



Universiteit
Leiden
The Netherlands

Vaccination and targeted therapy using liposomes : opportunities for treatment of atherosclerosis and cancer

Benne, N.

Citation

Benne, N. (2020, September 8). *Vaccination and targeted therapy using liposomes : opportunities for treatment of atherosclerosis and cancer*. Retrieved from <https://hdl.handle.net/1887/136519>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/136519>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/136519> holds various files of this Leiden University dissertation.

Author: Benne, N.

Title: Vaccination and targeted therapy using liposomes : opportunities for treatment of atherosclerosis and cancer

Issue Date: 2020-09-08

6

An Accelerated Prime-Boost Strategy with cdiGMP-Containing Liposomes Induces Large and Long-Lasting CD8 T Cell Responses Against Neoepitopes and Eliminates Tumors in Mice

Authors and affiliations

Naomi Benne^{a1}, Dmitrij Ostroumov^{b1}, Bram Slütter^a, Thomas Wirth^b

^a Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden, the Netherlands

^b Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Hannover, Germany

¹ Authors contributed equally

Manuscript in preparation

Abstract

The immune system is known to be involved in the resolution of cancer and many cells, including T cells, are important for suppressing tumor growth. Antigen-specific vaccination to prime T cells against tumor cells is an attractive treatment strategy for tumors. One possibility to elicit antigen-specific T cells is by injecting dendritic cells (DCs), loaded with neoantigens *ex vivo* and boosting with a mix of an agonistic anti-CD40 antibody (Co), antigen (A) and a TLR3 ligand (T) Poly I:C. While DC-CoAT vaccination does lead to strong immune responses, *ex vivo* priming of DCs is very labor-intensive and expensive, prohibiting widespread use. Using a delivery system to encapsulate a neoantigen, such as nanoparticles, can deliver the antigen to DCs *in situ* in an “off-the-shelf” approach. Here, we present a vaccination strategy using cationic liposomes composed of distearoylphosphatidylcholine (DSPC), dipalmitoyltrimethylammoniumpropane (DPTAP) and cholesterol (CHOL) as a platform for encapsulating neoantigens and TLR ligands. Vaccination effectiveness was tested by priming mice with liposomes and boosting with CoAT (LS-CoAT), and restimulating blood *ex vivo* with neoantigens. The resulting IFN γ production by CD8 $^{+}$ and CD4 $^{+}$ T cells indicated the magnitude of the antigen-specific immune response. We show using this strategy that the STING agonist cyclic-di-guanosine monophosphate (cdiGMP) is the best adjuvant for enhancing antigen-specific CD8 $^{+}$ T cell responses against the neoepitope Adpgk $_{mut}$, and we report extremely high levels (60%) of antigen-specific CD8 $^{+}$ T cells. Moreover, cdiGMP liposomes also induced high and long-lasting CD8 $^{+}$ T-cell responses against another neoepitope, Alg8 $_{mut}$, with around 12% and 4% of CD8 $^{+}$ T cells still recognizing Alg8 $_{mut}$ and Adpgk $_{mut}$, respectively, after 83 days. Furthermore, we evaluated whether co-administering CD8 $^{+}$ and CD4 $^{+}$ epitopes in cdiGMP liposomes would boost antigen-specific CD8 $^{+}$ T cell responses. We found no additional benefit of the CD4 $^{+}$ epitope MTAG85B. Finally, LS-CoAT with Adpgk $_{mut}$ was tested in a tumor mouse model. Mice were inoculated with the MC-38 cells and received LS-priming after 4 days of tumor growth, followed by CoAT-boosting one week later. We observed high antigen-specific responses to the antigen combined with tumor regression within 30 days and 100% survival. In conclusion, we show that our LS-CoAT strategy can induce extremely high frequencies of antigen-specific CD8 $^{+}$ T cells to at least two neoepitopes in a short time and that these T cells are very long-lasting. This allowed for rapid and complete tumor elimination in an MC-38 mouse tumor model.

Introduction

The immune system is known to be involved in the resolution of cancer. Many cells, including T cells, are important for suppressing tumor growth from the early stages of tumor development¹. While the main focus for tumor suppression is CD8⁺ T cells², CD4⁺ T cells have also been shown to be important³. Antigen-specific vaccination to prime CD8⁺ and CD4⁺ T cells against tumor cells is an attractive treatment strategy for tumors. In order for T cells to distinguish healthy cells from tumor cells, T cells detect surface expression of tumor antigens⁴. These can be tumor-associated antigens, which are epitopes that are expressed on healthy cells but over-expressed on tumor cells. However, vaccinating against these epitopes can lead to unwanted side effects when T cells attack healthy tissues⁵. The second class of tumor antigens is neoantigens. These result from non-synonymous somatic mutations that encode new peptides that can be presented on the cell surface. Since they are not expressed in healthy cells, they are highly immunogenic and a safer target for vaccination⁶. T cells are activated by antigen-presenting cells (APCs) that have taken up and processed an antigen. The antigen is presented on major histocompatibility complex (MHC)-I or MHC-II molecules to CD8⁺ and CD4⁺ T cells, respectively, and together with a costimulatory signal directs T cell differentiation and activation⁷. A strategy to elicit antigen-specific T cells is by injecting dendritic cells (DCs), a type of APC, loaded with neoantigens. Hereby, DCs are isolated and pulsed with antigens *ex vivo*, and subsequently reinjected⁸. These DCs can then present the tumor-specific epitopes to T cells and prime them to destroy the cancer cells. However, this response is often quite weak without the use of an adjuvant, so DCs are typically primed *ex vivo*⁹. APCs generally become activated upon recognition of danger signals via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and stimulator of interferon (IFN) genes (STING)¹⁰. Therefore, after priming with antigen-loaded DCs, boosting with a mix of an agonistic anti-CD40 antibody (Co), antigen (A) and a TLR ligand (T) Poly I:C, greatly enhances antigen-specific CD8⁺ and CD4⁺ T cell responses¹¹. Furthermore, this CoAT vaccination strategy could control tumor growth in an MC-38 tumor mouse model¹². While DC-CoAT vaccination does lead to strong immune responses, *ex vivo* priming of DCs is very labor-intensive and expensive, prohibiting widespread use⁸. Using a delivery system to encapsulate a neoantigen, such as nanoparticles, can deliver the antigen to DCs *in situ* in a simpler way¹³. Liposomes are a type of nanoparticle composed of phospholipids which form a bilayer around an aqueous core¹⁴. A major advantage of liposomes is that they can encapsulate one or multiple antigens and adjuvants, such as TLR ligands^{15,16} or STING agonists¹⁷, to further enhance the immune response. We and others have shown that cationic liposomes by themselves or formulated with a TLR ligand such as Poly I:C^{16,18-20} can induce strong antigen-specific pro-inflammatory CD8⁺ and CD4⁺ T cells responses. Furthermore, it is evident that liposomes with high rigidity can further enhance these responses²¹⁻²³.

We hypothesize that cationic liposomes can replace DC priming in DC-CoAT vaccination, providing a more broadly applicable vaccination strategy against neoepitopes. Here, we present a vaccination strategy using cationic liposomes composed of distearoylphosphatidylcholine (DSPC), dipalmitoyltrimethylammoniumpropane (DPTAP) and cholesterol (CHOL) as a platform for encapsulating neoantigens and TLR ligands. We show that the STING agonist cyclic-di-guanosine monophosphate (cdiGMP) is the best adjuvant for enhancing antigen-specific CD8⁺ T cell responses using the

neoepitope Adpgk_{mut} in a liposome-CoAT (LS-CoAT) vaccination strategy. In a head-to-head comparison, cdiGMP liposomes performed as well as DCs. Furthermore, cdiGMP liposomes also induced high and long-lasting CD8⁺ T-cell responses against another neoepitope, Alg8_{mut}. Moreover, we evaluated whether co-administering CD8⁺ and CD4⁺ epitopes in cdiGMP liposomes would boost antigen-specific CD8⁺ T cell responses. Finally, LS-CoAT with Adpgk_{mut} eliminated established MC-38 tumors in mice.

