

CO-DELIVERY OF GLUTAMIC ACID-EXTENDED PEPTIDE ANTIGEN AND IMIDAZOQUINOLINE TLR7/8 AGONIST VIA IONIZABLE LIPID NANOPARTICLES INDUCES PROTECTIVE ANTI-TUMOR IMMUNITY

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ABSTRACT

Cancer vaccines aim at generating cytotoxic CD8⁺ T cells that kill cancer cells and confer durable tumor regression. Hereto, CD8⁺ peptide epitopes should be presented by antigen presenting cells to CD8⁺ T cells in lymphoid tissue. Unfortunately, in unformulated soluble form, peptide antigens are poorly taken up by antigen presenting cells and do not efficiently reach lymph nodes. Hence, the lack of efficient delivery remains a major limitation for successful clinical translation of cancer vaccination using peptide antigens. Here we propose a generic peptide nanoformulation strategy by extending the amino acid sequence of the peptide antigen epitope with 10 glutamic acid residues. The resulting overall anionic charge

of the peptide allows encapsulation into lipid nanoparticles (peptide-LNP) by electrostatic interaction with an ionizable cationic lipid. We demonstrate that intravenous injection of peptide-LNP efficiently delivers the peptide to immune cells in the spleen. Peptide-LNP that co-encapsulate an imidazoquinoline TLR7/8 agonist (IMDQ) induce robust innate immune activation in a broad range of immune cell subsets in the spleen. Peptide-LNP containing the minimal CD8+ T cell epitope of the HPV type 16 E7 oncoprotein and IMDQ induces high levels of antigen-specific CD8+ T cells in the blood, and can confer protective immunity against E7-expressing tumors in both prophylactic and therapeutic settings.

INTRODUCTION

Cancer outgrowth occurs due to malfunctioning of the immune system. Therapeutic interventions that instigate immune defense mechanisms within tumors are, hence, of tremendous relevance.¹⁻⁷ Cancer vaccination aims at generating CD8+ T cells that kill cancer cells by recognizing MHC-I-bound peptide fragments from protein antigens that are over-expressed by cancer cells or neo-antigens that arise by somatic mutation.^{8,9} Hereto, protein or peptide fragments thereof need to be delivered to antigen-presenting cells where they are processed and trimmed into minimal epitope amino acids sequences, loaded onto MHC-I and transported onto the cell surface.

Administration of antigens in soluble form is extremely poor at generating robust CD8+ T cell responses⁹⁻¹¹ due to (1) the poor uptake by antigen presenting cells in lymphoid tissue and (2) the poor efficiency by which exogenous peptides are cross-presented onto MHC-I. Formulation of antigens into nanoparticles facilitates antigen trafficking to immune-inducing sites in lymphoid tissue, i.e. lymph nodes after local subcutaneous or intramuscular injection or the spleen after intravenous injection. Phagocytosis of nanoparticles strongly depends on the size and physicochemical properties of the nanoparticles, but often occurs to a higher extent than phagocytosis of soluble peptides.^{12,13} Nanoparticles can also co-deliver molecular adjuvants, such as Toll Like Receptor (TLR) agonists, that trigger innate activation pathways in antigen presenting cells. TLR triggering has been reported to enhance MHC-I presentation of phagocytosed antigens.¹⁴ TLR triggering in antigen presenting cells also induces upregulation of cell surface co-stimulatory ligands and cytokine secretion. This immune-stimulatory environment greatly improves the magnitude and quality of the adaptive T cells response against exogenous antigens.¹⁵ To this end, different formulation technologies, including lipid nanoparticle,^{16,17} liposomes,^{18,19} and polymeric nanoparticles²⁰⁻²¹ have been developed thus far. Recently, we took inspiration from the mRNA delivery field²²⁻²⁴ and used lipid nanoparticles (LNP), containing a cationic ionizable lipid, to formulate a peptide antigen and a molecular adjuvant through electrostatic interaction.²⁵ Peptides largely differ in physicochemical

properties and cannot simply be encapsulated into LNPs by charge interaction. Therefore, we conjugated the peptide antigen to poly-L-glutamic acid. The latter is a polyanionic polymer which provides sufficient anionic charge density to allow for encapsulation by electrostatic interaction with a cationic ionizable amine into LNP. An imidazoquinoline TLR7/8 agonist (IMDQ) was conjugated to poly-L-glutamic acid. IMDQ induces broad activation of innate immune cells subsets, including dendritic cells (DCs), macrophages and also B cells, and induces the secretion of IL-12 and Type I interferons (IFNs) that promote Th1 CD4⁺ and CD8⁺ T cell immunity.^{26,27} We demonstrated, using ovalbumin as a model antigen, that peptide/IMDQ-LNPs induce, relative to the soluble unformulated peptide antigen, a dramatic increase in antigen-specific T cell responses upon local subcutaneous injection by targeting the peptide and IMDQ to the lymphoid tissue.

In the present work, we further expanded our approach on the level of the (1) antigen design, (2) ionizable lipid, (3) route of administration and (4) therapeutic scope. **Scheme 1** displays the key features of our design. On the level of the antigen design, we engineered the minimal epitope sequence of a CD8⁺ T cell epitope antigen with an anionic glutamic acid oligomer. This is a much more defined approach, with a much higher translational value compared to random conjugation of peptide antigens to a poly-L-glutamic acid backbone. On the level of the ionizable lipid, we selected S-Ac7-DOG (chemical structure is displayed in **Scheme 1A**), an ionizable lipid, which we recently identified during a combinatorial synthesis campaign for mRNA delivery lipids²⁸. Whereas the ionizable lipid DIPADS, used in our previous work was found unable to transfect cells with mRNA, S-Ac7-DOG is a potent transfection reagent. Hence, S-Ac7-DOG LNPs hold the capacity to deliver a payload into the cytoplasm of cells, which is beneficial in the context of peptide antigen vaccinations.^{29,30} Notably, S-Ac7-DOG was designed as a dialkyl lipid in which the alkyl lipids are connected to a glycerol-mimicking linker region, which is then connected to the ionizable amine head-group through a reducible disulfide bond. This design introduces several options for degradability into the lipid structure. Furthermore, we observed *in vivo*, in mouse models, that S-Ac7-DOG LNPs show particularly low reactogenicity, which highlights its potential for the design of safe vaccines. Regarding the route of administration, we have elaborated in the present work on intravenous administration as an optimal route to reach a large number of immune cells in the spleen.³¹ In respect to the therapeutic scope, we selected the minimal CD8⁺ T cell epitope of the human papillomavirus (HPV) 16 oncoprotein E7. The latter is a critical driver of cellular malignant transformation, causing cervical cancer. E7 is constitutively expressed by cancer cells mutated by HPV infection, and hence, is an attractive target for immunotherapy.³²

We demonstrated that intravenous administration of E7 peptide antigen and TLR7/8 agonist in LNPs is highly effective at delivering the peptide to splenocytes and inducing robust

activation of antigen presenting cells, B cells and T cells. Co-delivery of the antigen and a TLR7/8 agonist in LNPs induces E7 antigen-specific T cells responses and mounts protective adaptive immunity against E7-expressing tumors in both prophylactic and therapeutic settings.

RESULTS AND DISCUSSION

Extension of peptide epitopes with a glutamic acid decamer enables their encapsulation in LNPs through electrostatic interactions with ionizable lipids.

We extended the N terminus of the minimal CD8+ T cell epitope of the HPV 16 oncoprotein E7, *i.e.*, RAHYNIVTF, with five amino acid residues from the natural E7 full protein sequence, *i.e.* QAEPD, two serines and ten glutamic acid residues. The glutamic acid decamer will be further denoted as E₁₀. The amino acid sequence of all peptides used in this work is listed in **Table 1**. The imidazoquinoline TLR7/8 agonist 1-(4-(aminomethyl)-benzyl)-2-butyl-1H-imidazo[4,5-c] quinolin-4-amine (IMDQ) was conjugated at its aliphatic amine position to the C-terminus of E₁₀. Fluorescently labelled E₁₀ was prepared by addition of a lysine at the C terminus followed by conjugation of NHS-activated Cy3, respectively Cy5, dyes to the amine in the lysine side chain. All peptides were synthesized by solid phase synthesis and listed in **Table 1**. For simplicity, the glutamic acid decamer will be further denoted as E₁₀ and the E₁₀-extended peptide antigen as Ag-E₁₀. Notably, extension of peptide antigens with cationic (*i.e.*, lysine- or arginine-rich) amino acid sequences has been reported previously to drive electrostatic self-assembly with anionic compounds^{33,34} or covalent crosslinking.³⁵

Table 1. Peptides used in this study. The amino acid sequence is listed from N to C terminus.

amino acid sequence	abbreviation	description
RAHYNIVTF	Ag	Minimal CD8+ T cell epitope of the HPV 16 oncoprotein E7
EEEEEEEEESSQAEPDRAHYNIVTF	Ag-E ₁₀	Antigen engineered for LNP encapsulation
EEEEEEEE-IMDQ	IMDQ- E ₁₀	IMDQ TLR7/8 agonist conjugated to glutamic acid decamer
EEEEEEEEEK-Cy3	Cy3-E ₁₀	Cy3 dye conjugated to glutamic acid decamer
EEEEEEEEEK-Cy5	Cy5-E ₁₀	Cy5 dye conjugated to glutamic acid decamer

The peptide was formulated in LNPs (**Scheme 1B**) in a similar way as reported for mRNA LNP formulation.³⁶ LNP were composed of (1) the ionizable cationic lipid S-Ac7-DOG to bind the anionic peptide by electrostatic complexation, (2) cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) aiding in LNP stabilization and endosomal membrane rupturing^{37,38} and (3) 1,2-dimyristoyl-rac-glycero-3-methoxypoly(ethylene glycol) (DMG-PEG, 2kDa PEG Mw) to shield the LNPs from aggregation. A 50/38.5/10/1.5 molar ratio of ionizable

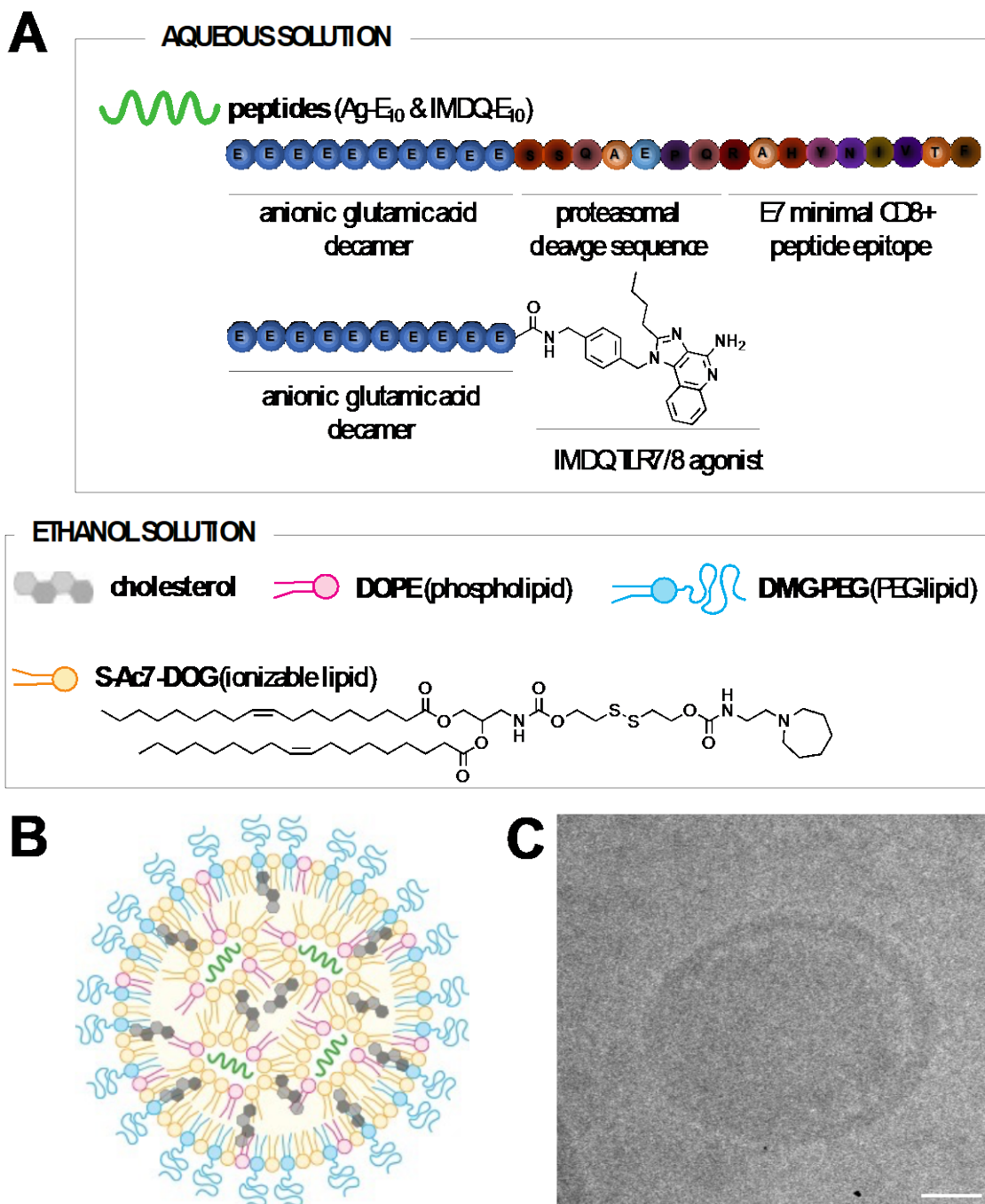
lipid/cholesterol/DOPE/DMG-PEG was used. S-Ac7-DOG was used as an ionizable cationic lipid. This ionizable lipid was recently developed in our lab in the context of a combinatorial synthesis campaign to identify ionizable lipids for mRNA LNP delivery. S-Ac7-DOG is a biodegradable ionizable lipid comprising a reducible disulfide,³⁹ a cyclic ionizable amine head group,²³ and two dioleoyl lipid tails connected via two ester bonds to the linker region (**Scheme 1A**). S-Ac7-DOG LNPs (without peptide) exhibited a pKa value of 6.96, as measured by 2-(p-toluidino) naphthalene-6-sulfonic acid (TNS) fluorescence titration assay (**Figure S1**). Hence, LNPs comprising S-Ac7-DOG feature a low charge density at neutral physiological pH, whereas they gain a high cationic charge density at endosomal pH of 5.5-6.5. This property mediates destabilization of the anionic endosomal membrane and promotes release of cargo into the cytosol.⁴⁰ The unsaturated oleic acid alkyl tails of S-Ac7-DOG can increase membrane fluidity and have a tendency to form a non-bilayer phase, which further facilitates endosomal membrane disruption and cargo release.⁴¹

To illustrate the ability of S-Ac7-DOG LNPs to deliver a payload into the cytoplasm of cells we formulated mRNA encoding for eGFP into S-Ac7-DOG LNPs and DIPADS LNPs at an N:P (N: ionizable amines, P: phosphate anions) molar ratio of 10:1. DIPADS is the ionizable lipid that we previously used for peptide-LNP formulation.²⁵ DIPADS is a non-degradable lipid comprising a diisopropylamine head and two saturated alkyl chains. DIPADS LNP have a pKa of 5.2 (**Figure S1**) which is lower than the reported optimal range of 6~7 to facilitate endosomal escape.⁴² Flow cytometry analysis of mRNA translation into eGFP by dendritic cells *in vitro*, which is a measure for cytoplasmic delivery of intact mRNA, indicated that DIPADS LNPs are much less potent than S-Ac7-DOG LNPs. (**Figure S2**)

Peptide-LNPs were fabricated by rapid mixing under vigorous stirring of an aqueous solution containing the peptide and an ethanolic solution containing all lipids. Within the aqueous solution, the molar ratio of IMDQ-E₁₀ and Ag-E₁₀ was 7: 3, resulting in an equal weight ratio of IMDQ to Ag (i.e. QAEPDRAHYNIVTF) peptide. Following mixing, peptide-LNPs were dialyzed against phosphate buffered saline (PBS) or HEPES buffer to remove the ethanol. Uniform peptide-LNPs with a mean diameter of 100 nm and a slightly negative zeta potential (**Table 2**) were obtained. Cryo-TEM imaging (**Scheme 1C**) of an individual LNP indicated an electron dense interior of the LNP akin to that of RNA-loaded LNP.

