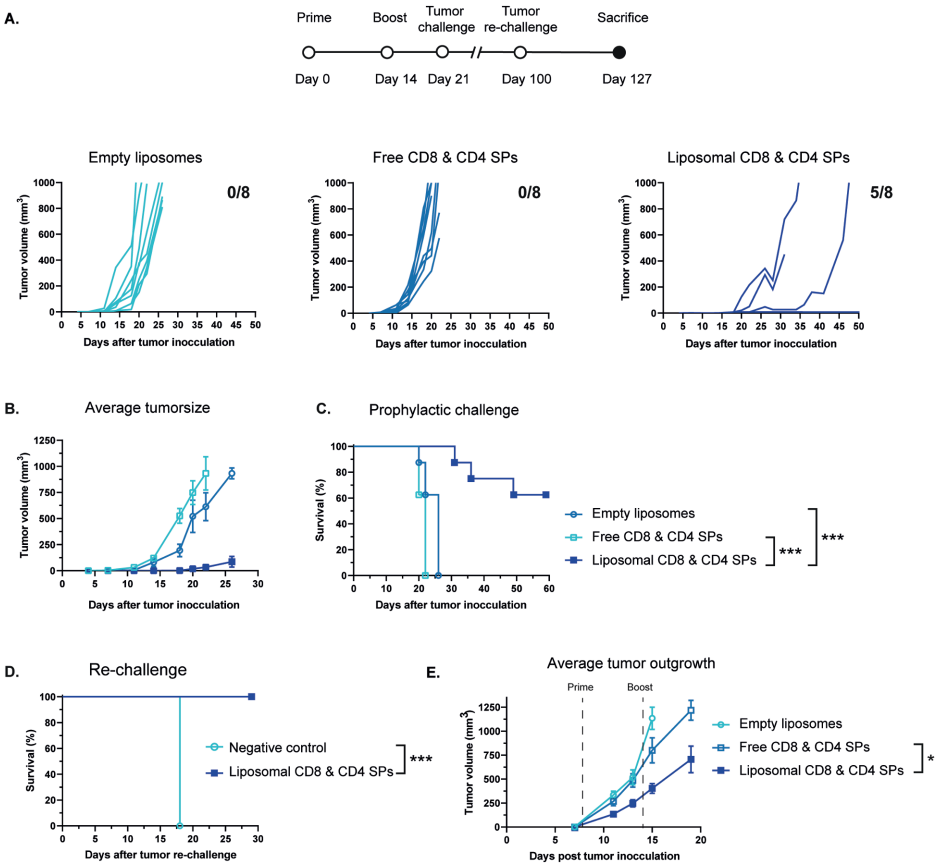


Figure 5. Tumor control by multi-neoepitope vaccination in prophylactic (A-D) and therapeutic setting (E).(A) Individual tumor growth curves per group with the number of surviving mice at the end of the experiment. **(B)** Average tumor sizes per group, data shown as mean ± SEM, n=8. **(C)** Overall survival curve, ***= p<0.001 determined by Log-Rank test and **(D)** Survival curve after tumor re-challenge at day 100 in which naïve mice served as the negative control in this experiment. Mice were vaccinated with 10 nmol of SP per neoepitope adjuvanted with 1 μg poly(I:C) per mouse. **(E)** The average tumor outgrowth after therapeutic vaccination. Mice received $3.5x10^5$ MC-38 cells. When tumors were palpable, mice received a prime vaccination followed by a booster 7 days later. Vaccines contained 10 nmol of each neoepitope and were adjuvanted with 1 μg poly(I:C) per mouse. Data shown as mean ± SEM, n=8. * = P<0.05 determined by an unpaired t-test.

DISCUSSION

In this work we have shown the potency of cationic liposomes as delivery system for neoepitope-based cancer vaccination. Encapsulation of MHC class I neoepitopes in the DOTAP-based liposomes improved priming of tumor-specific $CD8^+$ T-cells *in vitro* and *in vivo*, which was independent of the physicochemical characteristics of the SP. Addition of liposomal MHC class II neoepitopes significantly enhanced $CD8^+$ T-cell priming in naïve mice. The induced neoepitope-specific T-cells were able to inhibit tumor outgrowth of a lethal dose of MC-38 tumor cells and induced long term tumor immunity. The capacity of the liposomal vaccine to accommodate and efficiently deliver both MHC class I and MHC class II neoepitopes *in vivo* shows great potency for developing personalized cancer vaccines. Our data is in line with literature that for optimal anti-tumor immunity both $CD8^+$ and $CD4^+$ T-cells are required (19-22).