Methods

Liposome preparation

Cationic liposomes were prepared by the thin film dehydration-rehydration method, as described before²³. Briefly, DSPC, DPTAP, and CHOL were dissolved in chloroform and mixed in a 50 mL round-bottom flask at a molar ratio of 4:1:2, to a final lipid concentration of 10 mg/mL. The chloroform was evaporated under vacuum at 40°C for 30 min using a rotary evaporator (Rotavapor R-210, Büchi, Switzerland). The resulting lipid film was rehydrated with 1 mg peptide (Adpgk_{mut}: ASMTNMELM, Alg8_{mut}: ITYTWTRL, Adpgk_{mut} long: TGIPVHLELASMTNMELMSSIVH, MTAG85B: KFQDAYNAAGGHNAVF, Adpgk_{mut} long/MTAG85B fused: TGIPVHLELASMTNMELMSSIVHKFQDAYNAAGGHNAVF, all purchased from ProlImmune, Oxford, UK) dissolved in 1 mL 1:1 ACN/MilliQ (v/v) + 0.04% NH₄OH (w/v), or 1 mL 1:1 ACN/MilliQ (v/v) + 0.04% NH₄OH (w/v) for empty liposomes. To incorporate cdiGMP, 150 µg cdiGMP was dissolved in methanol and added to the lipid film before evaporation. For liposomes containing monophosphoryl lipid A (MPLA), 80 nmol MPLA was dissolved and added to the lipid mixture before evaporation. For Poly I:C liposomes, 99.5 µg Poly I:C and 0.5 µg Poly I:C-Rhodamine were dissolved in MilliQ at a concentration of 1 mg/mL and added dropwise to the lipid/peptide or lipid/MilliQ mix. After rehydration, glass beads were added and the suspension was homogenized for 30 minutes at 60°C. The dispersion was subsequently snap-frozen in liquid nitrogen and freeze-dried overnight (Christ alpha 1–2 freeze-dryer, Osterode, Germany). The resulting lipid cake was slowly rehydrated using 10 mM sodium phosphate buffer (PB), pH 7.4 at 60°C. Two volumes of 500 µL and one volume of 1,000 µL PB were successively added, with intervals of 30 min between each addition. The mixture was vortexed well between each hydration step, and the resulting dispersion was left to rehydrate for at least 1 hour. Sizing of the liposomes was performed using high-pressure extrusion at 60°C (LIPEX Extruder, Northern Lipids Inc., Canada) by passing the dispersion four times through stacked 400-nm and 200-nm pore size polycarbonate track-etched membranes (Whatman® Nuclepore™, GE Healthcare, Little Chalfont, UK). To separate non-encapsulated peptide and/or adjuvants, the dispersion was washed several times using a Vivaspin 2 centrifuge membrane concentrator (MWCO 300 kDa, Sartorius, Göttingen, Germany) by centrifugation at 1500 rpm and 4°C.

Liposome characterization

The Z-average diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS) using a NanoZS Zetasizer (Malvern Ltd., Malvern, UK). The same instrument was used to measure ζ-potential by laser Doppler electrophoresis. The liposomes were diluted 100-fold in PB to a total volume of 1 mL for measurement. To determine the concentration of encapsulated peptide and cdiGMP, samples were analyzed by reversed-phase UPLC (Waters ACQUITY UPLC, Waters, MA, USA). 20 µL of the liposome dispersion was dissolved in 180 µL methanol. 10 µL of this was injected into a 1.7 µm BEH C18 column (2.1 × 50 mm, Waters ACQUITY UPLC, Waters, MA, USA). The column temperature was 40°C. The mobile phases were Milli-Q water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). For separation, the mobile phases were applied in a linear gradient from 20% to 100% solvent B over 6 minutes at a flow rate of 0.25 mL/min. Peptides were detected by absorbance at 220 nm using an ACQUITY

UPLC TUV detector (Waters ACQUITY UPLC, Waters, MA, USA). cdiGMP was detected by absorbance at 254 nm. Poly I:C content was determined by fluorescence (Ex: 546 nm, Em: 576 nm) using a TECAN plate-reader (Salzburg, Austria). MPLA integrates readily into the lipid bilayer, so loading was assumed to be 100%²⁴.

Animals

6- to 8-week old C57BL6/J mice were bred at the Animal Care Facility of the Hannover Medical School, Germany. During experiments, mice were housed in the Animal Care Facility of the Hannover Medical School according to the required biosafety level. All animal experiments were performed according to German legal guidelines for animal care and experimentation (TierSchG) and were approved by institutional and governmental boards (LAVES).

DC-CoAT and LS-CoAT vaccinations

For DC-CoAT vaccination, splenic DCs were isolated from donor mice that received a subcutaneous injection of 5×10^6 B16 cells expressing Flt3L resuspended in 100 μ L PBS, as described previously²⁵. After DC harvest, cells were matured in vitro with LPS (0.5 μ g/ml) and incubated in the presence of peptides (2 μ g/ml) for approximately 2 hours. For vaccination, 1×10^6 DCs resuspended in 100 μ L PBS were injected intravenously into the mice. CoAT immunizations were performed as described previously¹². Briefly, individual mice received intravenous injection of 100 μ g soluble peptides (thinkpeptides, Oxford, UK), 200 μ g Poly I:C (Invivogen, San Diego, CA, USA) and 100 μ g of agonistic anti-CD40 antibody (clone 1C10, hybridoma kindly provided by Frances Lund, Department of Microbiology, University of Alabama at Birmingham, AL, USA). For LS-CoAT vaccinations, mice were injected intravenously with 100 μ L of 10 nmol peptide encapsulated in liposomes. CoAT boosting was the same as described above for both particles.

Quantification and phenotypic analysis of antigen-specific T-cells.

The magnitude of the epitope-specific CD8⁺ T-cell response was determined by IFN γ staining as described before²⁶. For antibody or tetramer staining, a small volume (~50 μ L) of blood was collected via submandibular bleeding. Extracellular antibody and intracellular cytokine staining were performed according to standard protocols. Intracellular cytokine staining for IFN γ was performed in total splenocytes and tumors of sacrificed mice. Mice were sacrificed and subjected to cardiac perfusion with PBS (Gibco, Germany) prior to harvesting the spleens. In order to obtain single-cell suspensions spleens were forced through 40 μ m cell strainers (Falcon, Colorado Springs, CO, USA) prior to extracellular antibody and intracellular cytokine staining. For preparation of cells from subcutaneous tumors, mice were euthanized and tumors were removed. Then tumor tissue was mechanically separated using a scalpel and afterwards enzymatically digested for 30 min at 37°C and 200 rpm. The digestion medium contained RPMI + GlutaMAX (Gibco), supplemented with 10% fetal calf serum (FCS, Biowest), 1% Penicillin-Streptomycin (100 U/ml Penicillin, 100 μ g/ml Streptomycin, Biochrom), Hyaluronidase (0.2 mg/ml), DNaseI (0.2 mg/ml), collagenase type IA (0.2 mg/ml), and collagenase type IV (0.2 mg/ml) (Sigma-Aldrich). Digestion was arrested by addition of RPMI + GlutaMAX supplemented with 10% FCS and 1% Penicillin-Streptomycin, and cell suspensions were passed through 40 μ m cell strainers and washed once with RPMI + GlutaMAX supplemented with 10%

FCS and 1% Penicillin-Streptomycin prior to extracellular antibody and intracellular cytokine staining. Antibodies used for FACS analysis were CD8 (53-6.7), CD4 (GK1.5), IFN γ (XMG1.2), CD90.2 (53-2.1) (eBioscience, San Diego, CA, USA), TruStain FcX™ (anti-mouse CD16/32) (Biolegend, San Diego, CA, USA), and appropriate isotype controls.