Peptide-LNPs were stable for at least one week at 4 °C in PBS (**Figure S3**). A close to neutral zeta potential value is favorable with regard to avoiding aggregation in the bloodstream upon systemic delivery administration.^{43,44} Bicinchoninic acid assay (BCA) analysis indicated an encapsulation efficiency of Ag-E₁₀ in LNP of 69 ± 3 %. Cy3-E₁₀ was taken as a mimic to measure the encapsulation of IMDQ-E₁₀ using the UV absorbance of Cy3, thereby indicating an encapsulation efficiency of 74 ± 2 %. Notably, the values are significantly higher than those

obtained by most of the nanoparticle systems such as PLGA and liposomes that rely on mere physical interaction.⁴⁵



Scheme 1. Peptide-LNP design. (A) E7 peptide antigen and imidazoquinoline TLR7/8 agonist were modified with E₁₀, i.e., a glutamic acid decamer. Peptides were assembled into lipid nanoparticles (LNP) by electrostatic interaction with S-Ac7-DOG as an ionizable lipid by rapid mixing of an ethanol and an aqueous solution. Phospholipid, cholesterol and PEG-lipid aid in the formation of colloiddally stable LNP and endosomal release of encapsulated payload. (B) Schematic illustration of peptide-LNP. (C) Cryo-TEM image of peptide-LNP. Scale bar represents 50 nm.

Table 2. Peptide-LNP composition and biophysical properties. Size was measured by dynamic light scattering (DLS), zeta-potential by electrophoretic mobility analysis in HEPES buffer (pH 7.4) and peptide encapsulation efficiency by BCA assay.

Ionizable lipids	Phospholipids	N:COOH	Size (nm)	PDI	Zeta potential (mV)	IMDQ-E ₁₀ Encapsulation efficiency (%)	E7Ag-E ₁₀ Encapsulation efficiency (%)
S-Ac7-DOg	DOPE	5:1	99	0.104	-3.3	73.5 ± 2.2	69.0 ± 3.3

We then screened whether adjusting the formulation parameters could further improve the encapsulation efficiency or alter the physicochemical properties of the LNP. The molar ratio of N:COOH was increased to 10:1. Distearoylphosphatidycholine (DSPC) was used as an alternative phospholipid to DOPE, as DSPC was reported to increase LNP stability, albeit at the cost of lower endosomal membrane rupturing.³⁶ **Table S1** shows that both interventions yielded stable peptide-LNPs, however, without any significant improvement to the encapsulation efficiency.

S-Ac7-DOG peptide-LNPs release peptide in response to a reductive trigger

We set out to investigate whether peptides encapsulated in LNP could be released in a reductive medium in response to cleavage of the disulfide bond of S-Ac7-DOG. Hereto we encapsulated Cy3- and Cy5-labelled, respectively, E₁₀ in LNP (**Figure 1A**). Co-encapsulation of Cy3-E₁₀ and Cy5-E₁₀ in LNP was expected to induce Förster resonance energy transfer (FRET) between Cy3 and Cy5 upon excitation of Cy3.⁴⁶ A decrease in fluorescence emission at the wavelength of maximum emission of Cy5 (*i.e.*, the so-called FRET signal) would suggest that the distance between both dyes increased, *e.g.* when being released from a nanoparticle. Here, we used this approach to measure alterations in the FRET signal upon exposure to glutathione as a mimic of the reductive cytoplasmic environment. Peptide-LNPs with S-Ac7-DOG as a reduction-sensitive ionizable lipid and LNPs with E-Ac7-DOG as a reduction-insensitive control ionizable lipid were prepared. Both ionizable lipids share the same structural features, but E-Ac7-DOG contains a stable ether bond instead of a reduction-sensitive disulfide bond (**Figure 1A**). The fluorescence emission spectra shown in **Figure 1B** give proof that the dyes in LNP (Cy3-E₁₀ + Cy5-E₁₀) indeed exhibited strong FRET coupling, as evidenced by a Cy5 emission peak at 675 nm (λ_{max} of Cy5) upon excitation at 550 nm (λ_{max} of Cy3). Mixtures of Cy3-E₁₀ and Cy5-E₁₀ in solution, and mixtures of both dyes in separate LNP, *i.e.*, LNP(Cy3-E₁₀) + LNP(Cy5-E₁₀), by contrast, were completely absent of FRET coupling.

LNPs (Cy3-E₁₀ + Cy5-E₁₀) containing S-Ac7-DOG or E-Ac7-DOG, respectively, were incubated at 37 °C in PBS (pH 7.4) or PBS supplemented with 10 mM glutathione, as a mimic on the intracellular environment. We then recorded the fluorescence emission spectra, upon excitation at 550 nm (λ_{max} of Cy3), of the samples over time (**Figure 1C**). S-Ac7-DOG LNPs (Cy3-E₁₀ + Cy5-E₁₀) exhibited a sharp decrease in FRET coupling in glutathione buffer, in contrast to buffer lacking glutathione. Notably, despite a small extent of peptide being released over time from S-Ac7-DOG LNPs in redox-neutral medium, our data demonstrate that in a reductive medium, peptides are released at a much faster rate.

E-Ac7-DOG LNPs (Cy3-E₁₀ + Cy5-E₁₀) remained invariant, irrespective of the presence of glutathione. The slight decrease in FRET coupling over time in PBS in absence of glutathione in S-Ac7-DOG LNPs could be due to disulfide exchange within the LNPs, allowing release of peptide. Notably, when the assay was performed at a pH value of 5.5, mimicking the endosomal pH, the FRET signal of S-Ac7-DOG LNPs did not change in presence of glutathione (data not shown), indicating that in endosomes, disulfide cleavage is less likely to occur than in the cellular cytosol.

Figure 1D depicts the evolution over time of the normalized FRET ratio. We defined the latter as the ratio of the emission of the acceptor Cy5 dye at 675 nm over the emission of the donor Cy3 dye at 580 nm. Note that normalization was performed by setting the maximum measured FRET ratio at 100% and absence of Cy5 emission at 0%. FRET coupling disappeared within a few hours in S-Ac7-DOG LNPs (Cy3-E₁₀ + Cy5-E₁₀) when exposed to a reductive milieu at pH 7.4. These observations suggest that, in response to a reductive milieu, Cy3-E₁₀ and Cy5-E₁₀ can become released from S-Ac7-DOG LNPs, whereas in control LNPs, lacking a disulfide bond, peptides remained encapsulated. Upon reduction of the disulfide bond in S-Ac7-DOG, the ionizable amine head group is no longer conjugated to the alkyl chains. Hence, the amine will likely diffuse outside the LNP and the LNP will be no longer capable of providing electrostatic complexation to the peptide. Notably, as the overall hydrophobic nature of the lipidic LNP constituents did not change, the LNPs retained their physical integrity (**Figure S5**), as measured by DLS. Of note, these experiments do not shed light on the exact sequence of events that will occur inside a cell and should only be considered as a proof-of-concept for the sensitivity of S-Ac7-DOG LNPs to a reductive trigger.

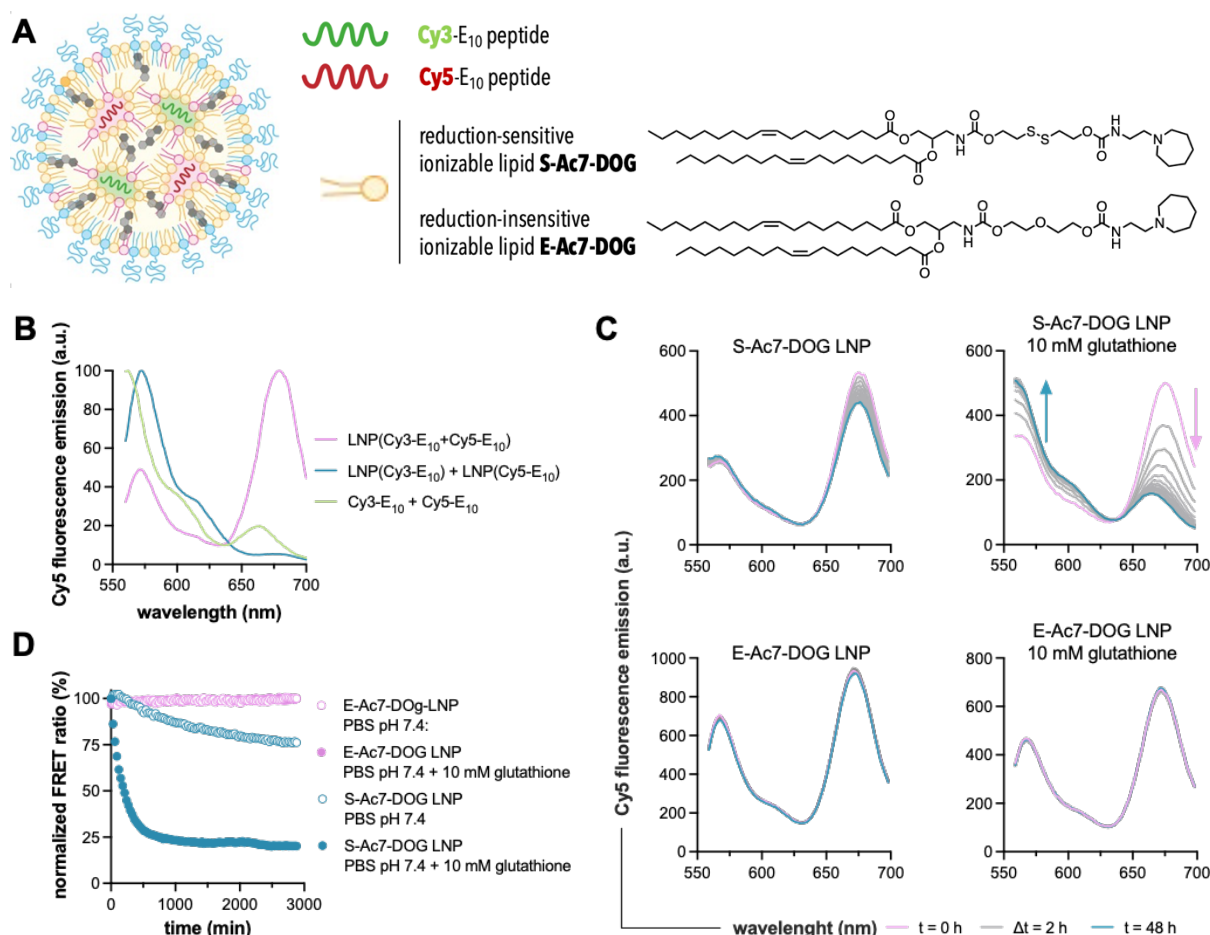


Figure 1. S-Ac7-DOG LNP release peptide in a reductive medium. (A) Schematic representation of the tested LNP (comprising the reduction-sensitive ionizable lipid S-Ac7-DOG and the non-reduction sensitive ionizable lipid E-Ac7-DOG, respectively) loaded with the FRET couple Cy3-E₁₀ and Cy5-E₁₀. (B) Fluorescence emission spectra upon excitation at 550 nm of Cy3-E₁₀ and Cy5-E₁₀ in solution, formulated in sperate LNP and in the same LNP. (C) Evolution over time of the fluorescence emission spectrum upon excitation at 550 nm of S-Ac7-DOG LNP and E-Ac7-DOG LNP in PBS at pH7.4 in absence or presence of 10 mM glutathione. (D) Corresponding evolution of the normalized FRET ratio.

