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

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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

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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 IFNγ 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 development[1]. While the main focus for tumor suppression is $CD8^+$ T cells[2], $CD4^+$ T cells have also been shown to be important[3]. 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 antigens[4]. 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 tissues[5]. 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 vaccination[6]. 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 activation[7]. 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 reinjected[8]. 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 responses[11]. Furthermore, this CoAT vaccination strategy could control tumor growth in an MC-38 tumor mouse model[12]. While DC-CoAT vaccination does lead to strong immune responses, *ex vivo* priming of DCs is very labor-intensive and expensive, prohibiting widespread use[8]. Using a delivery system to encapsulate a neoantigen, such as nanoparticles, can deliver the antigen to DCs *in situ* in a simpler way[13]. Liposomes are a type of nanoparticle composed of phospholipids which form a bilayer around an aqueous core[14]. A major advantage of liposomes is that they can encapsulate one or multiple antigens and adjuvants, such as TLR ligands[15,16] or STING agonists[17], 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 responses[21-23].

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 before[23]. 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 ProImmune, Oxford, UK) dissolved in 1 mL 1:1 ACN/MilliQ (v/v) + 0.04% NH_4OH (w/v), or 1 mL 1:1 ACN/MilliQ (v/v) + 0.04% NH_4OH (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® NucleporeTM, 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 µL PBS, as described previously[25]. After DC harvest, cells were maturated in vitro with LPS (0.5 µg/ml) and incubated in the presence of peptides (2 µg/ml) for approximately 2 hours. For vaccination, $1x10^6$ DCs resuspended in 100 µL PBS were injected intravenously into the mice. CoAT immunizations were performed as described previously[12]. Briefly, individual mice received intravenous injection of 100 µg soluble peptides (thinkpeptides, Oxford, UK), 200 µg Poly I:C (Invivogen, San Diego, CA, USA) and 100 µg 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 µL 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 IFNγ staining as described before[26]. For antibody or tetramer staining, a small volume (~50 µL) of blood was collected via submandibular bleeding. Extracellular antibody and intracellular cytokine staining were performed according to standard protocols. Intracellular cytokine staining for IFNγ 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 µm 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 µg/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 µm 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), IFNγ (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 µL 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 cm^3 or when tumors exulcerated considerably. Tumor volume was determined by the formula 0.5 × length (mm) × (width (mm))^2, which was published previously[27].

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 IFNγ production by $CD8^+$ T cells was measured. 7 days after prime, all mice received the CoAT boost, and 7 days after boost IFNγ production by $CD8^+$ T cells was determined (Figure 1A). Only mice receiving liposomes with cdiGMP showed measurable IFNγ responses before CoAT (Figure 1B). The CoAT boost enhanced IFNγ responses for all groups except the free peptide group, showing liposomal delivery significantly enhances the effect of CoAT boosting. The levels of IFNγ 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 IFNγ 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