Induction of subcutaneous tumors

Subcutaneous tumors in C57BL6/J mice were generated by subcutaneous injection with 5×10^6 MC-38 cells per mouse resuspended in 100 μ L PBS. MC-38 is a colon carcinoma cell line (kindly provided by Michael Neumaier, University of Mannheim, Germany). The growth of subcutaneous tumors was monitored three times a week and mice were sacrificed when tumor diameter exceeded 1.5 cm³ or when tumors exulcerated considerably. Tumor volume was determined by the formula $0.5 \times \text{length (mm)} \times (\text{width (mm)})^2$, which was published previously²⁷.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v8. All experiments were repeated at least once to ensure reproducibility. Asterisks indicate levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

cdiGMP is a superior liposomal adjuvant for vaccination in combination with CoAT

DSPC:DPTAP:CHOL (mol ratio 4:1:2) encapsulating the neoepitope Adpgk_{mut} were prepared using the dehydration-rehydration method. Without adjuvant, liposomes are around 200 nm in size and cationic. The loading efficiency of Adpgk_{mut} was 40% (Table 1). Inclusion of poly I:C increased the size of the liposomes and decreased the ζ -potential to 27 mV. The inclusion of MPLA resulted in a small reduction in size to around 180 nm. Liposomes adjuvanted with cdiGMP did not alter in size compared to non-adjuvanted liposomes, but ζ -potential decreased slightly to 32 mV. This formulation showed a 50% reduction in antigen loading efficiency (Table 1), suggesting competition of the antigen and adjuvant. To test whether liposomes encapsulating the neoepitope Adpgk_{mut} and different adjuvants could induce antigen-specific CD8⁺ T cell responses, mice were injected with Adpgk_{mut} liposomes (10 nmol peptide concentration), either without adjuvant or with Poly I:C, MPLA or cdiGMP. As controls, mice received empty liposomes or free Adpgk_{mut} (10 nmol). 6 days after injection, blood was collected from mice and restimulated with Adpgk_{mut}, and IFN γ production by CD8⁺ T cells was measured. 7 days after prime, all mice received the CoAT boost, and 7 days after boost IFN γ production by CD8⁺ T cells was determined (Figure 1A). Only mice receiving liposomes with cdiGMP showed measurable IFN γ responses before CoAT (Figure 1B). The CoAT boost enhanced IFN γ responses for all groups except the free peptide group, showing liposomal delivery significantly enhances the effect of CoAT boosting. The levels of IFN γ production are in fact among the highest ever reported for this peptide^{12,28,29}. All liposomal formulations with peptide, except for Poly I:C liposomes, showed significantly higher production of IFN γ compared to empty liposomes or free peptide (Figure 1C). There was no significant difference between adjuvant-free liposomes, MPLA liposomes, and cdiGMP liposomes. Importantly, mice showed no signs of distress due to vaccination. Since cdiGMP liposomes already elicited a response after prime and outperformed the other adjuvanted liposomes, we decided to continue with this formulation in combination with CoAT boosting, hereafter referred to as LS-CoAT.

Table 1: Physicochemical properties of DSPC:DPTAP:CHOL liposomes encapsulating Adpgk_{mut} and different adjuvants.

Adjuvant	Size (nm)	PDI	Z-potential (mV)	LE Adpgk _{mut} (%)	LE adjuvant (%)
None	209.2	0.158	39.4	40.8	-
Poly I:C	394.6	0.264	27.2	39.5	8.8
MPLA	178.7	0.281	36.6	57.8	Assumed 100%
cdiGMP	210.2	0.119	31.6	19.2	39.3

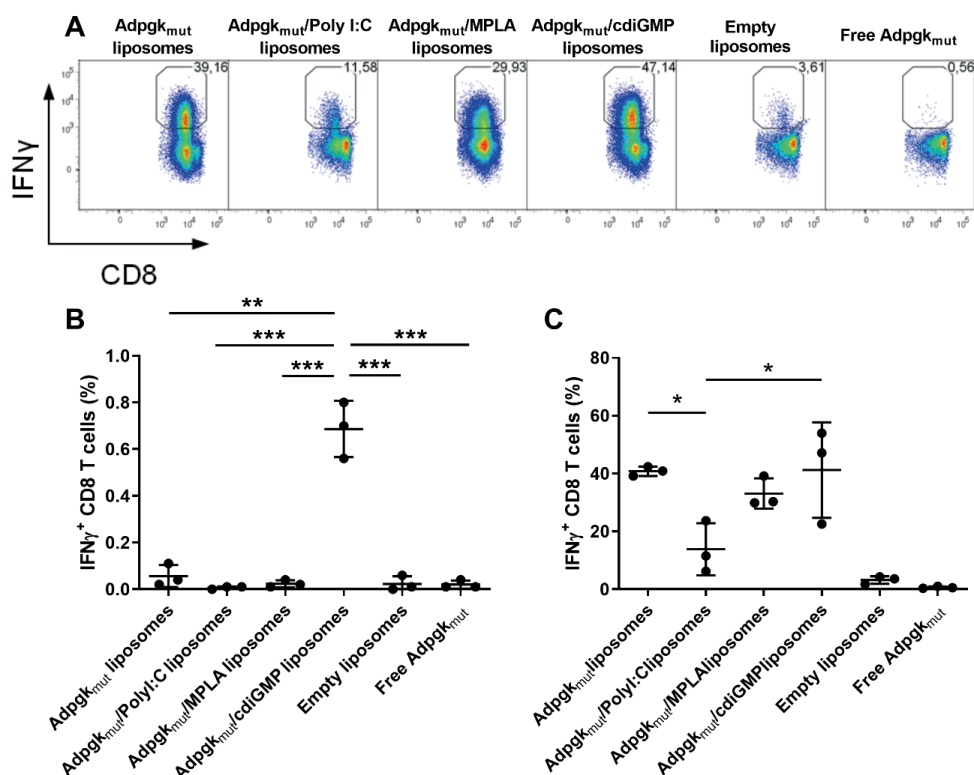


Figure 1: Effect of liposomal adjuvant on Adpgk $_{mut}$ -specific CD8 $^{+}$ T cell responses *in vivo*. Mice were injected with Adpgk $_{mut}$ -containing liposomes (10 nmol peptide concentration), either without adjuvant or with Poly I:C, MPLA or cdiGMP. As controls, some mice received empty liposomes or free Adpgk $_{mut}$ (10 nmol). All mice received a CoAT boost on day 7. (A) Representative FACS plots of blood restimulated with Adpgk $_{mut}$ 7 days after boost. IFN γ production by CD8 $^{+}$ T cells upon restimulation with Adpgk $_{mut}$ was measured in the blood (B) 6 days after prime and (C) 7 days after boost. Graphs show mean $\pm$ SD, *p < 0.05, **p < 0.01, ***p < 0.001 determined by one-way ANOVA with Tukey's multiple comparisons test.

cdiGMP liposome vaccination performs as well as DC-CoAT vaccination

As stated above, DC-CoAT vaccination is currently one of the best methods for inducing high numbers of antigen-specific CD8 $^{+}$ T cells. To test whether cdiGMP liposomes are a viable replacement for *ex vivo* primed DCs, we performed a direct comparison between cdiGMP liposome-CoAT and DC-CoAT vaccination. Non-adjuvanted DPTAP liposomes were used as control. Mice were injected with the different groups, and 6 days after prime, blood was collected and restimulated with Adpgk $_{mut}$ to measure IFN γ production by CD8 $^{+}$ T cells. We observed a significantly higher production of IFN γ in mice injected with cdiGMP liposomes compared to the other groups (Figure 2A). One day later, all mice received a CoAT boost and the immune response was measured in blood again after 7 days. Here, we saw that DC-CoAT and cdiGMP LS-CoAT significantly increased IFN γ production in mice compared to non-adjuvanted liposomes (Figure 2B).