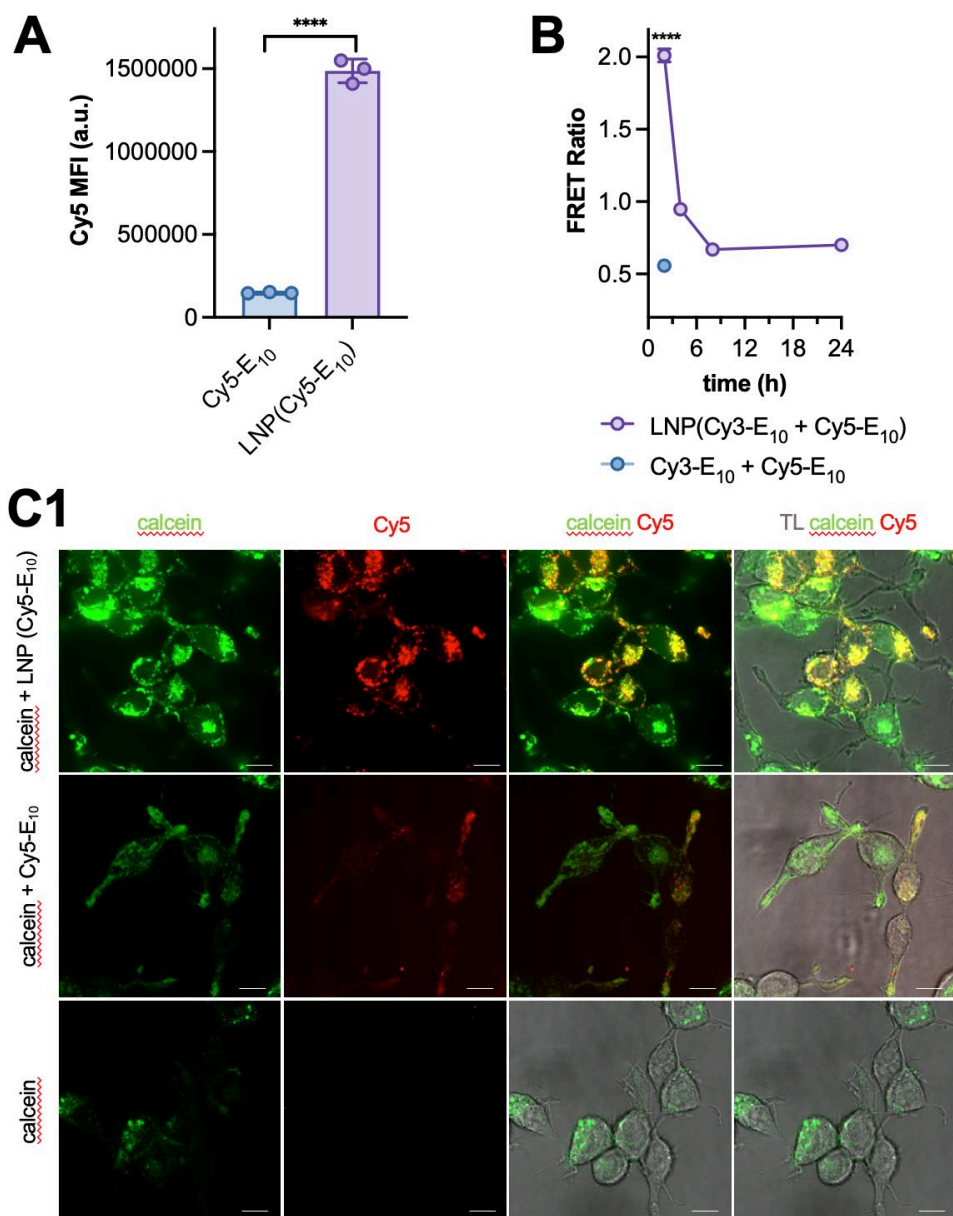
LNP formulation enhances intracellular peptide delivery

We first set out to investigate whether LNP formulation affects the cellular association of peptides. To this end, we formulated Cy5-E₁₀ into LNP and incubated it with DC2.4 cells. DC2.4 is an immortalized mouse dendritic cell line, that is frequently used as model for antigen presenting cells. Cells were treated with LNP(Cy5-E₁₀) or unformulated soluble Cy5-E₁₀, respectively, followed by flow cytometry analysis to measure cellular Cy5 fluorescence. Unformulated soluble Cy5-E₁₀ showed low cellular association, likely due to its fairly large hydrophilic structure and strong anionic charge density. Importantly, LNP-formulated Cy5-E₁₀ demonstrated a 10-fold increase in cellular fluorescence compared to the unformulated soluble Cy5-E₁₀ peptide (**Figure 2A**), thereby demonstrating the clear ability of LNP to efficiently mediate the cellular association of peptides.

We then aimed to investigate whether LNPs became internalized and, upon cellular internalization, whether peptides could be released from the LNPs to some extent. To this end, we analyzed the FRET signal as a function of time during cell culture, similar to the method detailed above in **Figure 1B-D**. DC2.4 cells were treated with LNP(Cy3-E₁₀ + Cy5-E₁₀) for 2h to allow for endocytosis to occur, followed by washing several times to remove unbound LNPs. Cells were then analyzed by flow cytometry at different timepoints, and the FRET ratio of the cellular fluorescence was calculated at each time point. More details about this experimental set-up to monitor intracellular disassembly of nanomaterials can be found in earlier work from our lab.^{46, 26} A soluble mixture of Cy3-E₁₀ and Cy5-E₁₀ was used as a control. As **Figure 2B** shown, at the earliest timepoint, strong FRET coupling in cells treated with LNPs (Cy3-E₁₀ + Cy5-E₁₀) was observed. A soluble mixture of Cy3-E₁₀ and Cy5-E₁₀, by contrast, exhibited minimal FRET coupling. As a function of time, the FRET ratio of cells treated with LNP (Cy3-E₁₀ + Cy5-E₁₀) rapidly decreased and reached a baseline level after 8h, close to the level of cells treated with a soluble mixture of Cy3-E₁₀ and Cy5-E₁₀. Hence, these findings suggested that upon cellular uptake, LNPs rapidly released their peptide payload. This is in line with our ongoing work on mRNA delivery with S-Ac7-DOG LNP where mRNA transfection is observed already within the first hours after treatment, both *in vitro* and *in vivo*.²⁸

To investigate whether peptide-LNPs can mediate cytoplasmic delivery of the encapsulated peptide, we loaded DC2.4 cells with calcein followed by treatment with LNP(Cy5-E₁₀) or unformulated soluble Cy5-E₁₀, respectively. Notably, calcein is a fluorophore with membrane-impermeable characteristics and is internalized into endosomal vesicles by endocytosis. Within these endosomal compartments, the fluorescence intensity of calcein is partially attenuated due to the high concentration and acidic microenvironment, yielding a distinct punctate fluorescence pattern. Upon vesicular disruption, the resulting calcein release into the cytoplasm manifests as an augmented and homogeneously distributed green fluorescent signal. Note that, while several semi-quantitative techniques exist for analysing endosomal rupturing, the calcein-release assay has been recognized as a simple yet effective approach.^{47, 72} Confocal microscopy indicated cellular uptake of the peptide and confirmed the enhanced uptake of the peptide-LNP, relative to unformulated soluble peptide, depicting a punctate pattern indicative of a predominant endosomal localization of the peptide (**Figure 2C1**). Furthermore, both untreated calcein-loaded cells and cells treated with unformulated soluble Cy5-E₁₀ displayed a dim punctate, green fluorescent, signal. By contrast, calcein-loaded cells treated with LNP(Cy5-E₁₀) exhibited a much brighter, green fluorescent, signal, which also spread throughout the entire volume of the cell, thereby indicating that some extent of endosomal rupture occurred. These observations were quantitatively confirmed by flow cytometry (**Figure 2C2**), showing an increase in calcein fluorescence in cells treated with

LNP(Cy5-E₁₀) compared to cells treated with unformulated soluble Cy5-E₁₀. Taken together, these findings highlight the potential of peptide-LNPs to increase cellular uptake of peptides and enable cytoplasmic delivery.



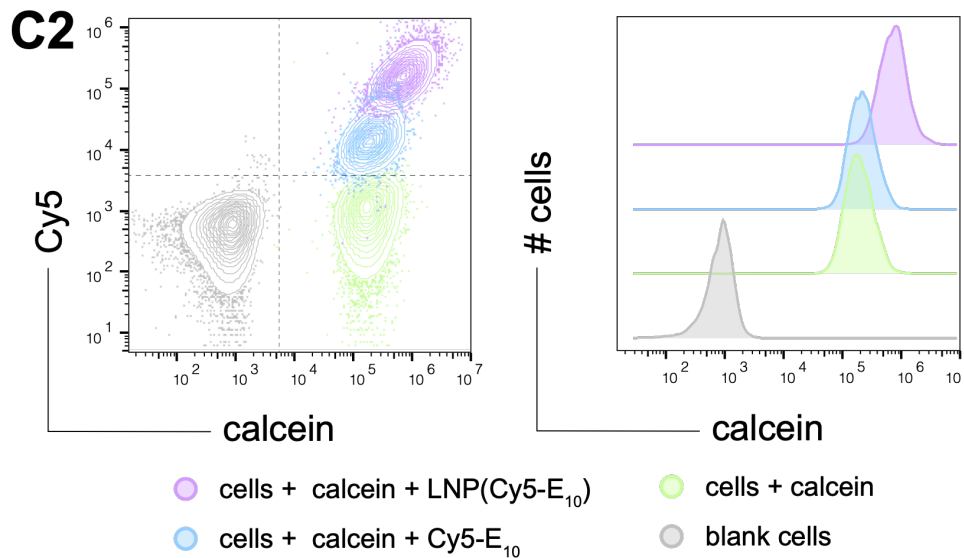


Figure 2. Peptide-LNP mediate intracellular peptide delivery (A) Flow cytometry analysis of cellular association of LNP(Cy5-E₁₀) and soluble Cy5-E₁₀, respectively, in DC 2.4 cells, 2h post incubation (n=3, mean $\pm$ SD; t-test: ****p < 0.0001). (B) FRET ratio measured at multiple time points of DC2.4 cells pulsed with LNP (Cy3-E₁₀ + Cy5-E₁₀) or soluble Cy3-E₁₀ + Cy5-E₁₀ (n=3; t-test: ****p < 0.0001). (C) Calcein release assay. (C1) Confocal microscopy of calcein-loaded DC2.4 cells pulsed with LNP(Cy5-E₁₀) or soluble Cy3-E₁₀. (C2) Flow cytometry scatterplots (left) and histograms of calcein-loaded DC2.4 cells pulsed with LNP(Cy5-E₁₀) or soluble Cy3-E₁₀.

LNP formulation increases the innate activation capacity of IMDQ-E₁₀

The RAW-Blue macrophage reporter cell line was used to investigate whether IMDQ-E₁₀ and LNP(IMDQ-E₁₀) can still trigger TLR7/8. RAW-Blue macrophages are genetically engineered cells that produce secreted embryonic alkaline phosphatase (SEAP) in response to triggering of various Toll like receptors (TLRs) and downstream signaling via the NF- κ B and AP-1 pathways. SEAP production was quantified by a high throughput multi-well colorimetric assay. Relative to native IMDQ, IMDQ-E₁₀ exhibited a strong loss in potency (**Figure 3A**).

E₁₀ is a negatively charged, hydrophilic, large (>1 kDa) molecule, which is poorly taken up by cells *in vitro*, as evidenced earlier in this work by measuring the cellular uptake of Cy5-E₁₀ by flow cytometry (**Figure 2A**) and microscopy (**Figure 2C1**). Hence, one can reasonably assume that conjugation to E₁₀ reduces cellular uptake of IMDQ. Additionally, conjugation of the primary aliphatic amino position of IMDQ inherently leads to a reduction in TLR-agonistic activity, likely due to reduced affinity for the receptor, which has been observed in several of our previous studies.^{26,48,49} Notably, all these studies also demonstrated that *in vivo*, by altering the pharmacokinetic profile of IMDQ through nanoparticle and amphiphile conjugation, a greatly improved therapeutic outcome could be achieved, which is also expected in the present work (*vide infra*).

As depicted in **Figure 3A**, LNP(IMDQ-E₁₀) showed increased potency compared to IMDQ-E₁₀, likely due to the increased cellular uptake and delivery into endosomal compartments (*cfr.*

microscopy images in **Figure 2C1**) where TLR7/8 is located on the endosomal phospholipid membrane. Empty LNPs, lacking IMDQ-E₁₀ were fully quiescent in this assay. When comparing the currently used peptide-LNPs, *i.e.*, comprising S-Ac7-DOG as an ionizable lipid, to our previously reported LNP system, which comprised DIPADS as an ionizable lipid,²⁵ we observed that S-Ac7-DOG LNPs (IMDQ-E₁₀) were slightly more potent in the RAW-Blue assay (**Figure S9**). Of note, replacing DOPE by DSPC and increasing the N:COOH ratio from 5 to 10 did not further improve TLR7/8 activation, thereby suggesting that in the latter LNP formulations, IMDQ might be less available for TLR triggering upon endocytosis of the LNP (**Figure S6**). In parallel we measured the influence of all samples on the cell viability and did not observe any signs of toxicity within the tested experimental window (**Figure 3B**).

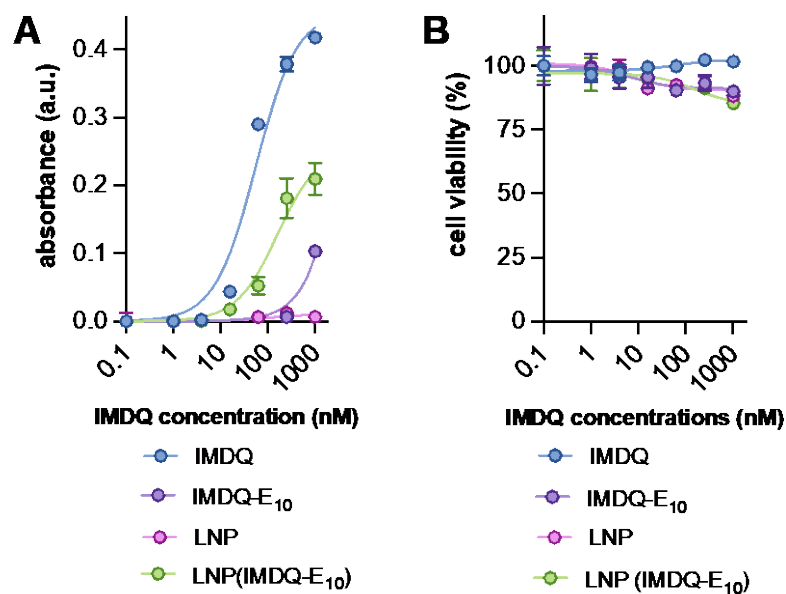


Figure 3. Peptide-LNPs induce innate activation *in vitro*. (A) Dose-response innate immune NF-kB activation measured using the RAW-Blue reporter cell assay. Secreted embryonic alkaline phosphatase (SEAP) served as reporter protein and was quantified by colorimetric analysis, measuring the absorbance at 620 nm. (n = 5, mean $\pm$ SD). (B) Cell viability measured by MTT assay (n = 5, mean $\pm$ SD).

Intravenous injection of LNP(IMDQ-E₁₀) induces a more damped innate immune activation compared to soluble IMDQ

Here we aimed at screening the effect of E₁₀-conjugation and LNP encapsulation on the innate activation capacity of IMDQ on a macroscopic level *in vivo*. As a route of administration of our LNP vaccine we opted for intravenous (IV) injection. This IV administration route was chosen based on findings by several prominent labs. For example, the Seder lab, reporting on peptides conjugated with an IMDQ analogue, found that immunization via the IV route elicited a much stronger antigen-specific immune response compared to subcutaneous and intramuscular routes. This effect was ascribed to the enhanced transcriptional quality of

antigen-specific CD8⁺ T cells after delivery to the spleen via intravenous administration.³¹ Furthermore, IV injection was also reported as the preferred route by Sahin and coworkers,⁷³ based on mouse and human patient data. Indeed, IV injection enhances LNP effectiveness by delivering them directly into the bloodstream, allowing rapid, widespread distribution and improved access to lymphoid tissues. In contrast, SC injection limits LNPs to the injection site and nearby lymph nodes, potentially reducing their distribution and exposure of the antigen to the immune system.