For all evaluated neoepitopes in this study, both MHC class I and MHC class II, liposomal encapsulation outperformed the analyzed mixtures of empty liposomes and free SPs. Encapsulation most likely supports efficient uptake of both antigen and adjuvants (cationic lipids and poly(I:C)) by the same dendritic cell, resulting in optimal T-cell priming (3, 4, 23). In comparison, the cationic adjuvant formulation (CAF) is an established adjuvant platform in which the antigen is admixed with the cationic liposomes (24, 25). This is in line with our observations, but we show that for synthetic peptides, antigen encapsulation is crucial for eliciting effective T cell immune responses and tumor control. In addition, differences in liposomal composition, antigen format, adjuvant dosing and administration routes play a role in the potency of different delivery platforms. Currently, further mechanistic studies are on-going in our group to gain more insight into the immunological mechanism of action of SP loaded DOTAP:DOPC liposomes.

Several nano-sized delivery systems have recently been evaluated in the MC-38 model. Similar to our liposomal platform the rationale is to co-deliver neoepitopes and adjuvants in the same APC to induce a cellular anti-tumor immune response (16, 26). Self-assembling nanoparticles based on antigenic SPs conjugated to TLR-7/8a ligands have been evaluated in a prophylactic MC-38 tumor setting. The tumor challenge in vaccinated mice resulted in delayed outgrowth of the MC-38 tumors, however, no overall survival data was reported (26). Lipid based nano-discs loaded with antigenic SPs and the TLR-9 ligand CpG showed tumor regression in a therapeutic MC-38 model when the vaccine was combined with multiple administrations of anti PD-1 treatment (16). The administration of anti-PD-1/PD-L1 blocking antibodies results in the removal of inhibitory signals for tumor-specific T-cells and multiple reports have shown that MC-38 tumors are sensitive for this treatment (27-29). Our therapeutic tumor experiments showed a significant delay in tumor outgrowth in mice vaccinated with the liposomal vaccine but limited long term survival. For instance, liposomal multi-neoepitope vaccination combined with checkpoint therapy could be a potential treatment for tumors with an immune

suppressive microenvironment expressing co-inhibitory molecules (2, 3, 30). Further studies in neoepitope model systems are required to develop optimal immunotherapy regimens including liposomal neoepitope-based vaccines.

An important advantage of the liposomal platform is the simple particle composition and feasibility of production process. The liposomal platform consists of only two lipids, DOTAP and DOPC, a synthetic peptide and the clinically used adjuvant poly(I:C). Previously we have reported the optimization of SP loading which allows loading of a wide range of physicochemical different SPs (14). Hereby the SPs can be rapidly formulated yielding liposomes with a particle size under 200 nm, allowing sterile filtration of the vaccine after production. From a clinical perspective, the production time of personalized cancer vaccines should be as short as possible and GMP compatible. Liposomes have proven to be the most translatable category of nanoparticles from bench to bedside so far (31-33). Therefore, a framework of adequate technologies and regulatory requirements will allow further clinical development of our cationic liposomal platform.

CONCLUSION

In conclusion, we describe a cationic liposomal platform that is suitable for the formulation of personalized SP-based cancer vaccines. Both $CD8^+$ as well as $CD4^+$ T-cell neoepitopes can be individually loaded in DOTAP:DOPC liposomes. Independent of the physicochemical properties of the peptide, all peptide were encapsulated in cationic nanoparticles with comparable physicochemical characteristics. The SP-loaded cationic liposomes efficiently induced priming of functional neoepitope-specific $CD8^+$ T-cells upon intradermal injection which is strongly enhanced in the presence of CD4+ T cell neoepitopes. The liposomal multi-neoepitope vaccine efficiently protected mice against a lethal dose of tumor cells and induced long term tumor protection.