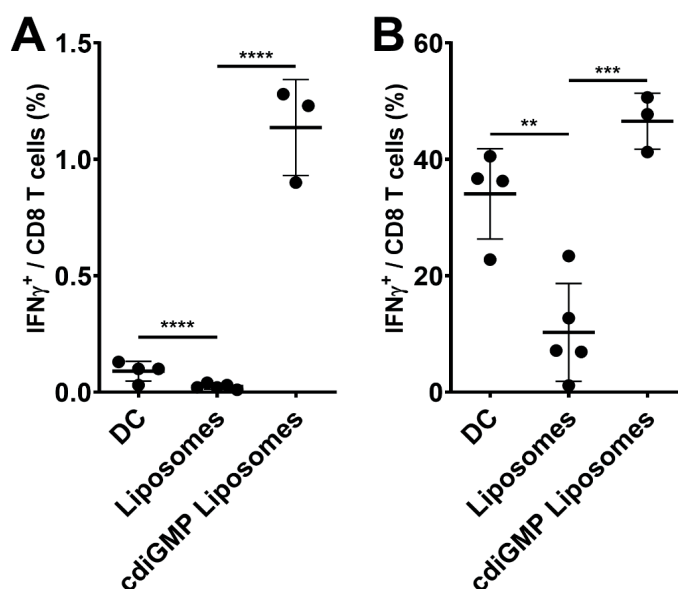


Figure 2: Comparison of liposomal formulations to DC-CoAT vaccination *in vivo*. Mice were injected with Adpgk_{mut}-pulsed DCs, Adpgk_{mut} liposomes, or Adpgk_{mut} cdiGMP liposomes. All mice received a CoAT boost on day 7. IFN γ production by CD8⁺ T cells upon restimulation with Adpgk_{mut} was measured in the blood (A) 6 days after prime, and (B) 7 days after boost. Graphs show mean $\pm$ SD, **p < 0.01, ***p < 0.001, ****p < 0.0001 determined by one-way ANOVA with Tukey's multiple comparisons test.

LS-CoAT induces high, long-lasting antigen-specific CD8⁺ T cell responses against Adpgk_{mut} and Alg8_{mut}

Next, we aimed to assess whether the LS-CoAT could also induce responses against other neopeptides, such as Alg8_{mut}, and how long these responses would be sustained. For good anti-tumor responses, it is important that CD8⁺ T cells persist over a long time. Furthermore, different tumor cells will have different antigens, so it is important to test the LS-CoAT approach for another neopeptide. We prepared Alg8_{mut} liposomes, Adpgk_{mut} liposomes, Alg8_{mut}/cdiGMP liposomes, and Adpgk_{mut}/cdiGMP liposomes (Table 2), and injected these in mice. 6 days after prime, blood was collected and restimulated with either Alg8_{mut} or Adpgk_{mut}. Again, we observed a measurable antigen-specific IFN γ response in CD8⁺ T cells, only for mice that received cdiGMP-adjuvanted liposomes (Figure 3A). 7 days after prime mice were boosted with CoAT, and responses were measured again after 7 days. For both non-adjuvanted liposomes, the IFN γ response was around 6%. Incorporation of cdiGMP enhanced this response significantly to around 30% for Alg8_{mut}, and 45% for Adpgk_{mut} (Figure 3B). This response was very long-lasting, and after 83 days (the last time-point for the experiment), we measured 12% IFN γ ⁺ CD8⁺ T cells for mice injected with Alg8_{mut}/cdiGMP liposomes, and 4% for Adpgk_{mut}/cdiGMP liposomes (Figure 3C). Non-adjuvanted liposomes showed shorter-lasting responses and lower peak numbers of around 1% on day 7 (Figure 3C).

Table 2: Physicochemical properties of DSPC:DPTAP:CHOL liposomes encapsulating Alg8_{mut} with and without cdiGMP.

Liposome	Size (nm)	PDI	Z-potential (mV)	LE Alg8 _{mut} (%)	LE cdiGMP (%)
Alg8 _{mut}	178.1	0.078	38.6	60.0	-
Alg8 _{mut} /cdiGMP	166.8	0.081	40	53.7	67.6

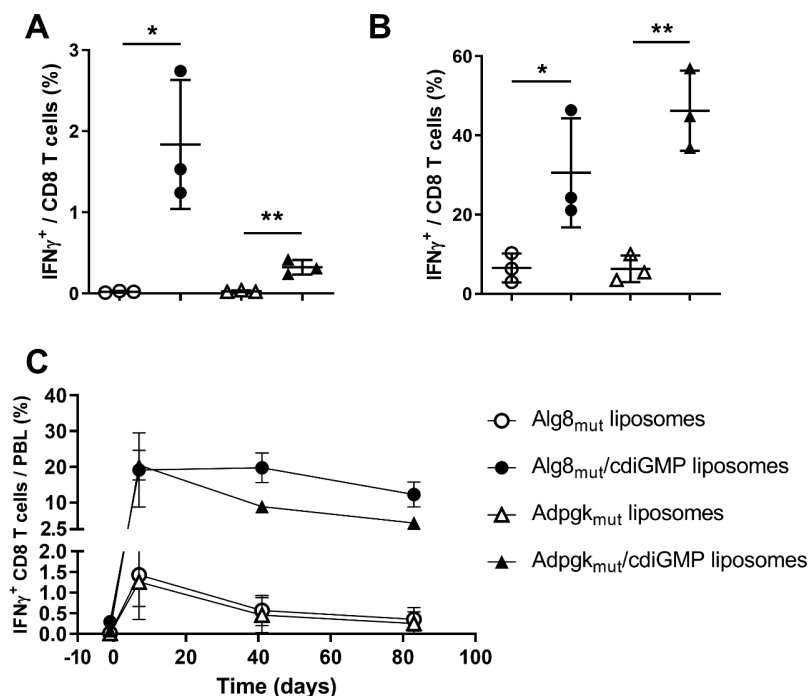


Figure 3: Long-lasting antigen-specific responses after LS/CoAT with Adpgk_{mut} and Alg8_{mut}. Mice were injected with Alg8_{mut}- or Adpgk_{mut}-containing liposomes (10 nmol peptide, circles, and triangles, respectively), with or without cdiGMP (black and open symbols, respectively). All mice received a CoAT boost after 7 days. IFN γ production by CD8⁺ T cells upon restimulation with Alg8_{mut} or Adpgk_{mut} was measured in the blood (A) 6 days after prime, and (B) 7 days after boost. (C) Antigen-specific CD8⁺ T cells in the PBLs were followed over a period of 83 days. Graphs show mean $\pm$ SD, *p < 0.05, **p < 0.01, determined by one-way ANOVA with Tukey's multiple comparisons test.

Mixing Adpgk_{mut} and MTAG85B liposomes enhance CD8⁺ T cell responses in LS-CoAT vaccination

CD4⁺ T cell help is pivotal for CD8⁺ T cell expansion and acquisition of effector function, so the addition of MHCII restricted peptides may improve the CD8⁺ T-cell response³. Moreover, long peptides (LP) have been shown to exhibit stronger CD8⁺ T cell activation than short peptides (SP), due to the need for LP to undergo endosomal processing³⁰. Therefore, we tested whether exchanging the Adpgk_{mut} SP for LP would enhance the immune response. We also assessed whether mixing Adpgk_{mut} SP or LP cdiGMP liposomes with cdiGMP liposomes encapsulating the CD4⁺ epitope MTAG85B,