We used transgenic Balb/c IFN $\beta^{+/\Delta\beta-luc}$ mice to assess the intensity and biodistribution of the type I interferon cytokine response upon intravenous injection of the respective samples (**Figure 4A1**). This mouse model produces firefly luciferase under the control of the IFN- β promoter which can be triggered by a TLR7/8 agonist.⁵⁰ LNP(IMDQ-E₁₀), IMDQ-E₁₀ and native IMDQ were injected intravenously in mice into the tail vein at an equal IMDQ dose (*i.e.*, 10 μ g). Quantification of the full-body bioluminescence images (**Figure 4A2**) suggests that native IMDQ and LNP(IMDQ-E₁₀) elicited a similar IFN- β response at 4h post injection. This response was transient for LNP(IMDQ-E₁₀) as the bioluminescence signal strongly decreased at the 24 h timepoint. IMDQ-E₁₀ was significantly less potent at inducing IFN- β at both time points. A shorter duration of the innate response could potentially be beneficial to avoid over-stimulation and anergy.^{51,52} Notably, in soluble form, IMDQ caused a strong IFN- β response in the neck area. Using LNP containing near-infrared dye-labelled DSPE (*i.e.*, DSPE-Cy7), we also confirmed that LNPs indeed accumulated in the spleen, in addition to accumulating in the liver (**Figure 4B**).

Next, we assessed the expression levels of a panel of inflammatory cytokines 6h post administration of PBS, IMDQ, empty LNP and LNP(IMDQ-E₁₀), respectively. Cytokines of interest included: TNF- α , pivotal for inflammation and immune cell activation; IFN- α , enhancing antigen presentation and activation of CD8⁺ T cells; IFN- γ , activating macrophages and enhancing CD8⁺ T cell cytotoxicity; IL-12, stimulating NK cells and CD8⁺ T cells for increased cytotoxicity; and IL-6, modulating immune responses and inflammation, particularly in activating anti-tumoral CD8⁺ T cells. LNP(IMDQ-E₁₀) potently induced these cytokines (**Figure 4C**), suggesting their potential to instigate anti-tumor immunity. Furthermore, the chemokine CXCL10, which attracts activated T cells, including cytotoxic CD8⁺ T cells, to tumor sites, exhibited increased production in response to both soluble IMDQ and LNP(IMDQ-E₁₀).^{53–55} Furthermore, empty LNPs only induced a slight response beyond the background detection levels.

In parallel, serum samples were collected and analyzed for the presence of aspartate aminotransferase (AST) as a marker of liver damage. Mice treated with soluble IMDQ had elevated AST levels suggesting that innate immune activation by soluble IMDQ can result in

hepatocellular stress during the early phase of the response. However, mice injected with empty LNPs and LNP(IMDQ-E₁₀) showed lower AST levels (**Figure 4D**). Taken together, formulation of IMDQ in LNPs reduced systemic responses, relative to unformulated soluble IMDQ, which has previously been coined as “wasted inflammation” and prolonged systemic toxicity. This has been an important limitation thus far for clinical development of small molecule TLR7/8 agonists.²⁶ Formulation of small molecule TLR7/8 agonists, such as IMDQ, in LNPs is, hence, a powerful strategy to mitigate these issues and alter the pharmacokinetic profile.⁵⁶

Intravenous injection of LNPs delivers peptide and TLR7/8 agonist to antigen presenting cells in the spleen and induces broad innate immune activation

We then set out to investigate the uptake of LNPs and immune activation at a cellular level. Hereto, LNP(IMDQ-E₁₀/Cy5-E₁₀) were injected intravenously into the tail vein of mice and the biodistribution of the Cy5 signal and the innate activation were analyzed by flow cytometry. As controls, we selected LNP(Cy5-E₁₀), IMDQ-E₁₀/Cy5-E₁₀, native IMDQ and PBS, comprising an equal IMDQ (*i.e.*, 10 µg) and/or Cy5 (*i.e.*, 2 µg) dose. Spleens were harvested one day post injection and analyzed by flow cytometry for cellular Cy5 fluorescence and expression of maturation/activation markers by a panel of immune cell subsets. Fluorescence from soluble Cy5-E₁₀ peptide could not be detected above background levels. By contrast, LNP-formulated Cy5-E₁₀ peptide was strongly associated to splenic macrophages and DCs (**Figure 5A**). Within the DC population, up to 25% of the conventional dendritic cells type 1 (cDC1) sub-population became positive for LNP, whereas only a minor fraction of 2-3% of the conventional dendritic cells type 2 (cDC2) population became associated with LNP. cDC1 cells are highly capable of cross-presenting exogenous antigens to MHC I complex and drive the generation of cytotoxic T cells. Hence, cDC1 cells are an attractive target cell population for vaccine delivery.^{57,58} The presence of IMDQ-E₁₀ in LNP increased LNP uptake by macrophages and cDC1s, but not cDC2s. Notably, the fraction of LNP+ cDC2s was a 10-fold lower than the fraction of LNP+ cDC1s. Also, a significant fraction of B cells was positive for LNP, in particular when LNP contained IMDQ. T cells, being non-phagocytic cells, were positive for LNP only to a very minor extent.

Next, we analyzed the DC population for their upregulation of the chemokine receptor CCR7 (**Figure 5B**), which regulates migration of mature DCs to secondary lymphoid tissue, where they subsequently can present antigens to T cells.⁵⁹ CCR7 expression was not affected by administration of soluble Cy5-E₁₀, but was significantly upregulated in all other groups, in both cDC1 and cDC2 subsets. Empty LNP and IMDQ-E₁₀/Cy5-E₁₀ induced intermediate

upregulation while native IMDQ and LNP(IMDQ-E₁₀/ Cy5-E₁₀) induced high expression levels of CCR7.

The ability of empty LNPs to induce innate immune activation has recently been reported by the Pardi lab ⁶⁰ and ascribed to the induction of IL-6. Lipids and polymers featuring a similar azepanyl-based ionizable amine,⁶¹ as used in the design of S-Ac7-DOG, have been reported to activate STING (stimulator of interferon genes). We tested the capacity of empty S-Ac7-DOG LNP to activate STING on a mouse STING reporter cell assay (**Figure S8**), but did not observe a significant activity. Although this does not fully rule out a contribution of STING activation to the observed innate activation, its contribution is likely very minor.

Native IMDQ was more potent than unformulated IMDQ-E₁₀, which is in line with our *in vitro* data (**Figure 3A**) that show loss in activity of IMDQ upon E₁₀ conjugation. A similar trend was observed for the upregulation of the costimulatory ligands CD80 and CD86, which play a critical role in the initial steps of T cell activation (**Figure 5B**).⁶²⁶³ CD86 and CD80 were not upregulated in mice receiving Cy5-E₁₀, but were upregulated in all other groups. LNP(IMDQ-E₁₀/Cy5-E₁₀) induced the highest upregulation of both markers. Hence, LNP formulation of IMDQ-E₁₀, which exhibits moderate *in vitro* innate activation capacity, induces robust innate activation *in vivo*, likely by mediating enhanced uptake by innate immune cells upon systemic administration. The flow cytometry density plots (**Figure 5C**) of the Cy5 signal versus the respective maturation markers CCR7, CD80 and CD86 clearly show that cDC1s that took LNP(IMDQ-E₁₀/Cy5-E₁₀) were fully mature. Although visually less clear due to a lower extent of LNP (IMDQ-E₁₀/Cy5-E₁₀) uptake, also Cy5+ cDC2s were fully mature. Large fractions of cDC1s and cDC2s that did not take up LNP(IMDQ-E₁₀/Cy5-E₁₀) (*i.e.*, Cy5- cells in the Q1 quadrant) were also mature.

CD69, a ligand of early lymphocyte activation on B and T cells ⁶⁴ was strongly activated in B and T cells in groups that received IMDQ, IMDQ-E₁₀ and LNP(IMDQ-E₁₀/Cy5-E₁₀) (**Figure 5D**). Empty LNP did not stimulate CD69 upregulation. LNP(IMDQ-E₁₀/Cy5-E₁₀) was more potent than IMDQ-E₁₀, but less potent than native IMDQ. B cells express TLR7/8 and were associated to LNP to a certain extent. However, it is unsure whether B cells also endocytosed LNP. T cells showed almost no association to LNP and do not express TLR7/8. Hence, we hypothesize that DCs, B and T cell become activated in response to type I interferon cytokines produced by DCs, macrophages and possible other TLR7/8-expressing cells that endocytosed LNP.

Taken together, our data demonstrate that encapsulating the peptide and TLR7/8 agonist in LNPs efficiently delivers both compounds to antigen presenting cells in the spleen and triggers an innate immune response leading to broad activation of multiple splenocyte subsets.

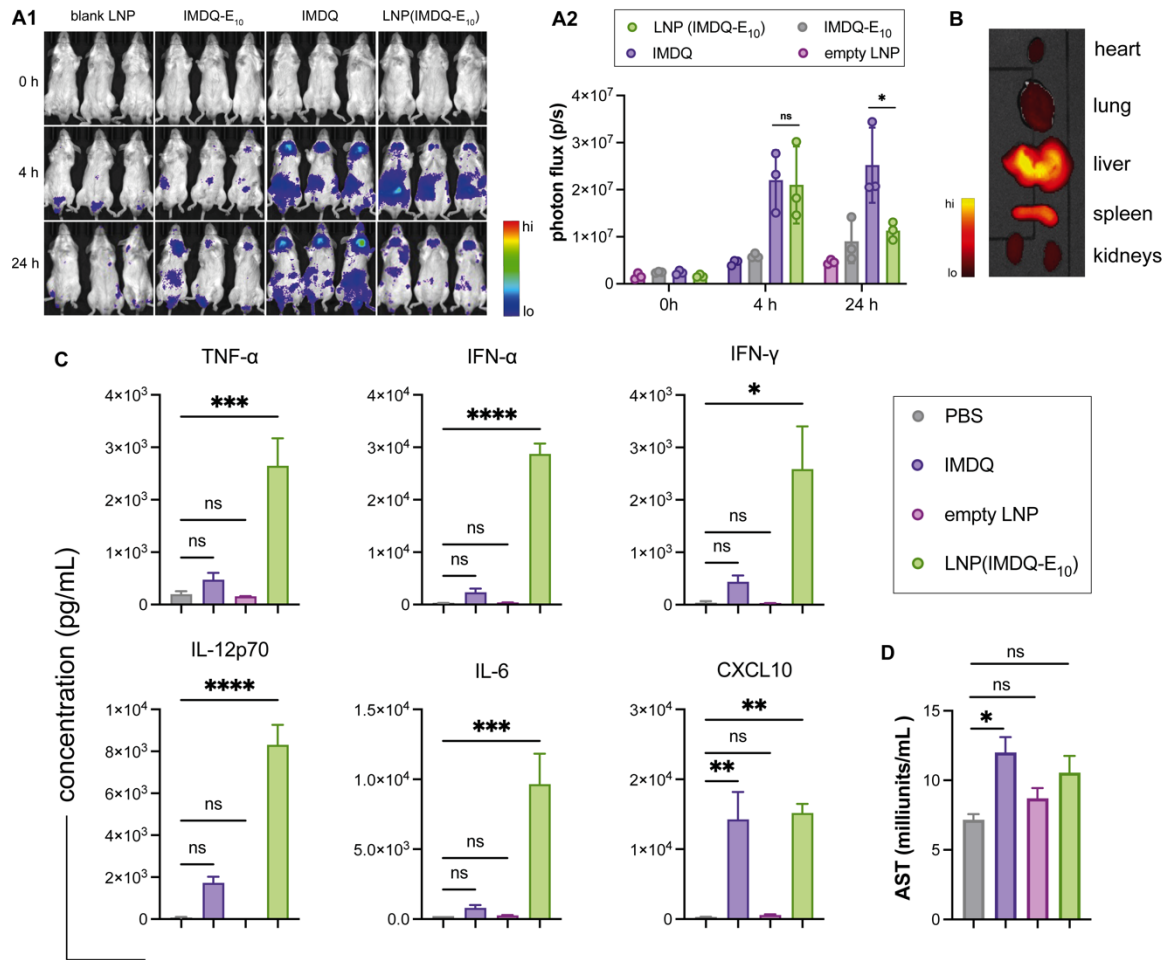
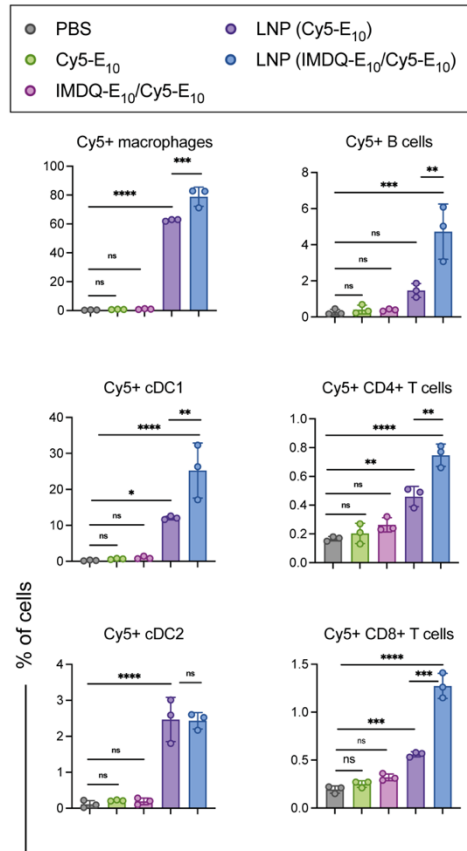
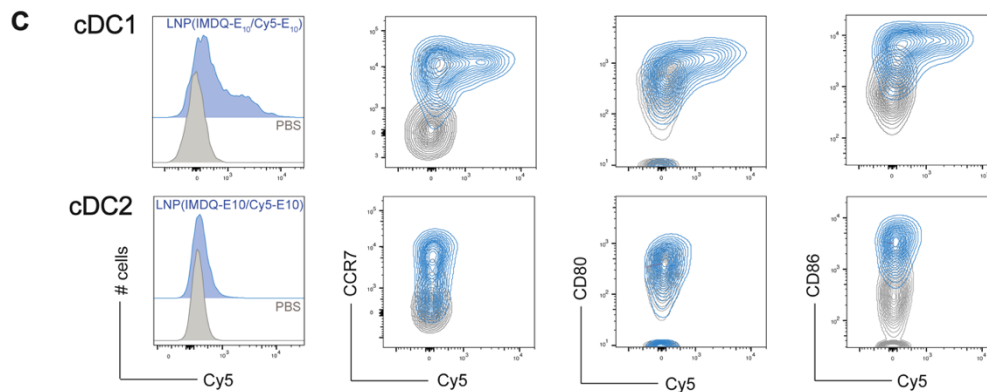
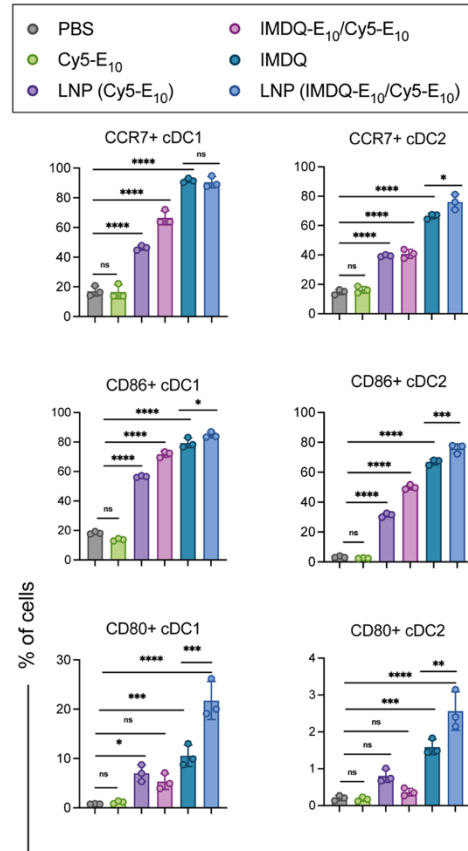