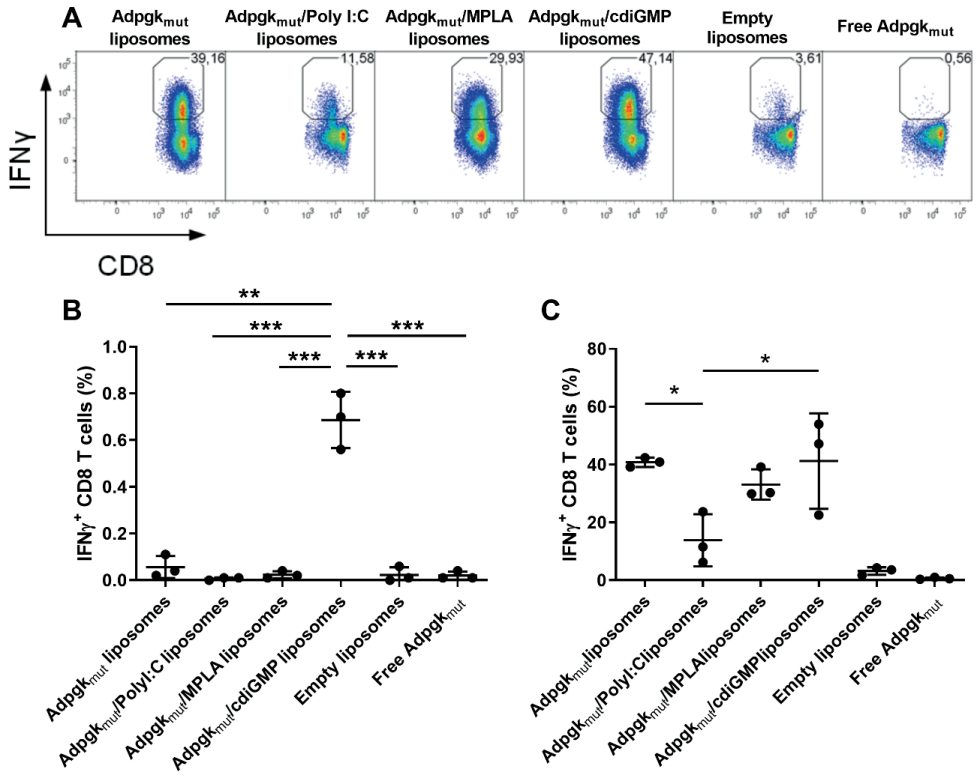

Figure 1: Effect of liposomal adjuvant on Adpgk$_{mut}$-specific CD8$^+$ T cell responses *in vivo*. Mice were injected with Adpgk$_{mut}$-containing liposomes (10 nmol peptide concentration), either without adjuvant or with Poly I:C, MPLA or cdiGMP. As controls, some mice received empty liposomes or 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. IFNγ 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 ± 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 IFNγ production by CD8$^+$ T cells. We observed a significantly higher production of IFNγ 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 IFNγ production in mice compared to non-adjuvanted liposomes (Figure 2B).

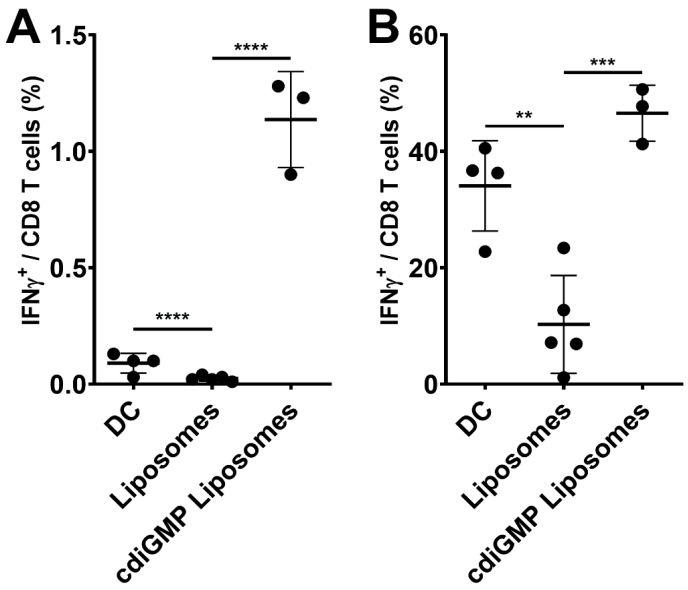

Figure 2: Comparison of liposomal formulations to DC-CoAT vaccination *in vivo*. Mice were injected with Adpgk$_{mut}$-pulsed DCs, Adpgk$_{mut}$ liposomes, or Adpgk$_{mut}$ cdiGMP liposomes. All mice received a CoAT boost on day 7. IFNγ production by CD8$^+$ T cells upon restimulation with Adpgk$_{mut}$ was measured in the blood (A) 6 days after prime, and (B) 7 days after boost. Graphs show mean ± 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 neoepitopes, 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 neoepitope. 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 IFNγ 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 IFNγ 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% IFNγ$^+$ 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