or cdiGMP liposomes containing a fused Adpgk_{mut} LP/MTAG85B peptide would increase the CD8⁺ T cell response (Table 3). Mice were primed with either Adpgk_{mut} SP/cdiGMP liposomes, Adpgk_{mut} LP/cdiGMP liposomes, a mixture of Adpgk_{mut} SP/cdiGMP liposomes and MTAG85B/cdiGMP liposomes, a mixture of Adpgk_{mut} LP/cdiGMP liposomes or Adpgk_{mut} LP/MTAG85B fusion peptide/cdiGMP liposomes. In all cases, peptide dose was 10 nmol. 6 days after priming, IFN γ production by CD8⁺ T cells in the blood was measured after restimulation with Adpgk_{mut} SP (Figure 4A), and Adpgk_{mut} LP (Figure 4B) for mice receiving Adpgk_{mut} LP. IFN γ production by CD4⁺ T cells was analyzed after MTAG85B restimulation in the mice receiving MTAG85B (Figure 4C). 7 days after priming mice were injected with CoAT with the corresponding peptide(s), and 7 days later, IFN γ production in CD8⁺ and CD4⁺ T cells was measured again in the blood (Figure 4D, E, and F). To measure long-term CD4⁺ and CD8⁺ T cell stimulation, the same responses were measured after 92 days (Figure 4G, H, and I). Before boosting, IFN γ responses after restimulation with Adpgk_{mut} SP or MTAG85B were not altered between any of the groups, but IFN γ production by CD8⁺ T cells was significantly higher for mice injected with cdiGMP liposomes encapsulating the fused Adpgk_{mut} LP/MTAG85B peptide compared to Adpgk_{mut} LP alone (Figure 4B). One week after boosting, CD8⁺ T cell responses were unchanged between any of the groups, but CD4⁺ T cells had a significantly higher production of IFN γ after injection with fused peptide liposomes compared to a mix of cdiGMP/Adpgk_{mut} LP liposomes and cdiGMP/MTAG85B liposomes (Figure 4F). This persisted until day 92 (Figure 4I). Using LS-CoAT in combination with CD4⁺ epitopes induces potent CD4⁺ T cell responses, and for this, the fused peptide is more effective. However, for CD8⁺ T cell responses against the minimal Adpgk_{mut} epitope, the CD4⁺ epitope has no benefit.

Table 3: Physicochemical properties of cdiGMP-adjuvanted DSPC:DPTAP:CHOL liposomes encapsulating different peptides

Peptide	Size (nm)	PDI	Z-potential (mV)	LE peptide (%)	LE cdiGMP (%)
Adpgk _{mut} LP	213.1	0.180	35.7	35.4	76.7
MTAG85B	217.2	0.119	34.9	12.8	33.8
Fused Adpgk _{mut} LP/ MTAG85B	235.1	0.210	36.5	28.4	58.8

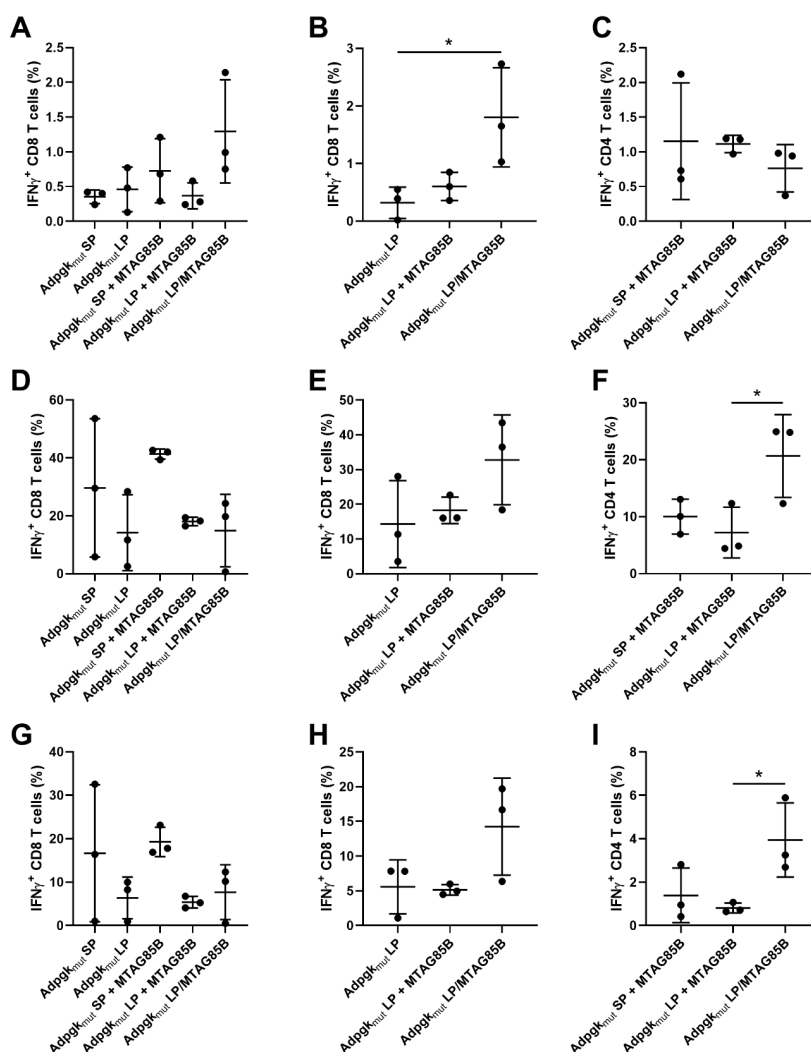


Figure 4: Effect of long and short Adpgkmut peptides, and the CD4⁺ epitope MTAG85B on antigen-specific CD8⁺ and CD4⁺ T cell responses in vivo. Mice were injected with liposomes encapsulating Adpgkmut SP or LP, a mix of Adpgkmut SP and MTAG85B liposomes, a mix of Adpgkmut LP and MTAG85B liposomes, or liposomes containing a fused Adpgk-mut LP/MTAG85B peptide (10 nmol peptide concentration). All liposomes were adjuvanted with cdiGMP. All mice received a CoAT boost with the corresponding peptide(s) on day 7. Blood was collected at different time points. One day before boost, IFN γ production by CD8⁺ T cells in the blood was measured after restimulation with (A) Adpgkmut SP, and (B) Adpgkmut LP. (C) CD4⁺ T cell responses were measured after restimulation with MTAG85B. 7 days after boost IFN γ production by CD8⁺ T cells in the blood was measured after restimulation with (D) Adpgkmut SP, and (E) Adpgkmut LP. (F) CD4⁺ T cell responses were measured after restimulation with MTAG85B. 92 days after boost IFN γ production by CD8⁺ T cells in the blood was measured after restimulation with (G) Adpgkmut SP, and (H) Adpgkmut LP. (I) CD4⁺ T cell responses were measured after restimulation with MTAG85B. Graphs show mean $\pm$ SD, * p < 0.05, determined by one-way ANOVA with Tukey's multiple comparisons test.

LS-CoAT and LS-CoT eliminate tumors in an MC38 tumor model

Since the Adpgkmut-loaded cdiGMP liposomes elicited strong antigen-specific CD8+ T cell responses, we address their potential to treat tumors. Mice were inoculated with MC-38 tumor cells and injected 4 days later with liposomes. As controls, mice were injected with antigen-free cdiGMP liposomes (Table 4), or untreated tumor-bearing mice. The tumor-free control mice also received Adpgkmut/cdiGMP liposomes. 6 days after prime, blood was collected and IFN γ responses were measured after restimulation of CD8+ T cells with Adpgkmut. Mice that received Adpgkmut/cdiGMP liposomes showed a significantly higher number of IFN γ + CD8+ T cells compared to unvaccinated controls, reaching more than 60% of the total CD8+ T-cell population (Figure 5A). On day 11 mice that received Adpgkmut/cdiGMP liposomes were boosted with CoAT, and mice that received cdiGMP liposomes without antigen were boosted with CoT, that only contained agonistic CD40 antibody and Poly I:C, and no peptide antigen. After the boost immunization mice that received LS-CoT had only a small but still measurable CD8+ T cell response against Adpgkmut, while mice receiving LS-CoAT had significantly high IFN γ responses of around 60% (Figure 5B). There were no differences between the tumor-inoculated and the tumor-free control mice, suggesting the CD8 T-cell response was not impeded by the tumor. Interestingly, LS-CoT produced significantly high IFN γ production by CD8+ T cells upon restimulation with MC38 cells (Figure 5C). Tumor size and mouse mortality were monitored for 40 days, starting from tumor inoculation. After treatment with LS-CoAT or LS-CoT, tumor size shrank, and tumors disappeared within 26 days for all mice in these groups (Figure 5D and E). The untreated control mice had massive outgrowth of the tumors and all had to be sacrificed according to humane end-points within 36 days after tumor inoculation (Figure 5F). All mice receiving LS-CoAT or LS-CoT survived until the end of the experiment (Figure 5G). We also observed high endogenous Adpgkmut responses in tumor cells of untreated tumor-bearing mice (Figure 6).