Figure 4. LNP(IMDQ-E₁₀) induce cytokine production *in vivo*. Balb/c mice were injected intravenously with formulations containing (or not) 10 ug of IMDQ. **(A1)** Bioluminescence imaging of Balb/c IFN $\beta^{+/\Delta\beta-luc}$ mice, 4 h and 24 h post intravenous (IV) injection. **(A2)** Quantification of the total luminescence (n=3; one-way ANOVA test: *p < 0.05, **p < 0.01). **(B)** C57BL/6 mice were injected with DSPE-Cy7 labeled LNP(E7Ag-E₁₀/IMDQ-E₁₀). After 6 hours, major organs were harvested and Cy7 fluorescence was measured using IVIS imaging. **(C)** A panel of inflammatory cytokines was assessed 6 hours following IV administration of samples in C57BL/6 mice using an LEGENDplex™ Multi-Analyte Flow Assay Kit. **(D)** Serum aspartate aminotransferase (AST) levels were assessed 6 hours post IV administration of samples in C57BL/6 mice using an AST Activity Assay Kit. (n=3; one-way ANOVA test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

A - cellular Cy5 fluorescence



B - DC activation



D - B cell and T cell activation

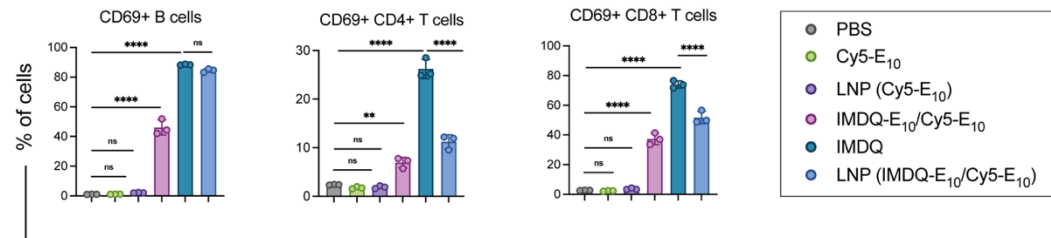


Figure 5. LNP(IMDQ-E₁₀/Cy5-E₁₀) induce broad immune activation in splenocytes. C57BL/6 mice were injected intravenously with formulations containing (or not) 10 ug of antigen and 10 ug of IMDQ. FACS analysis of (A) Cy5 association by splenic immune cell subsets, (B) expression of maturation markers on splenic DCs, (C) corresponding contour plots and (D) expression of activation markers on splenic B and T cells. Spleens were dissected 24h post IV injection (n = 3, mean ± SD; one-way ANOVA test : *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

LNP encapsulated E7 antigen and TLR7/8 agonist induce antigen-specific T cell responses and protective immunity against E7-expressing tumors

A peptide antigen comprising E₁₀ and the minimal epitope from HPV 16 oncogene E7, *i.e.*, E7Ag-E₁₀ was co-encapsulated with IMDQ-E₁₀ into LNP (*i.e.*, LNP(E7Ag-E₁₀/IMDQ-E₁₀)) and injected intravenously in mice at a 10 µg E7Ag dose and a 10 µg IMDQ dose. Other groups included LNP(E7Ag-E₁₀), E7Ag/IMDQ, E7Ag and PBS. Mice were given a prime-boost immunization with a two-week interval **Figure 6A**. Seven days post each immunization, the presence of antigen-specific E7-tetramer positive CD8⁺ T cells was detected in peripheral blood by flow cytometry. In prophylactic setting, after prime immunization (**Figure 6B**), the percentage of tetramer positive CD8⁺ T cells was low and detection above the background level was only possible in mice immunized with LNP(E7Ag-E₁₀/IMDQ-E₁₀). After boost immunization, the percentage of tetramer positive CD8⁺ T cells increased up to 10 % in mice immunized with LNP(E7Ag-E₁₀/IMDQ-E₁₀), whereas in all other groups no antigen-specific T cells were detected (**Figure 6B**). These results suggested that delivery of antigen and TLR7/8 agonist by LNP enhanced cross-presentation of exogenous antigen on MHC I by antigen presenting cells to CD8⁺ T cells *in vivo*. LNP(E7Ag-E₁₀) without IMDQ were unable to generate detectable levels of antigen-specific T cells in the blood. Hence, it is clear that TLR7/8 triggering by IMDQ is required to create a milieu that drives differentiation and proliferation of antigen-specific CD8⁺ T cells.

As only mice immunized with LNP(E7Ag-E₁₀/IMDQ-E₁₀) exhibited detectable levels of E7 antigen-specific T cells beyond the background detection threshold, we selected this group for investigating the anti-tumor response against E7-expressing tumors in a prophylactic and a therapeutic setting, respectively. In a prophylactic setting, mice were implanted subcutaneously in the right rear flank with E7-expressing (mouse lung epithelial) TC-1 cancer cells, 2 weeks post booster immunization. Mice were either sacrificed within a 21-day observation period, with regular monitoring of the tumor volume, or when the tumor size exceeded 1500 mm³. The individual tumor growth curves depicted in **Figure 6C** illustrate that non-immunized mice (PBS group) exhibited rapid tumor growth. Conversely, mice immunized with LNP(E7Ag-E₁₀/IMDQ-E₁₀) demonstrated 100% protection against TC-1 tumor challenge. Although two mice developed minor tumors during the observation period, they promptly regressed. Notably, when performing the immunization through subcutaneous administration (**Figure S11**), mice were not protected against a tumor challenge, which highlights the efficacy of intravenous immunization to mount a broad and potent anti-tumor immunity, in line with recent reports of the Seder lab.³¹

In a therapeutic setting, mice were implanted with TC-1 cells followed by two consecutive treatments with LNP(E7Ag-E₁₀/IMDQ-E₁₀) with a one-week interval starting at 7 days post-implantation. Monitoring of the tumor volume revealed that LNP(E7Ag-E₁₀/IMDQ-E₁₀)-immunized mice, initially displayed growth kinetics akin to those observed in the PBS control group (**Figure 6C**). However, in response to the prime immunization, the majority of tumors started to regress. The subsequent boost immunization augmented the therapeutic effect, ultimately culminating in the complete elimination of tumors.

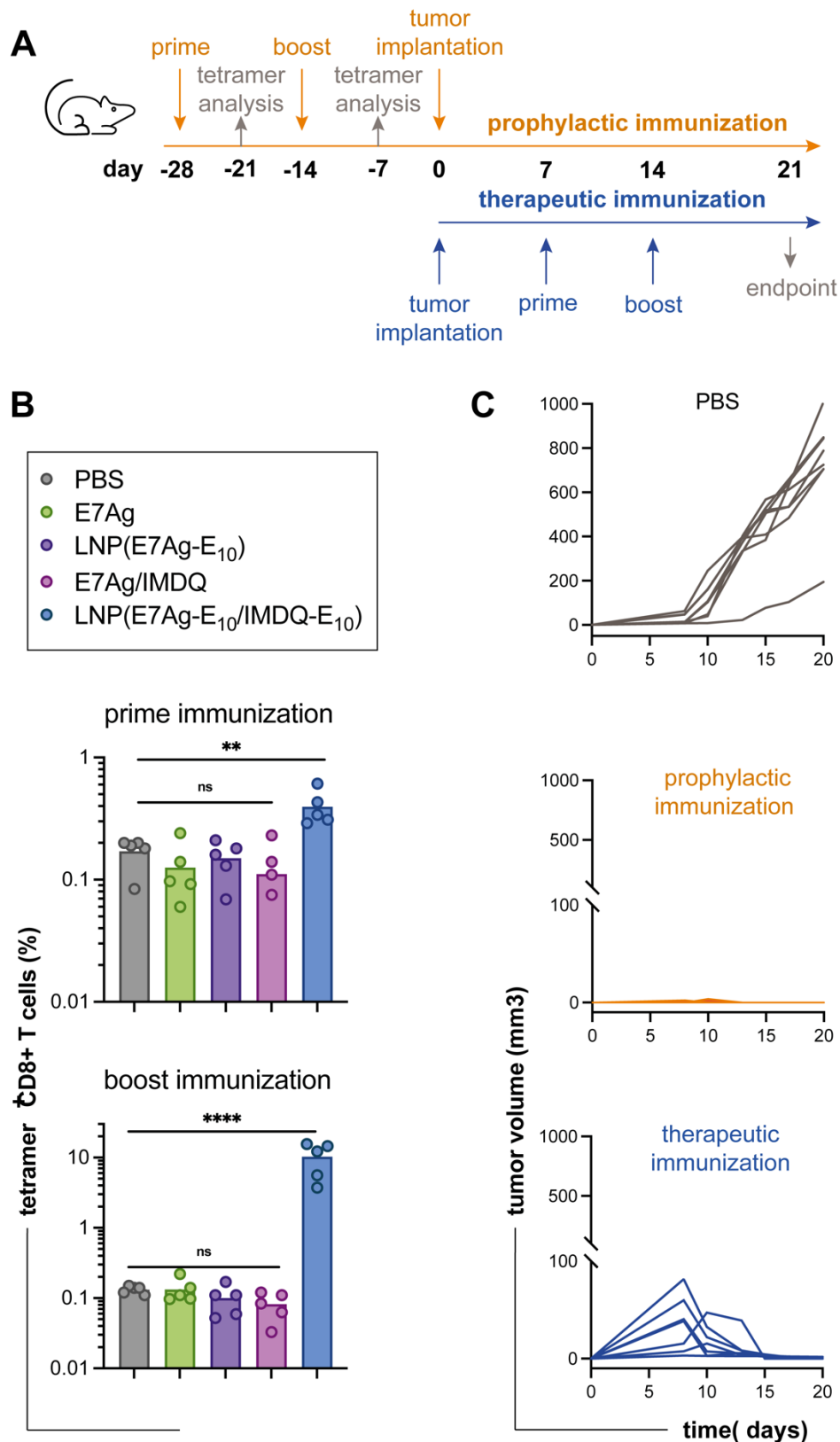


Figure 6. LNP(E7Ag-E₁₀/IMDQ-E₁₀) induces antigen specific T cells responses and mount anti-tumor immunity. (A) Experimental outline. C57BL/6 mice were injected intravenously with formulations containing (or not) 10 ug of antigen and 10 ug of IMDQ. **(B)** Frequency of tetramer+ CD8+ T cells in the blood, 5 days post boost immunization (n=5; one-way ANOVA test: ****p < 0.0001). **(C)** Individual TC-1 tumor growth curves (n≥6). The endpoint was reached when tumor volume reached 1500 mm³ or when the weights lost was > 20%.

To deepen our understanding of the specific mechanism responsible for the observed anti-tumor response, we examined through flow cytometry CD8⁺ T cells in the blood post-boost immunization and at the experimental endpoint. In the prophylactic treatments, a pronounced increase in antigen-specific (E7-tetramer⁺) CD8⁺ T cells was observed. They constituted 13% of total CD8⁺ T cells after the initial boost, decreasing to 3% at 21 days post-tumor implantation (**Figure 7A**). Remarkably, a significant increase in the expression of CX3CR1, coupled with augmented CD69, indicated a higher activation of CD8⁺ T cells following LNP treatment (**Figure 7B-C**). Simultaneously, an elevated expression of immune checkpoint markers PD-1 and Lag3 (**Figure 7D-E**) in LNP-treated mice suggested a potential state of CD8⁺ T cell exhaustion, despite reduced TOX1 expression (**Figure 7F**). Moreover, the heightened expression of CX3CR1 on CD8⁺ T cells in the blood, known to correlate with a predictive response to immune checkpoint inhibitors (ICI) implied that ICI treatment could alleviate exhaustion, potentially leading to improved outcomes in the therapeutic setting.⁶⁵ Furthermore, the larger population of effector memory CD44⁺ CD62L⁻ CD8⁺ T cells indicated the potential persistence of antigen memory (**Figure 7G**).

A similar pattern of activation and exhaustion was observed in the CD8⁺ T cell population in the therapeutic setting. In this case, antigen-specific E7-tetramer⁺ CD8⁺ T cells accounted for 7% of the total CD8⁺ T cell population (**Figure 8A**). We observed again an increase in the expression of the activation markers, CD69 and CX3CR1 (**Figure 8B-C**), although to a lesser extent compared to the prophylactic setting. Also in this case, the expression of exhaustion markers PD-1, Lag3 and TOX1 was higher in LNP-treated mice (**Figure 8D-F**), and a larger population of effector memory CD8⁺ T cells was observed (**Figure 8G**).

Taken together, our findings underscore a robust antigen-specific CD8⁺ T cell response induced by E7Ag and IMDQ-loaded LNPs in both the therapeutic and the prophylactic treatments.