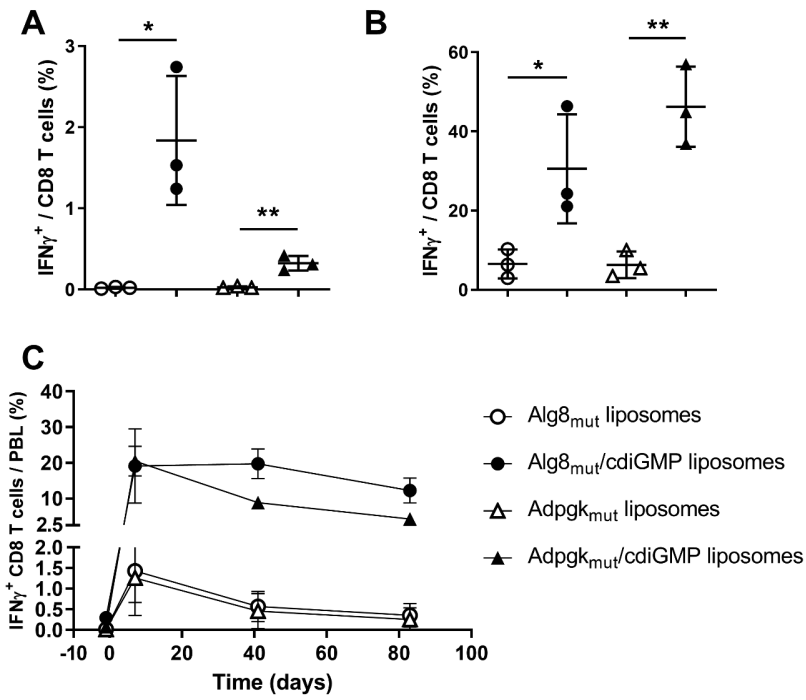

Figure 3: Long-lasting antigen-specific responses after LS/CoAT with $Adpgk_{mut}$ and $Alg8_{mut}$. Mice were injected with $Alg8_{mut}$- or $Adpgk_{mut}$-containing liposomes (10 nmol peptide, circles, and triangles, respectively), with or without cdiGMP (black and open symbols, respectively). All mice received a CoAT boost after 7 days. IFNγ 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 ± 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 response[3]. 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 processing[30]. 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, IFNγ 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. IFNγ 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, IFNγ 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, IFNγ responses after restimulation with $Adpgk_{mut}$ SP or MTAG85B were not altered between any of the groups, but IFNγ 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 IFNγ 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