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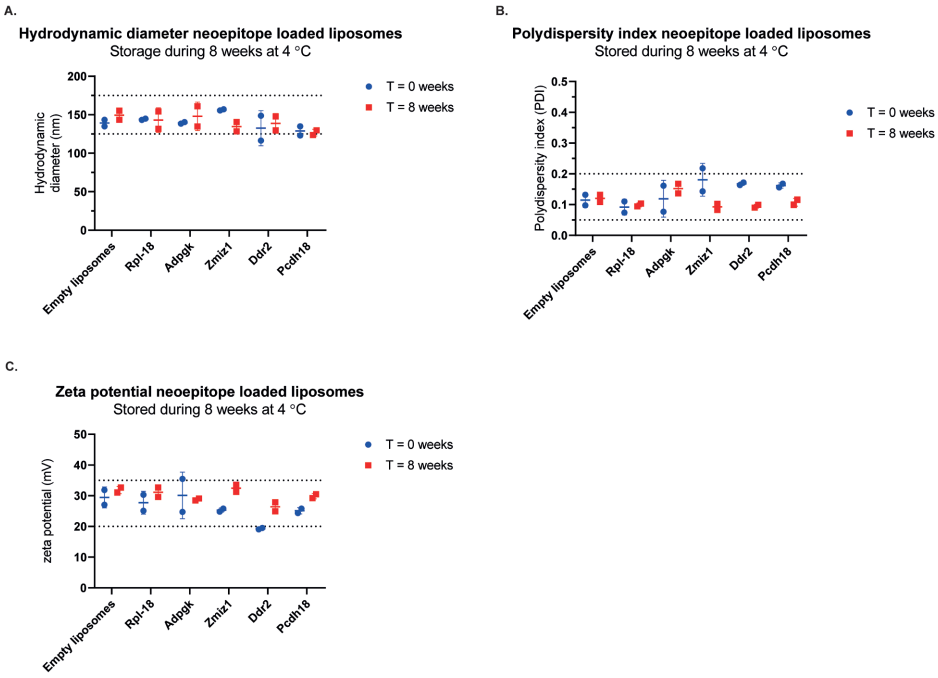
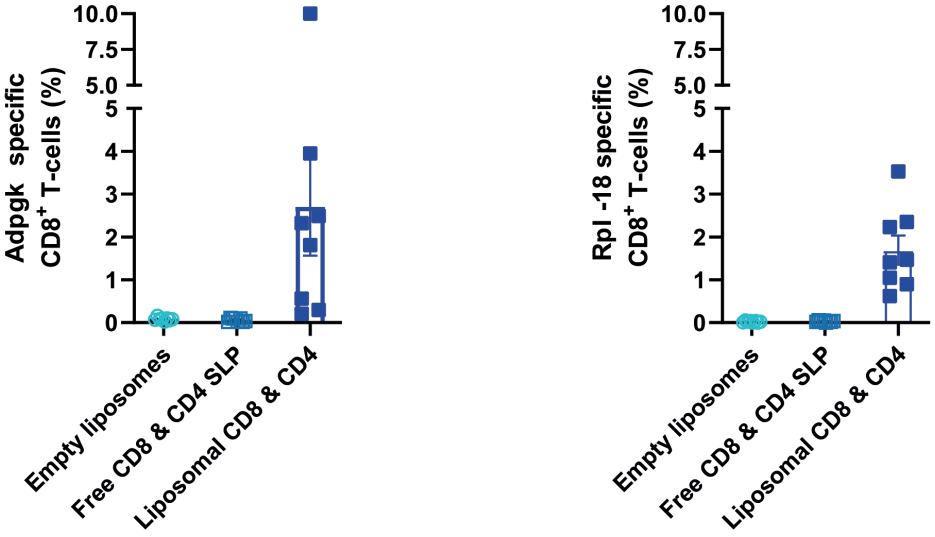
SUPPLEMENTARY FIGURES



Supplementary figure 1. Physicochemical stability of two independently prepared liposome batches containing 5 different neoepitopes after production (T = 0 weeks) and after 8 weeks of storage at 4°C (T = 8 weeks). **(A)** Z-average diameter and **(B)** polydispersity index were determined by dynamic light scattering, and **(C)** zeta potential by laser Doppler velocimetry. Data of two independent batches are displayed (3 measurements per batch) ± range (of two different batches).



Supplementary figure 2. Induction of Adpgk and Rpl-18 specific $CD8^+$ T-cells in blood of mice 2 days prior to the tumor cell inoculation. Data shown as mean ± SEM, n=8.