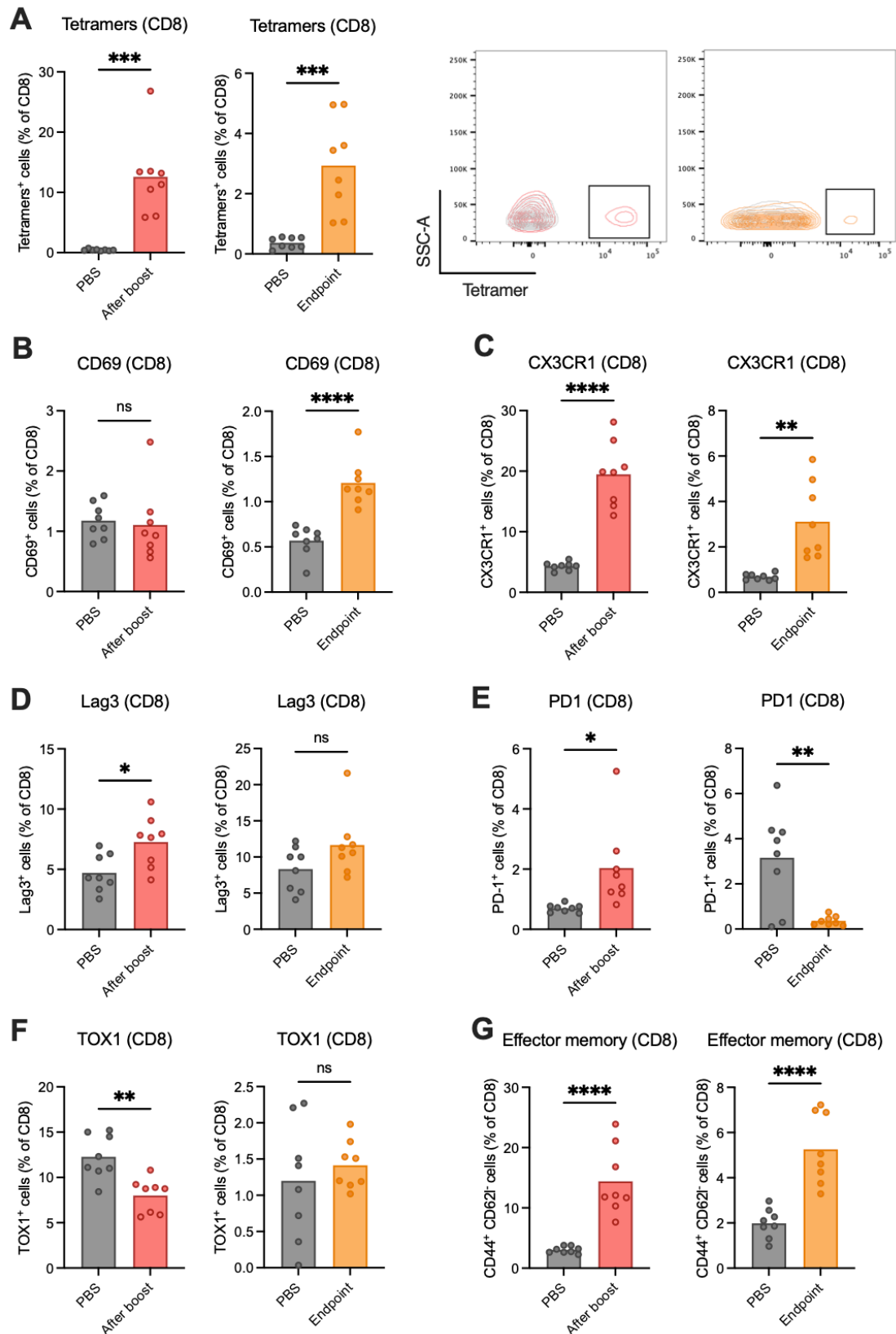


Figure 7. LNP(E7Ag-E₁₀/IMDQ-E₁₀) induces antigen specific CD8⁺ T cell activation in a prophylactic setting. (A) Frequency (left) and density plots (right) of tetramer⁺ CD8⁺ T cells (B-G) Expression of activation and exhaustion marker in CD8⁺ T cells in the blood, 5 days post boost immunization and at endpoint in the prophylactic setting. (n=8, mean $\pm$ SEM; unpaired t-test: *p < 0.05, **p < 0.01, *p < 0.001, ****p < 0.0001).**

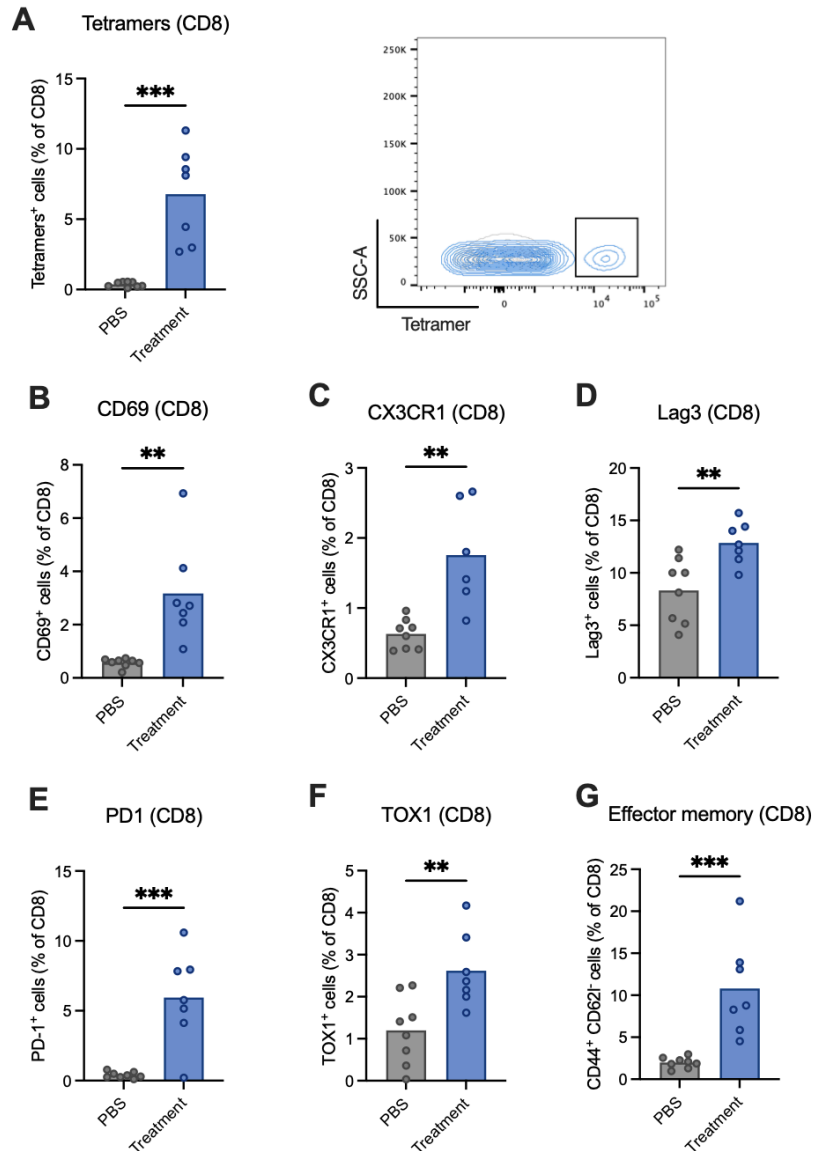


Figure 8. LNP(E7Ag-E₁₀/IMDQ-E₁₀) induces antigen specific CD8⁺ T cell activation in a therapeutic setting. (A) Frequency (left) and density plots (right) of tetramer⁺ CD8⁺ T cells **(B-G)** expression of activation and exhaustion marker in CD8⁺ T cells in the blood at endpoint in the therapeutic setting (n = 7-8, mean ± SEM; unpaired t-test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

CONCLUSION

In conclusion, we presented a simple, yet efficient, method to formulate peptide antigen and an imidazoquinoline TLR7/8 agonist into LNP. Our strategy is based on extending the peptide epitope with 10 glutamic acid residues. The latter endows the peptide with an overall anionic charge to allow for electrostatic interaction with an ionizable cationic lipid during an ethanol-water solvent exchange process to form LNP. Intravenously injected LNP are taken up by

antigen presenting cell in the spleen and the TLR7/8 agonist induced broad activation in splenocytes. Co-delivery of antigen and TLR7/8 agonist mounts a robust antigen specific CD8⁺ T cells response after prime-boost immunization and offers protective immunity against an antigen-expressing tumor challenge in a prophylactic and therapeutic setting. Our LNP approach holds flexibility towards encapsulation of different cargoes that shape the amplitude and quality of the immune response, and towards surface-modification for targeting of specific immune cells subsets.⁶⁶

MATERIALS AND METHODS

Materials

Peptides were custom synthesized by Genscript. IMDQ was synthesized according to literature⁶⁷⁴. IMDQ-E₁₀ was synthesized by standard Fmoc-based solid-phase peptide synthesis and purified (>90%) by high-performance liquid chromatography (HPLC) (Merck, Hitachi LaChrom, Tokyo, Japan). S-Ac7-DOG and E-Ac7-DOG were synthesized in our labs.²⁸ Phospholipids, cholesterol, PEG-lipids were purchased from Avanti. eGFP-encoding mRNA-LNP was obtained from Cellerna Bioscience, Germany. The STING agonist diABZI was purchased from Invivogen.

Cells

DC 2.4 cells⁶⁸ were a kind gift from Dr. Kenneth Rock (University of Massachusetts, Boston, US). The RAW Blue reporter cell line QUANTI-Blue™ and 293-Dual™ mSTING cells were obtained from InvivoGen. TC-1 cells were obtained from ATCC. PBS, cell culture media and supplements were obtained from ThermoFisher. The calcein molecule was purchased from Sigma-Aldrich.

Mice

C57BL/6 female mice were purchased from Janvier. IFN- β reporter mice (IFN- β Δ -luc/ Δ -luc) were provided by Dr. Stefan Lienenklaus (Hannover Medical School, Hannover, Germany⁶⁹). Mice were housed in individual ventilated cages, and given ad libitum access to food and water. All experiments were approved by the ethical committee of Ghent University (EC application number: 2022-062).

Cell culture

DC2.4 cells (immortalized dendritic cell line) were cultured in RPMI-glutamax, supplemented with 10 % FBS, 1 % penicillin/streptomycin and 1 mM sodium pyruvate. RAW-Blue™

macrophages were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 1 % penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 200 µg/mL Zeocin. TC-1 Cells were cultured in RPMI-glutamax medium supplemented with 10% FBS, 1 % penicillin/streptomycin, 1mM sodium pyruvate and 0.4 mg/mL geneticin. Cells were incubated at 37 °C in a controlled, sterile environment of 95 % relative humidity and 5 % CO₂.

LNP formulation

E7Ag-E₁₀, IMDQ-E₁₀, Cy3-E₁₀ or Cy5-E₁₀ were dissolved in an aqueous 25 mM acetate buffer (pH 5.2). Ionizable lipid (S-Ac7-DOG), phospholipid (DOPE or DSPC), cholesterol and DMG-PEG were dissolved in ethanol at a molar ratio 50:10:38.5:1.5. An N/COOH (N: amine from ionizable lipid, COOH: carboxylic acid from glutamic acid) ratio of 5:1 and 10:1 were tested. The ethanol solution was injected into a triple volume of aqueous solution under vigorous mixing on a bench top vortexer. LNP were then dialyzed against PBS in 3.5 K MWCO Slide-A-Lyzer (ThermoFisher) dialysis cassettes for at least 2 h before use. LNP were concentrated, if necessary, using an Amicon Ultra 10K NMWL centrifugal filter (ThermoFisher).

LNP characterization

Dynamic light scattering (DLS) and electrophoretic mobility measurements were performed on a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.) equipped with a HeNe laser (λ = 633 nm) and detection at scattering angle of 173°. Z-average and polydispersity index were given by the CONTIN particle size distribution. Zeta potential values were calculated from the electrophoretic mobility. LNP suspension was diluted in sterile 10 mM HEPES buffer (pH 7.4) before analysis. For LNP stability assessment, LNP samples were incubated in PBS buffer (pH 7.4) at 4°C for one week, followed by a subsequent DLS analysis.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Sample containing 1 mg/mL of LNP (E7Ag-E₁₀/IMDQ-E₁₀) in PBS buffer was applied onto a Quantifoil R2/1 holey carbon film supported by a TEM copper grid (300 mesh, Quantifoil Micro Tools GmbH, Grossbloebichau, Germany). Prior to application, the grid underwent glow discharge treatment for 1 min at 6 mA using the ELMO Glow Discharge System (Agar Scientific Ltd, Essex, UK) to facilitate optimal sample settling. Excess liquid was removed from the grid by blotting with Whatman 1 filter paper (Whatman plc, Maidstone, UK) for 1 s. The sample was then rapidly plunged into liquid ethane at -176 °C using Cryoplunge 3 with GentleBlot system (Gatan Inc., Pleasanton, CA, USA) operating at 100% relative humidity. The vitrified samples were subsequently transferred using a Gatan 626 cryoholder (Gatan Inc., Pleasanton, CA, USA) and examined with a JEOL 1400+ transmission electron microscope

(Jeol Ltd., Tokyo, Japan) equipped with a LaB6 filament and TVIPS F416 charge-coupled device camera at 140 kV under low-dose conditions ($\approx 10 \text{ e}^- \text{Å}^{-2} \text{s}^{-1}$) at $\times 60,000$ magnification.

pKa determination by TNS assay

pKa values were measured by TNS assay as described previously.⁷⁰ pH buffers from 3.5 to 11.5 in 0.5 increments were prepared by adjusting a solution of 20 mM sodium phosphate, 25 mM citrate, 20 mM ammonium acetate, and 150 mM NaCl with 0.1 N NaOH and 0.1 N HCl. 100 μL of the respective buffers were added to black 96-well (3 replicated units). TNS reagent (300 μM stock solution) was added to reach a final concentration of 6 μM . LNP were added at an ionizable lipid concentration of 75 μM . Fluorescence emission intensities were recorded on a Perkin Elmer Enight multimode platereader at an excitation of 322 nm and an emission of 431 nm. The data points were fitted by a sigmoidal nonlinear curve using the GraphPad software package. The pKa of the respective LNP was calculated as EC50 values.

In Vitro mRNA transfection

DC 2.4 cells were seeded in 96-well plates at 2×10^4 cells per well. After overnight incubation, cells were transfected with eGFP-encoding mRNA-LNP at 200 ng per well dose of mRNA for 24 h. Unformulated mRNA was used as a negative control. eGFP transfection efficiency was measured on a BD Accuri C6 flow cytometer. Data were analysed using the FlowJo software package.