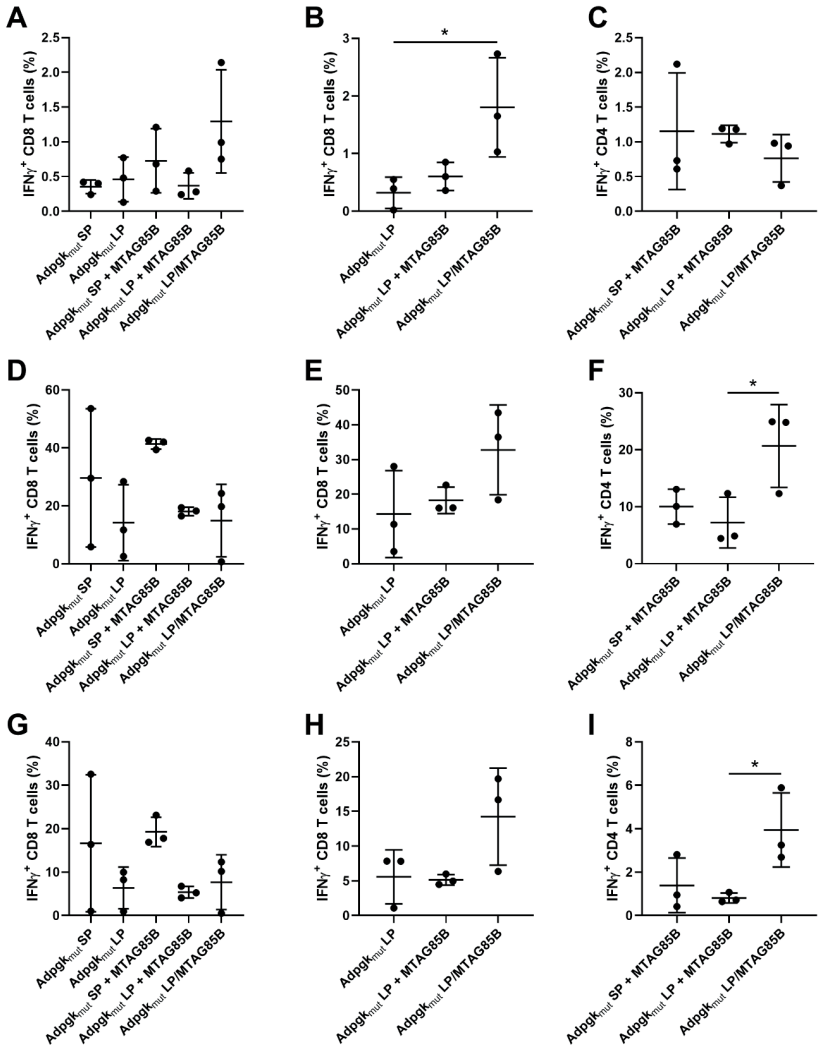

Figure 4: Effect of long and short Adpgkmut peptides, and the CD4+ epitope MTAG85B on antigen-specific CD8+ and CD4+ T cell responses in vivo. Mice were injected with liposomes encapsulating Adpgkmut SP or LP, a mix of Adpgkmut SP and MTAG85B liposomes, a mix of Adpgkmut LP and MTAG85B liposomes, or 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, IFNγ 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 IFNγ 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 IFNγ 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 ± 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 IFNγ responses were measured after restimulation of CD8+ T cells with Adpgkmut. Mice that received Adpgkmut/cdiGMP liposomes showed a significantly higher number of IFNγ+ 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 IFNγ 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 IFNγ 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