Table 4: Physicochemical properties of antigen-free cdiGMP-containing DSPC:DPTAP:CHOL liposomes

Size (nm)	PDI	Z-potential (mV)	LE cdiGMP (%)
217.4	0.075	31.5	41.6

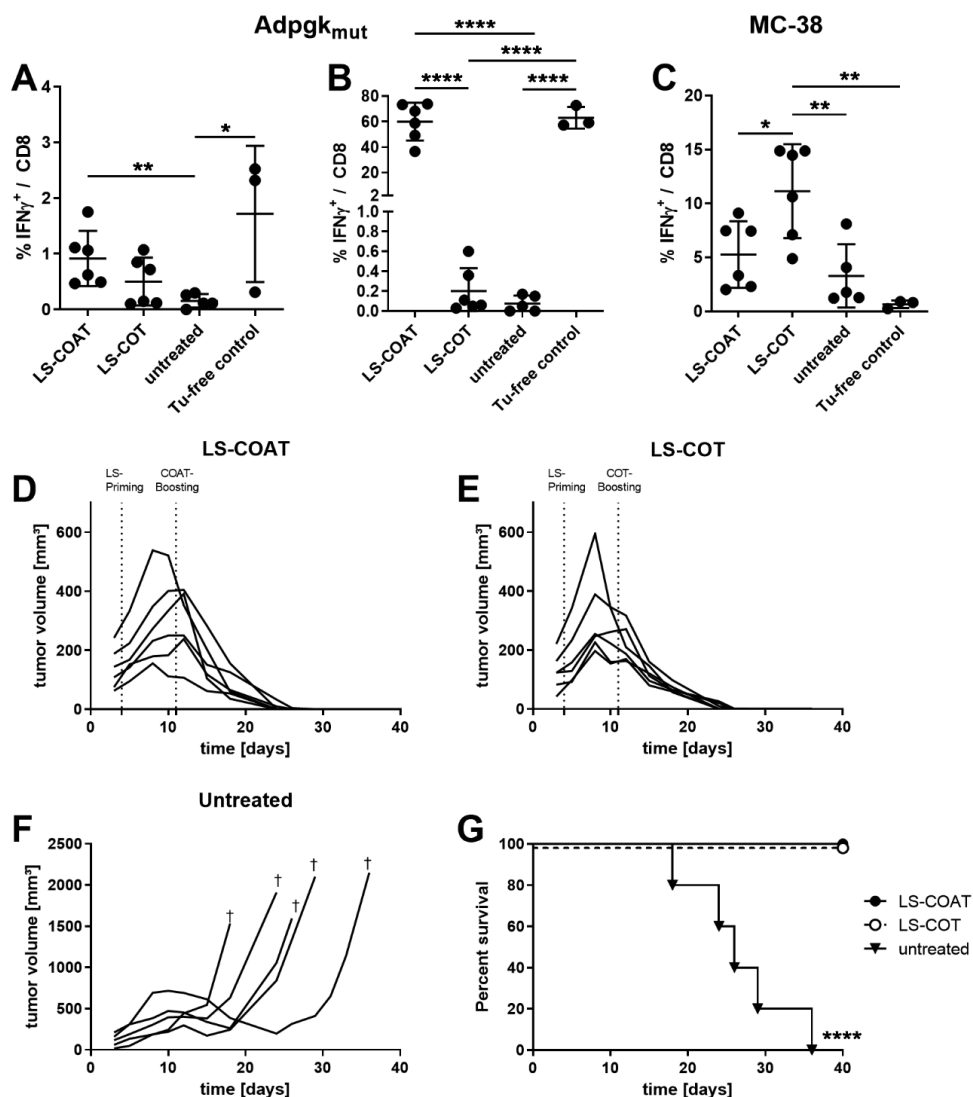


Figure 5: Tumor growth inhibition by cdiGMP liposomes with or without antigen. Mice were inoculated with 5×10^6 MC-38 cells, and tumors were allowed to grow for 4 days. One group did not receive tumor cells. Mice were primed with Adpgk_{mut}-containing cdiGMP liposomes (tumor and tumor-free mice), antigen-free cdiGMP liposomes or untreated. After 7 days, mice were boosted with CoAT, CoT or untreated. IFN γ production by CD8⁺ T cells upon restimulation with Adpgk_{mut} was measured in the blood (A) 6 days after prime, and (B) 7 days after boost. (C) IFN γ production by CD8⁺ T cells upon restimulation with MC-38 cells was measured in blood 7 days after boost. Tumor growth was measured during treatment with (D) LS-CoAT, (E) LS-CoT, and (F) untreated. (G) Survival of mice during tumor experiment. Graphs (A-C) show mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ determined by one-way ANOVA with Tukey's multiple comparisons test. (G) *** $p < 0.0001$ as determined by log-rank Mantel-Cox test.

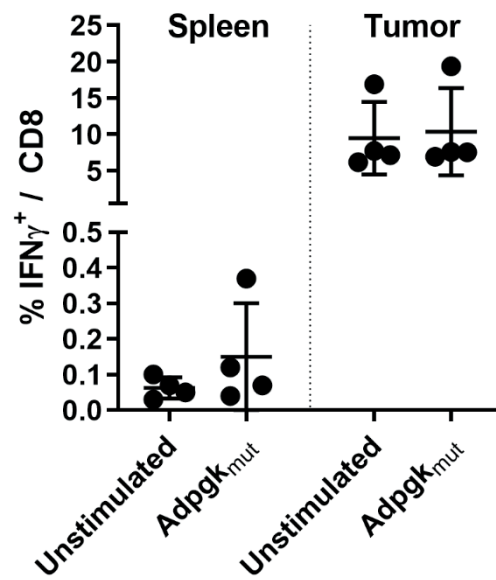


Figure 6: Endogenous CD8+ T cell responses in spleens and tumors of mice inoculated with MC-38 cells.

Discussion

Previously, DC-CoAT vaccination against the neoepitope Adpgkmut elicited around 20-60% IFN γ production by CD8 $^{+}$ T cells after restimulation with the peptide in a very short time period (2 weeks). Furthermore, DC-CoAT vaccination-induced tumor regression within 2 weeks in an MC-38 subcutaneous cancer model and eliminated tumors within 50 days¹². However, ex vivo priming of DCs is very labor-intensive and expensive⁸. Therefore, we aimed to replace the DC priming in the DC-CoAT vaccination scheme with liposomes. Cationic and rigid liposomes are known to elicit pro-inflammatory responses required for anti-tumor effects^{20,21}. Liposomes can be formulated with adjuvants to enhance this response. We compared non-adjuvanted Adpgkmut liposomes with liposomes containing the TLR3 ligand Poly I:C, the TLR4 agonist MPLA or the STING agonist cdiGMP (Figure 1), as all of these have been shown to be effective in cancer treatment¹⁵⁻¹⁷. Interestingly, LS/cdiGMP priming already induced a measurable response (Figure 1A). Targeting the STING pathway is especially interesting since it has been found to be the major pathway for APC activation in tumors¹⁰. STING can directly bind cyclic dinucleotides, such as cdiGMP, leading to the production of pro-inflammatory cytokines and type 1 IFNs, which are important for the activation of pro-inflammatory T cells^{31,32}. STING agonists have been used effectively as an adjuvant in liposomes for cancer vaccination^{33,34}. After boosting with CoAT, non-adjuvanted liposomes, MPLA liposomes, and cdiGMP liposomes had higher IFN γ responses compared to empty liposomes or free peptide (Figure 1B), approaching the high response seen with DC-CoAT vaccination. Non-adjuvanted liposomes had extremely high responses in this experiment (~40%); however, this response was not consistent among different experiments (Figures 2 and 3). Poly I:C liposomes showed much lower responses; this was probably because the poly I:C liposomes were much larger than the other liposomes (around 400 nm vs 200 nm, Table 1). Since poly I:C is negatively charged, it may aggregate with the cationic liposomes. Inclusion of cdiGMP in the liposomes reduced the antigen load by about a half; there is likely competition between the antigen and cdiGMP for loading. We minimized competition by loading cdiGMP in the lipid bilayer, but the loss of antigen could not be completely avoided. Due to the success of the cdiGMP liposomes and the interesting effects of cdiGMP reported in literature, we continued with this formulation in further experiments. Crucially, in a direct comparison between DC-CoAT and LS-CoAT with cdiGMP liposomes, liposomes performed as well as DCs (Figure 2).