IMDQ-E₁₀ encapsulation efficiency in LNP

Cy3-E₁₀ was used as a mimic to measure encapsulation of IMDQ-E₁₀ in LNP. LNP (E₁₀-Cy3) were subjected to high-speed centrifugation (*i.e.*, 20,000 G for 30 min). Then, the supernatant was aspirated and the absorbance at 550 nm was measured on a Perkin Elmer Enight multimode platereader. Empty LNP were served as a blank control. The concentration of non-encapsulated Cy3-E₁₀ in the supernatant was calculated using a calibration curve of unformulated Cy3-E₁₀. The encapsulation efficiency of Cy3-E₁₀ was calculated according to the following equation:

$$\text{Encapsulation efficiency} = \frac{\text{Total Cy3-E}_{10} - \text{Free Cy3-E}_{10}}{\text{Total Cy3-E}_{10}} \times 100\%$$

Peptide encapsulation efficiency in LNP

LNP (E7Ag-E₁₀) and empty LNP, as a control, were subjected to high-speed centrifugation (20,000G, 30mins, 4°C). Then, supernatant was aspirated and transferred to 96-well plates. BCA working reagent was added and the plates were placed on a shaker. After 30 min incubation at 37 °C, the absorbance at 562 nm was measured on a Perkin Elmer Enight

multimode platereader. Empty LNP were served as a blank control. The concentration of non-encapsulated E7Ag-E₁₀ in the supernatant was calculated using a calibration curve of unformulated E7Ag-E₁₀. The encapsulation efficiency of E7Ag-E₁₀ was calculated according to the following equation:

$$\text{Encapsulation efficiency} = \frac{\text{Total E7Ag-E}_{10} - \text{Free E7Ag-E}_{10}}{\text{Total E7Ag-E}_{10}} \times 100\%$$

FRET Assay

Cy3-E₁₀ and Cy5-E₁₀ were encapsulated into LNP at a 1:1 ratio. LNP were formulated with S-Ac7-DOG or E-Ac7-DOG, respectively. LNP were diluted in PBS buffer with or without 10 mM glutathione in black 96 well plates and incubated at 37 °C. Fluorescence emission spectra upon excitation at 550 nm were recorded every 30 min for 48h using an Agilent fluorescence spectrophotometer. The spectra of a mixture of unformulated Cy3-E₁₀ and Cy5-E₁₀, LNP (Cy3-E₁₀) and LNP (Cy5-E₁₀) were recorded as controls. The FRET ratio was calculated as the ratio of the emission at 670 nm (wavelength of maximum emission of Cy5) to 562 nm (wavelength of maximum emission of Cy3).

In vitro FRET assay

DC2.4 cells were seeded in 24-well culture plates at 1×10⁵ cells per well in 480 µL of cell culture medium and incubated overnight. The next day, 20 µL of 1 mg/mL of LNP (Cy3-E₁₀ + Cy5-E₁₀), LNP (Cy3-E₁₀) and LNP (Cy5-E₁₀) or PBS was added to the cells and mixed. After 2h incubation, the culture medium was aspirated, the cells were washed with PBS twice. Subsequently, part of the cells were dissociated using a cell scraper and transferred into centrifuge tubes. After centrifugation, the cell pellet was resuspended in PBS and analysed by on a BD Accuri C6 flow cytometer. The remaining cells were continuously incubated and dissociated at different time points (*i.e.*, 4, 8, 12 and 24 h), and analysed by flow cytometry. Cy3 fluorescence was detected in the FL2 channel, Cy5 was detected in the FL4 channel. The FRET signal was detected in the FL3 channel. Data were processed using the FlowJo software package. The FRET ratio was calculated as the ratio of the FL3/FL2 mean fluorescence intensity (MFI).

Calcein Release Assay by Confocal Microscopy

DC 2.4 cells were seeded at a density of 2 × 10⁴ cells per well in an IBIDI® µ-Slide 8 Well and incubated overnight in a CO2 incubator at 37 °C. The following day, cells were exposed to media containing Cy5-tagged peptide (0.01 mg/mL) and/or calcein (0.15 mg/mL) for 4 hours. Subsequently, cells were washed with PBS and subjected to confocal imaging using a Leica DMI6000B microscope equipped with a 63 × 1.40 NA objective. Imaging was performed with

an AndorDSD2 confocal scanner and a Zyla 5.5 CMOS camera. Image analysis and processing were conducted using the ImageJ software package.

Calcein Release Assay by Flow Cytometry

DC 2.4 cells were cultured in 24-well plates at a seeding density of 1×10^5 cells per well (480 μ L per well) and allowed to incubate overnight. Following incubation, cells were pulsed with 20 μ L/well of media containing Cy5-tagged peptide (25 μ g/mL) and/or calcein (0.15 mg/mL) for 4 hours. Subsequently, the supernatant was removed, cells were washed with PBS, and detached using cell dissociation buffer. The well contents were transferred to tubes, centrifuged (5 min, 300 G, 4 °C), and after removal of the supernatant, the cell pellets were resuspended in PBS. Analysis was performed using a BD Accuri C6 flow cytometer, with each sample conducted in quadruplicate (n=4). Data analysis was carried out using the FlowJo software package.

RAW Blue Assay

RAW Blue™ macrophages were seeded in a flat-bottom 96-well plate with density of 2×10^4 cells per well, in 180 μ L of culture medium. After overnight incubation at 37 °C, cells were pulsed with 20 μ L sample per well at IMDQ concentrations of, respectively 0, 1, 4, 16, 64, 256, 1024, 4096 nM and then incubated for another 24h. Subsequently, 50 μ L of supernatant was transferred to new flat-bottom 96-well plates followed by addition of 150 μ L of QUANTI-Blue reagent solution, prepared according to the manufacturer's instruction (InvivoGen). After 30 min to 2h (depending on visual inspection of the colour of the samples) at 37 °C, the secreted embryonic alkaline phosphatase (SEAP) levels were determined by UV-vis spectrophotometry at 620 nm using a Perkin-Elmer Ensign multimode platereader. Activity was determined by an increase in optical density relative to the negative control treated with PBS. All experiments were conducted at n = 5.

STING activation reporter cell assay

mSTING cells were seeded in a flat-bottom 96-well plate with density of 1×10^5 cells per well, in 180 μ L of culture medium. After overnight incubation at 37 °C, cells were pulsed with 20 μ L sample per well at corresponding samples (LNP or diABZI) concentrations (n=5) and then incubated for another 24h. Subsequently, 20 μ L of supernatant was transferred to new flat-bottom 96-well plates followed by addition of 180 μ L of QUANTI-Blue reagent solution (InvivoGen). After 1-3 hours incubation at 37 °C, the secreted embryonic alkaline phosphatase (SEAP) levels were determined by UV-vis spectrophotometry at 620 nm using a plate reader. Activity was determined by an increase in optical density relative to the negative control treated with PBS.

MTT Assay

The RAW Blue assay was combined with a MTT assay. After 50 μ L of supernatant was collected for SEAP level detection, the cells were pulsed with 40 μ L of 5 mg/mL MTT reagent dissolved in PBS and incubated at 37 °C. After 1h, the solution was removed gently and the formed formazan crystals were dissolved by addition of 100 μ L DMSO, the microplate then shook for a while for the better dissolution. Quantification was done by measuring the absorbance at 590 nm using a Perkin-Elmer Ensignht multimode platereader. A PBS groups was used as a negative control. All experiments were conducted at n = 5.

In Vitro Cellular Uptake Assay

LNP (Cy5-E₁₀) and unformulated Cy5-E₁₀ samples were prepared at a concentration of 0.05 mg/mL Cy5-E₁₀ in PBS. DC 2.4 cells were seeded in 24-well culture plates (1 $\times$ 10⁵ cells per well, 480 μ L per well) and incubated overnight. Subsequently the cells were pulsed with 20 μ L/well sample or PBS and incubated 2 h at 37 °C. Afterwards, the supernatant was removed, cells were washed with PBS, and detached with cell dissociation buffer. The content of the wells was transferred to a tube and centrifuged (5 min, 300 G, 4 °C). After aspiration of the supernatant, the cell pellets were resuspended in PBS and analyzed on a BD Accuri C6 low cytometer. Every sample was conducted at n=3. Data were processed using the FlowJo software package.

In Vivo Biodistribution of LNP

C57BL/6 WT mice received intravenous injections of LNP(E7Ag-E₁₀/IMDQ-E₁₀) labeled with DSPE-Cy7. Following a 6-hour post-injection period, the mice were euthanized, and major organs, including the heart, lungs, liver, spleen, and kidneys, were harvested for fluorescence measurement using IVIS imaging (n=3).

IFN β Luciferase Reporter Bioluminescence Imaging

Empty LNP, IMDQ-E₁₀, native IMDQ or LNP (IMDQ-E₁₀) were injected at an dose equivalent to 10 μ g IMDQ in 100 μ L (n=3). Prior imaging at the given time points (*i.e.*, 0, 4 and 24 h post injection), Balb/c IFN β ^{+/ Δ β -luc} mice were injected subcutaneously with 200 μ L of D-luciferin, and *in vivo* luminescence images were recorded after 12 min. Mice were anaesthetized by inhaling isoflurane before imaged by the IVIS Lumina II imaging system (PerkinElmer, USA). Local and full-body luminescence were quantified using the Living Image 4.4 software.

Analysis of Immunocytes Targeting and Activation in the Spleen.

IMDQ, Cy5-E₁₀, IMDQ-E₁₀/Cy5-E₁₀, LNP (Cy5-E₁₀) or LNP (IMDQ-E₁₀/Cy5-E₁₀) (100 μ L, containing an equivalent of 2 μ g Cy5 dose or 10 μ g IMDQ dose in PBS buffer) were injected

intravenously in the tail vein of female C57BL/6 WT mice (n=3). One day post injection, mice were sacrificed, spleens were isolated and collected in ice-cold PBS for further process. A single-cell suspension was prepared by smashing the spleens through 70 μ m cell strainers and washing them with PBS. Afterwards, splenocytes were stained for 3 min at 4 °C with the following primary labelled antibodies: CD11b, Ly6c, MHCII, F4_80, CD64, CD3, CD19, CD11c, CD172a, XCR1, CD80, CD86, CCR7, CD4, CD8, CD69. Live/dead ratios were determined by staining with fixable dead/live staining, and 123count eBeads were added to determine cellularity prior to analysis by a BD FACS Quanto flow cytometer. Data were processed using the FlowJo software package. All staining and beads were obtained from BioLegend.

In Vivo Analysis of Immune Cytokines and Aspartate Aminotransferase (AST).

C57BL/6 WT mice (n=3 per group) were intravenously administered with PBS, soluble IMDQ, empty LNP, and LNP (IMDQ-E₁₀) at a dose of 10 μ g IMDQ in 100 μ L. Six hours post-administration, blood samples (100 μ L) were collected from the tail veins of the mice. Serum samples were prepared by allowing the blood to clot for at least 30 minutes, followed by centrifugation for 20 minutes at 1,000 G. One serum sample was partitioned for the evaluation of immune cytokines and aspartate aminotransferase (AST) separately.

13 immune cytokines were quantified simultaneously using a LEGENDplex™ Multi-Analyte Flow Assay Kit (Biolegend). Specifically, this assay utilizes bead-based immunoassays, similar to sandwich immunoassays, where beads coated with specific capture antibodies selectively bind analytes in the sample. Subsequently, a biotinylated detection antibody cocktail forms sandwich complexes with the captured analytes. Streptavidin-phycoerythrin (SA-PE) generates fluorescent signals directly proportional to analyte levels, enabling the quantification of PE fluorescence through flow cytometry (BD Accuri C6). Finally, analyte concentrations are determined using a standard curve established within the assay platform.

The other serum sample was employed to quantify AST using an AST Activity Assay Kit (Sigma-Aldrich). This assay involves the transfer of an amino group from aspartate to α -ketoglutarate, resulting in the generation of glutamate and a colorimetric (450 nm) product proportional to AST enzymatic activity. One unit of AST corresponds to the amount of enzyme that generates 1.0 μ mole of glutamate per minute at pH 8.0 at 37 °C. Quantification was performed by measuring the absorbance at 450 nm using a Perkin-Elmer Ensign multimode plate reader, and glutamate standards were utilized to construct a calibration curve for colorimetric detection.

Immunization and Tetramer Staining.

C57BL/6 WT mice (n=5 per group) were immunized IV with PBS, E7Ag, E7Ag/IMDQ, LNP (E7Ag-E₁₀), LNP (E7Ag-E₁₀/IMDQ-E₁₀) (E7Ag dose:10 µg, IMDQ dose: 10 µg in 100 µL), respectively, following a prime-boost scheme with 2-week interval. Mice were bled on days 5, and 20 to obtain 100 µL of blood for flow cytometry analysis with tetramer staining. The antibodies used for flow cytometry are as follows: CD45(30-F11), CD3(145-2C11), and CD4(RM4-5) were obtained from eBioscience; CD8(53-6,7) from BD Biosciences; fixable viability dye Live/Dead Efluor 506 from Invitrogen; and MHC tetramer H-2Kb E7 RAHYNIVTF from MBL. 123count eBeads (eBioscience) were added to 100 µL of blood prior to staining to determine cellularity. Acquisition of the samples was done on a BD Fortessa flow cytometer. Data were processed using the FlowJo software package.

Tumor Experiments.

For the prophylactic tumor challenge experiment, C57BL/6 WT mice (4-6 weeks, female, n=8) were administrated intravenously either with PBS or LNP (E7Ag-E₁₀/IMDQ-E₁₀) (E7Ag dose:10 µg, IMDQ dose:10 µg in 100 µL), following a prime-boost scheme with 2-week interval. Two weeks post boost immunization, 5×10^5 TC-1 cells suspended in 100 µL PBS were injected subcutaneously in the right rear flank. The dimension (D) of the tumor at the site of injection were measured every two or three days in three perpendicular directions with a caliper, and the volume of the tumor was estimated by the formula $\frac{4}{3} R^3$, R is average of 2/D. The monitoring endpoint was set at day 21 post-tumor implantation, or when tumor volume exceeded 1500 mm³, or when weight loss exceeded 20%. For the therapeutic model, mice (4-6 weeks, female, n=7) were firstly inoculated subcutaneously with 5×10^5 TC-1 cells. Subsequently, mice were vaccinated on days 7 and 14 via subcutaneous injection as prime and boost immunization. Tumor growth was monitored as described above, and the endpoint criteria were consistent with those of the tumor prophylactic experiment.