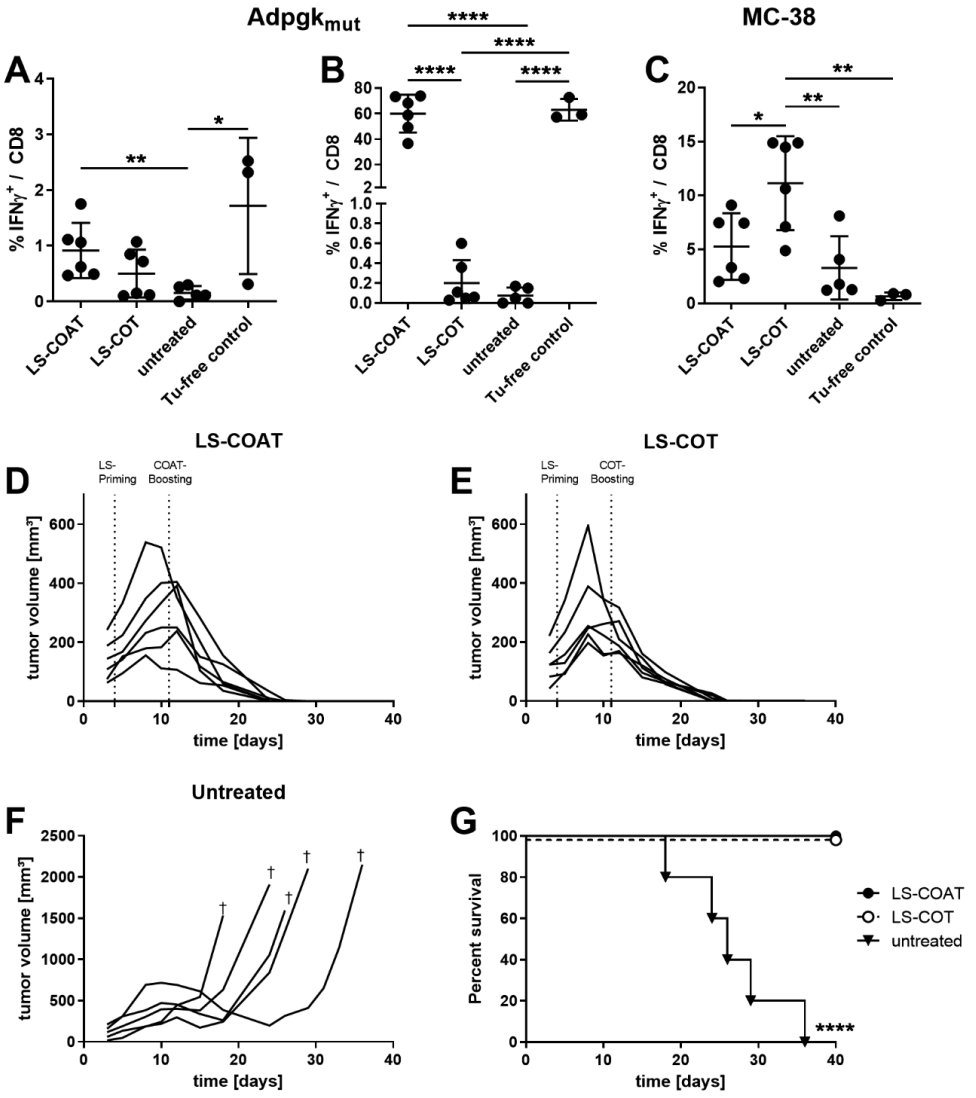

Figure 5: Tumor growth inhibition by cdiGMP liposomes with or without antigen. Mice were inoculated with 5 x 106 MC-38 cells, and tumors were allowed to grow for 4 days. One group did not receive tumor cells. Mice were primed with Adpgkmut-containing cdiGMP liposomes (tumor and tumor-free mice), antigen-free cdiGMP liposomes or untreated. After 7 days, mice were boosted with CoAT, CoT or untreated. IFNγ production by CD8+ T cells upon restimulation with Adpgkmut was measured in the blood (A) 6 days after prime, and (B) 7 days after boost. (C) IFNγ 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 ± 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.

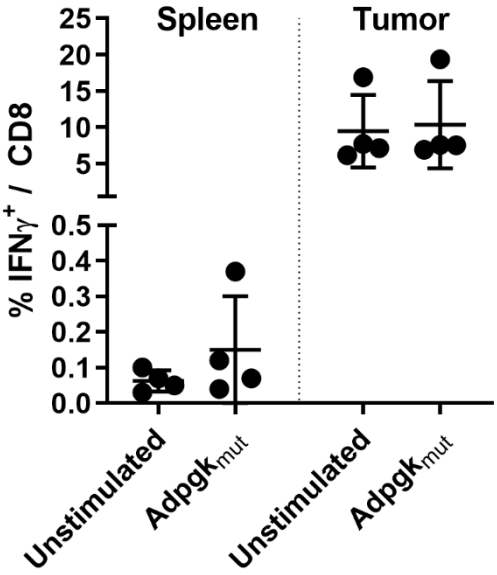

Figure 6: Endogenous CD8+ T cell responses in spleens and tumors of mice inoculated with MC-38 cells.

Discussion

Previously, DC-CoAT vaccination against the neoepitope Adpgkmut elicited around 20-60% IFNγ 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 effects20,21. 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 cells31,32. STING agonists have been used effectively as an adjuvant in liposomes for cancer vaccination33,34. After boosting with CoAT, non-adjuvanted liposomes, MPLA liposomes, and cdiGMP liposomes had higher IFNγ 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 IFNγ 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 IFNγ 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 injection24,40 and even eliminate tumors after intratumoral injection39,41. 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 injection39,43 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.

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