For a more widely applicable tumor vaccination strategy, we aim to address whether the cdiGMP liposomes could elicit CD8 $^{+}$ T cell responses against another peptide. Therefore, we encapsulated the neoepitope Alg8mut in the same liposomes to test the responses. Both neoepitopes have a high affinity for MHC-I (predicted affinity of Adpgkmut = 2 nM³⁵, and Alg8mut = 3 nM³⁶), and they showed comparable levels of IFN γ production by CD8 $^{+}$ T cells after restimulation (Figure 3). For both peptides, incorporation of cdiGMP in the liposomes clearly boosted the immune response and led to very long-lasting responses, which were similar to those achieved with DC-CoAT¹².

Next, we wanted to improve our vaccination by eliciting CD4 $^{+}$ T cell help³ and selecting a long version of Adpgkmut peptide³⁰. Vaccination with long peptides to trigger CD8 $^{+}$ T cell responses is generally very potent [ref]. In our experiment, the long peptide did not enhance the CD8 $^{+}$ T cell response against the minimal epitope

(Figure 4). However, it may still be useful to vaccinate with longer epitopes, since it is not prerequisite to know what the minimal epitope is [ref]. Furthermore, long peptides lead to processing by DCs and cross-presentation, which is important in the context of peptide presentation by tumor cells [ref]. We also saw no evidence of the MTAG85B-induced CD4⁺ T cell activation boosting the CD8⁺ responses (Figure 4), so it seems in this case that CD4⁺ T cell activation and using a long peptide has no additional value. It may be that the liposomes and/or cdiGMP direct the response away from CD4⁺ T cells to CD8⁺ T cells, and already enhance potency so much so as to abolish the potential benefits of a long peptide. In addition, the potent effects of the boost containing agonistic anti-CD40 antibody may eliminate the need for CD4⁺ T cell help, since it mimics CD40L and can thereby replace the function of CD4⁺ T cells³⁷. Due to the lack of improvement of the Adpgkmut responses with the addition of a CD4⁺ T cell epitope or a long peptide, we decided to perform a tumor study using only the minimal Adpgkmut peptide in cdiGMP liposomes.

In the MC-38 tumor model, we again observed high antigen-specific responses to the antigen with or without tumor inoculation (Figure 5A and 4B), and we saw tumor regression within 30 days and 100% survival in mice receiving the LS-CoAT vaccination (Figure 5D and 5G). Interestingly, tumor regression started in most mice before boost at around 6 days after priming with liposomes. Wang and Celis studied the effect of cdiGMP in a slightly altered CoAT vaccination approach as presented here. Mice were inoculated with B16-F10 cells and primed on day 8 with an iv injection of 100 µg peptide, 50 µg anti-CD40 antibody, 25 µg Poly I:C, and 100 µg cdiGMP followed by a boost 9 days later with an iv injection of 100 µg peptide, 25 µg anti-CD40 antibody, 25 µg Poly I:C, and 100 µg cdiGMP. They report high levels of tetramer⁺ CD8⁺ T cells after boost (up to 80%), and significant halting, but not the elimination of tumor progression compared to controls in a B16 melanoma model³⁸. Koshy et al. encapsulated cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) in DOTAP:CHOL:DSPE-PEG liposomes as a treatment in the B16-F10 melanoma model. Mice were inoculated with cells and injected intratumoral on days 7, 9, 14, and 16 with a dose of 1 µg cGAMP in liposomes. They observed tumor elimination in 50% of the mice. At day 60, the surviving mice were challenged with B16-F10 cells, which resulted in 100% survival, indicating long-lasting immunity. They also showed high retention of cGAMP in the tumor site 24 hours after injection of liposomes, which was mostly co-localized with MHC-II-expressing cells³⁹. Surprisingly, we saw complete tumor regression and mouse survival for mice receiving LS-CoT (Figure 5E and 5G), and this vaccination strategy was the only one to produce significantly higher IFN γ upon restimulation with MC-38 cells compared to untreated control (Figure 5C). Free STING agonists have been shown to significantly reduce tumor growth after iv injection^{24,40} and even eliminate tumors after intratumoral injection^{39,41}. However, these studies required many injections of high doses of STING agonist (up to 500 µg/mouse/injection). Encapsulating compounds in liposomes leads to targeting immune cells and reduction of the number of injections and dosage⁴². We provided a single liposomal injection followed by a boost, and the cdiGMP dose was 2 µg/mouse. Other types of cationic formulations encapsulating STING agonists can display long-lasting protection after intratumoral injection^{39,43} and iv injection⁴⁴, also compared to free STING agonists. In all aforementioned studies, it seems that the efficacy of the STING agonist in the absence of antigen is due to effects in the tumor

microenvironment. However, it may be that for other tumor models than the one tested here, antigen-specific therapy is necessary. Indeed, we saw high endogenous Adpgkmut responses in tumor cells of untreated tumor-bearing mice (Figure 6), supporting the hypothesis that the MC-38 tumor model is highly susceptible to immunotherapy.

In conclusion, we have developed an extremely potent cationic liposomal formulation adjuvanted with cdiGMP that performs as well as DC-CoAT at inducing antigen-specific CD8⁺ T cell responses. The generated immune response after liposome vaccination is very long-lasting and thereby enables the elimination of established tumors. In future, it would be interesting to test the LS-CoAT strategy in other tumor models in order to evaluate the wider application of the liposomes.

References

- 1 Ostroumov, D., Fekete-Drimusz, N., Saborowski, M., Kuhnel, F. & Woller, N. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. *Cell Mol Life Sci* 75, 689-713, doi:10.1007/s00018-017-2686-7 (2018).
- 2 Appay, V., Douek, D. C. & Price, D. A. CD8+ T cell efficacy in vaccination and disease. *Nat Med* 14, 623-628, doi:10.1038/nm.f.1774 (2008).
- 3 Shankaran, V. et al. IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410, 1107-1111, doi:10.1038/35074122 (2001).
- 4 Coulie, P. G., Van den Eynde, B. J., van der Bruggen, P. & Boon, T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer* 14, 135-146, doi:10.1038/nrc3670 (2014).
- 5 Johnson, L. A. et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114, 535-546, doi:10.1182/blood-2009-03-211714 (2009).
- 6 Lu, Y. C. & Robbins, P. F. Cancer immunotherapy targeting neoantigens. *Semin Immunol* 28, 22-27, doi:10.1016/j.smim.2015.11.002 (2016).
- 7 Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20, 621-667, doi:10.1146/annurev.immunol.20.100301.064828 (2002).
- 8 Chen, P. et al. Dendritic cell targeted vaccines: Recent progresses and challenges. *Hum Vaccin Immunother* 12, 612-622, doi:10.1080/21645515.2015.1105415 (2016).
- 9 Bol, K. F. et al. The clinical application of cancer immunotherapy based on naturally circulating dendritic cells. *Journal for immunotherapy of cancer* 7, 109, doi:10.1186/s40425-019-0580-6 (2019).
- 10 Corrales, L., McWhirter, S. M., Dubensky, T. W., Jr. & Gajewski, T. F. The host STING pathway at the interface of cancer and immunity. *J Clin Invest* 126, 2404-2411, doi:10.1172/JCI86892 (2016).
- 11 Llopiz, D. et al. Combined immunization with adjuvant molecules poly(I:C) and anti-CD40 plus a tumor antigen has potent prophylactic and therapeutic antitumor effects. *Cancer Immunology, Immunotherapy* 57, 19-29, doi:10.1007/s00262-007-0346-8 (2008).
- 12 Nimanong, S. et al. CD40 Signaling Drives Potent Cellular Immune Responses in Heterologous Cancer Vaccinations. *Cancer Res* 77, 1918-1926, doi:10.1158/0008-5472.CAN-16-2089 (2017).
- 13 Jia, J. et al. Interactions Between Nanoparticles and Dendritic Cells: From the Perspective of Cancer Immunotherapy. *Front Oncol* 8, 404, doi:10.3389/fonc.2018.00404 (2018).
- 14 Pattni, B. S., Chupin, V. V. & Torchilin, V. P. New Developments in Liposomal Drug Delivery. *Chem Rev* 115, 10938-10966, doi:10.1021/acs.chemrev.5b00046 (2015).
- 15 Boks, M. A. et al. MPLA incorporation into DC-targeting glycoliposomes favours anti-tumour T cell responses. *J Control Release* 216, 37-46, doi:10.1016/j.jconrel.2015.06.033 (2015).

- 16 Varypataki, E. M., van der Maaden, K., Bouwstra, J., Ossendorp, F. & Jiskoot, W. Cationic liposomes loaded with a synthetic long peptide and poly(I:C): a defined adjuvanted vaccine for induction of antigen-specific T cell cytotoxicity. *AAPS J* 17, 216-226, doi:10.1208/s12248-014-9686-4 (2015).
- 17 Nakamura, T. et al. Liposomes loaded with a STING pathway ligand, cyclic di-GMP, enhance cancer immunotherapy against metastatic melanoma. *J Control Release* 216, 149-157, doi:10.1016/j.jconrel.2015.08.026 (2015).
- 18 Korsholm, K. S. et al. Induction of CD8+ T-cell responses against subunit antigens by the novel cationic liposomal CAF09 adjuvant. *Vaccine* 32, 3927-3935, doi:10.1016/j.vaccine.2014.05.050 (2014).
- 19 Varypataki, E. M., Benne, N., Bouwstra, J., Jiskoot, W. & Ossendorp, F. Efficient Eradication of Established Tumors in Mice with Cationic Liposome-Based Synthetic Long-Peptide Vaccines. *Cancer Immunol Res* 5, 222-233, doi:10.1158/2326-6066.CIR-16-0283 (2017).
- 20 Heuts, J. et al. Cationic Liposomes: A Flexible Vaccine Delivery System for Physicochemically Diverse Antigenic Peptides. *Pharm Res* 35, 207, doi:10.1007/s11095-018-2490-6 (2018).
- 21 Benne, N., van Duijn, J., Kuiper, J., Jiskoot, W. & Slutter, B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. *J Control Release* 234, 124-134, doi:10.1016/j.jconrel.2016.05.033 (2016).
- 22 Anselmo, A. C. & Mitragotri, S. Impact of particle elasticity on particle-based drug delivery systems. *Adv Drug Deliv Rev* 108, 51-67, doi:10.1016/j.addr.2016.01.007 (2017).
- 23 Benne, N. et al. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. *J Control Release* 291, 135-146, doi:10.1016/j.jconrel.2018.10.028 (2018).
- 24 Alving, C. R., Rao, M., Steers, N. J., Matyas, G. R. & Mayorov, A. V. Liposomes containing lipid A: an effective, safe, generic adjuvant system for synthetic vaccines. *Expert Rev Vaccines* 11, 733-744, doi:10.1586/erv.12.35 (2012).
- 25 Schmidt, N. W. et al. Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proc Natl Acad Sci U S A* 105, 14017-14022, doi:10.1073/pnas.0805452105 (2008).
- 26 Badovinac, V. P., Messingham, K. A., Jabbari, A., Haring, J. S. & Harty, J. T. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat Med* 11, 748-756, doi:10.1038/nm1257 (2005).
- 27 Tomayko, M. M. & Reynolds, C. P. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer chemotherapy and pharmacology* 24, 148-154, doi:10.1007/bf00300234 (1989).
- 28 Aurisicchio, L. et al. Poly-specific neoantigen-targeted cancer vaccines delay patient derived tumor growth. *Journal of Experimental & Clinical Cancer Research* 38, 78, doi:10.1186/s13046-019-1084-4 (2019).
- 29 Menares, E. et al. Tissue-resident memory CD8+ T cells amplify anti-tumor immunity by triggering antigen spreading through dendritic cells. *Nature Communications* 10, 4401, doi:10.1038/s41467-019-12319-x (2019).

- 30 Melief, C. J. & van der Burg, S. H. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat Rev Cancer* 8, 351-360, doi:10.1038/nrc2373 (2008).
- 31 Woo, S. R. et al. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 41, 830-842, doi:10.1016/j.immuni.2014.10.017 (2014).
- 32 Barber, G. N. STING: infection, inflammation and cancer. *Nat Rev Immunol* 15, 760-770, doi:10.1038/nri3921 (2015).
- 33 Hanson, M. C. et al. Nanoparticulate STING agonists are potent lymph node-targeted vaccine adjuvants. *J Clin Invest* 125, 2532-2546, doi:10.1172/JCI79915 (2015).
- 34 Miyabe, H. et al. A new adjuvant delivery system ,cyclic di-GMP/YSK05 liposome' for cancer immunotherapy. *J Control Release* 184, 20-27, doi:10.1016/j.jconrel.2014.04.004 (2014).
- 35 Yadav, M. et al. Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. *Nature* 515, 572-576, doi:10.1038/nature14001 (2014).
- 36 Matsushita, H. et al. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* 482, 400-404, doi:10.1038/nature10755 (2012).
- 37 Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R. & Melief, C. J. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393, 480-483, doi:10.1038/31002 (1998).
- 38 Wang, Z. & Celis, E. STING activator c-di-GMP enhances the anti-tumor effects of peptide vaccines in melanoma-bearing mice. *Cancer Immunol Immunother* 64, 1057-1066, doi:10.1007/s00262-015-1713-5 (2015).
- 39 Koshy, S. T., Cheung, A. S., Gu, L., Graveline, A. R. & Mooney, D. J. Liposomal Delivery Enhances Immune Activation by STING Agonists for Cancer Immunotherapy. *Adv Biosyst* 1, doi:10.1002/adbi.201600013 (2017).
- 40 Li, T. et al. Antitumor Activity of cGAMP via Stimulation of cGAS-cGAMP-STING-IRF3 Mediated Innate Immune Response. *Sci Rep* 6, 19049, doi:10.1038/srep19049 (2016).
- 41 Corrales, L. et al. Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell Rep* 11, 1018-1030, doi:10.1016/j.celrep.2015.04.031 (2015).
- 42 Schwendener, R. A. Liposomes as vaccine delivery systems: a review of the recent advances. *Ther Adv Vaccines* 2, 159-182, doi:10.1177/2051013614541440 (2014).
- 43 Demaria, O. et al. STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity. *Proc Natl Acad Sci U S A* 112, 15408-15413, doi:10.1073/pnas.1512832112 (2015).
- 44 Cheng, N. et al. A nanoparticle-incorporated STING activator enhances antitumor immunity in PD-L1-insensitive models of triple-negative breast cancer. *JCI Insight* 3, doi:10.1172/jci.insight.120638 (2018).