Analysis of T Cell Response.

To assess the CD8⁺ T cell response triggered by lipid nanoparticles (LNPs) encapsulating the E7Ag-E₁₀ antigen and IMDQ-E₁₀, blood samples were collected from mice following prophylactic and therapeutic treatments. Approximately 100 µL of blood was obtained via tail veins post boost immunization and at the experiment's endpoint. Whole blood underwent triple treatment with ACK lysis buffer (Gibco), followed by washing, resuspension in cold flow cytometry staining buffer (Biolegend), and staining with a panel of antibodies: Fixable Viability Stain 575V (BD), APC-labeled MHC tetramer H-2Kb E7 RAHYNIVTF (Biolegend), PE/Cy7-TCRβ (H57-597, BD), BUV496-CD8a (5H10-1, BD), APC/Fire750-CD69 (H1.2F3, Biolegend), PE/CF594-PD-1 (J43, BD), BUV66-Lag3(C9B7W, BD), PE-TOX1(TXRX10, ThermoFisher), BUV395-CD44(IM7, BD), BV786-CD62L(MEL-14, BD), BV421-CX3CR1(SA011F11,

Biolegend). Following staining, cells were washed, resuspended in flow cytometry staining buffer with counting beads (eBioscience), and analyzed using a BD Fortessa flow cytometer. Data analysis was performed using the FlowJo software package.

Red blood cell lysis was performed by using a home-made red blood cell lysis buffer (155 mM NH₄Cl, 0.5 mM EDTA, 10 mM KHCO₃, pH 7.2). Single cell suspensions were resuspended in HBSS and incubated with Fixable Viability Dye BV570 (BD) for 30 minutes at 4°C. Next, cell suspensions were washed with FACS buffer (HBSS containing 0.5% fetal calf serum (FCS) and 2 mM EDTA), incubated with fluorescently labeled antibodies for 20 minutes at 4°C and then washed with FACS buffer. The following fluorochrome-conjugated antibodies were used: PE-Cy7-TCR β (H57-597, BD) BUV496-CD8a (53-6.7, BD) MHC tetramer H-2Kb E7 RAHYNIVTF (APC, Immunodex), APC/Fire750-CD69 (H1.2F3, Biolegend), PE/CF594-PD-1 (J43, BD), BUV66-Lag3(C9B7W, BD), PE-TOX1(TXRX10, ThermoFisher), BUV395-CD44(IM7, BD), BV786-CD62L(MEL-14, BD), BV421-CX3CR1(SA011F11, Biolegend). Flow cytometry data were acquired using BD FACS Symphony A3 and analyzed by FlowJo (TreeStar).

ASSOCIATED CONTENT

Supporting information containing additional experimental data is available.

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DATA AVAILABILITY STATEMENT

Data are available from the authors upon request.

CONFLICT OF INTEREST DISCLOSURE

YC, SDK and BDG are inventor of a patent on the LNP technology used in this paper. SDK is an employee of a company commercializing this technology.

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SUPPORTING INFORMATION

CO-DELIVERY OF GLUTAMIC ACID-EXTENDED PEPTIDE ANTIGEN AND IMIDAZOQUINOLINE TLR7/8 AGONIST VIA IONIZABLE LIPID NANOPARTICLES INDUCES PROTECTIVE ANTI-TUMOR IMMUNITY

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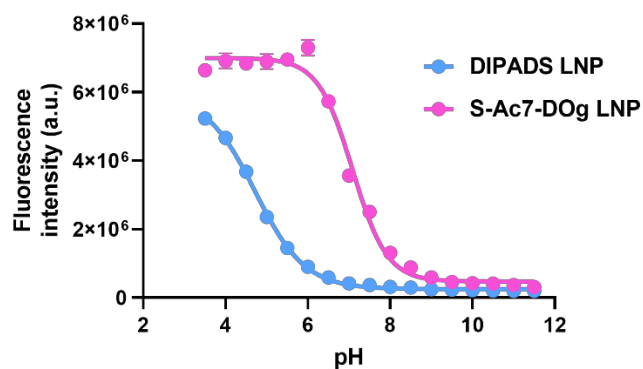


Figure S1. TNS binding assay. pKa values were calculated as EC50 values by fitting the TNS fluorescence intensity curves (dots) to a sigmoidal nonlinear equation (solid lines).

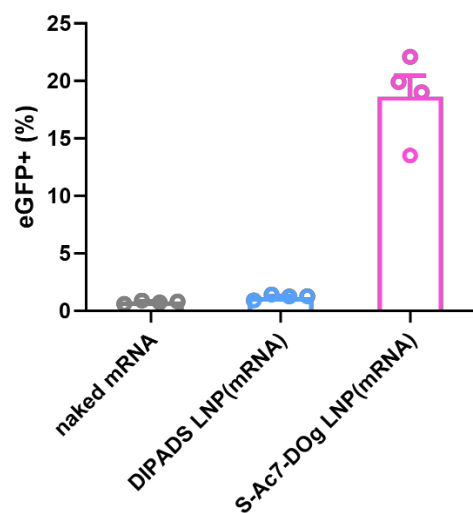


Figure S2. Transfection efficiency of unformulated, DIPADS LNP-formulated mRNA, and S-Ac7-DOg LNP-formulated mRNA in DC 2.4 cells (n=4).

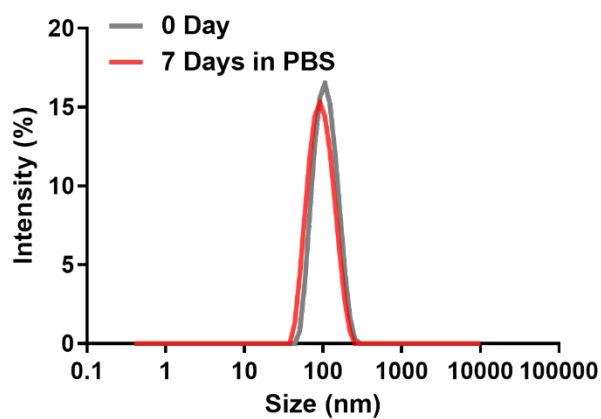


Figure S3. Size distribution of LNP in PBS on days 0 and 7 measured by DLS

Ionizable lipids	Phospholipids	N: COOH	Size (nm)	PDI	Zeta potential (mV)	IMDQ-E ₁₀ Encapsulation efficiency (%)	E7Ag-E ₁₀ Encapsulation efficiency (%)
S-Ac7-DOg	DOPE	10: 1	104	0.121	0.3	72 ± 2	74 ± 4
S-Ac7-DOg	DOPE	5: 1	99	0.104	-3.3	74 ± 2	69 ± 3
S-Ac7-DOg	DSPC	10: 1	134	0.103	3.5	75 ± 3	69 ± 8
S-Ac7-DOg	DSPC	5: 1	138	0.113	0.5	72 ± 8	68 ± 5
E-Ac7-DOg	DOPE	5: 1	107	0.159	5.0	75 ± 5	68 ± 3

Table S1. Composition and physicochemical Properties of LNP with different formulations

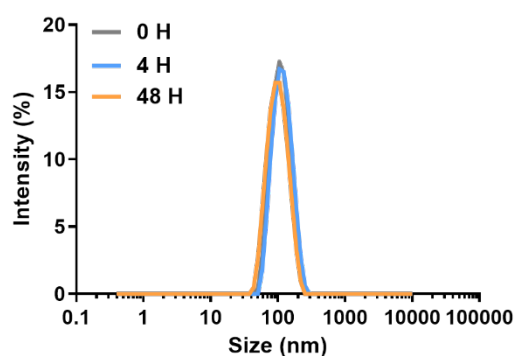


Figure S5. Size distribution of S-Ac7-DOG LNP incubated in 10 mM glutathione solution (in PBS buffer) over time.

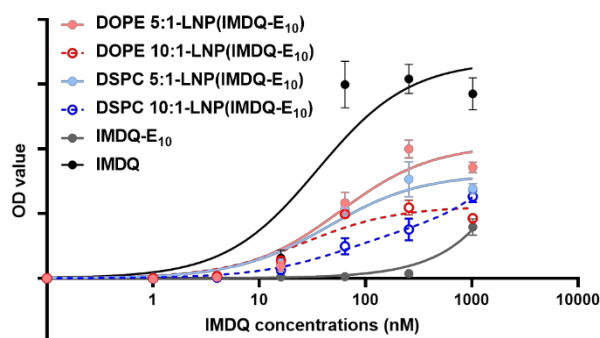


Figure S7. Dose-response innate activation of LNP(IMDQ-E₁₀) with different formulations (phospholipids: DOPE or DSPC; N:COOH ratio: 10:1 or 5:1) measured by the RAW Blue reporter cell assay (n = 5)

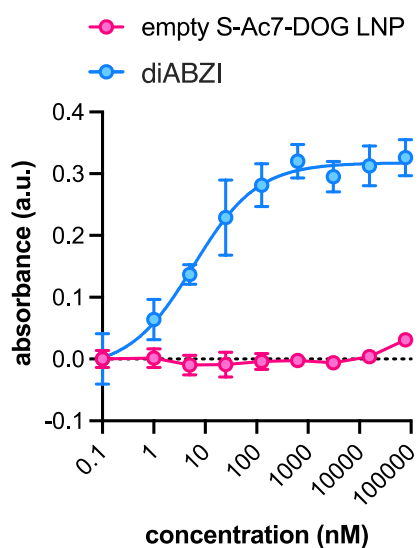


Figure S8. STING agonistic activity of empty S-Ac7-DOG LNP measured using the 293-Dual™ mSTING reporter cell assay.

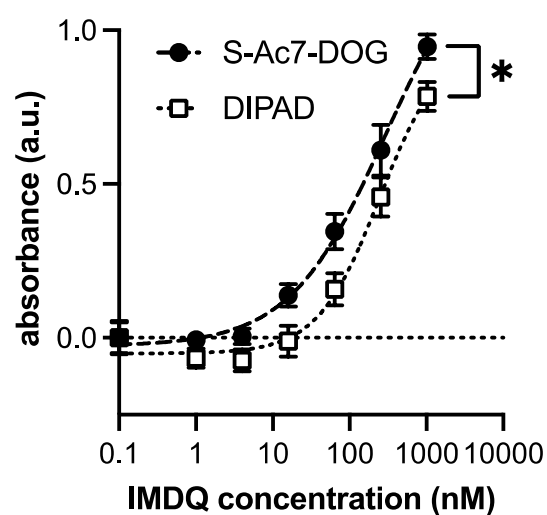


Figure S9. Dose-response innate activation, measured by the RAW Blue reporter cell assay, of LNP(IMDQ-E₁₀) with LNP containing S-Ac7-DOG or DIPADS, respectively, as an ionizable lipid (n = 5; Mann-Whitney test of hill slope: *p<0,05).

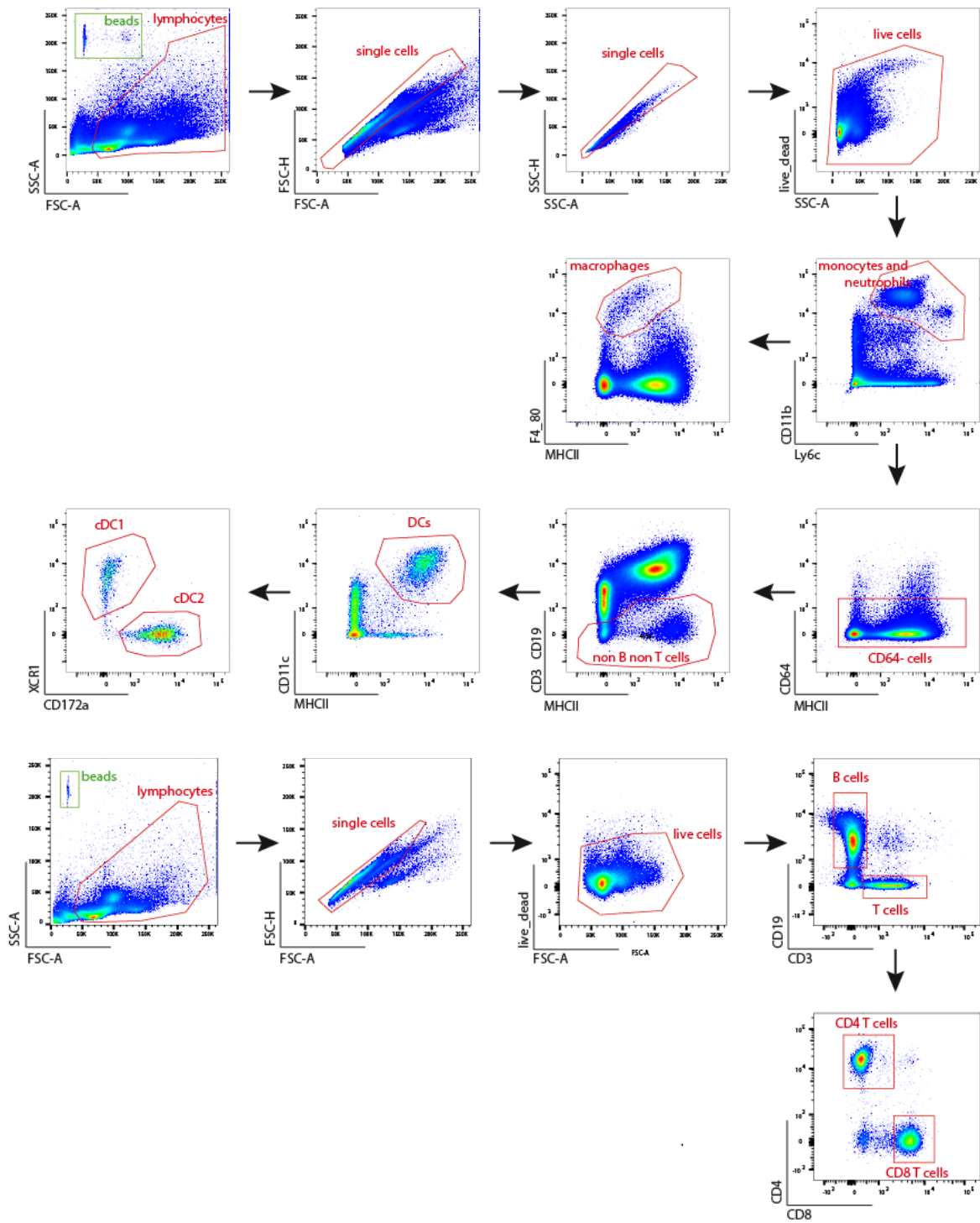


Figure S10. Flow cytometry gating strategy used for Figure 5.

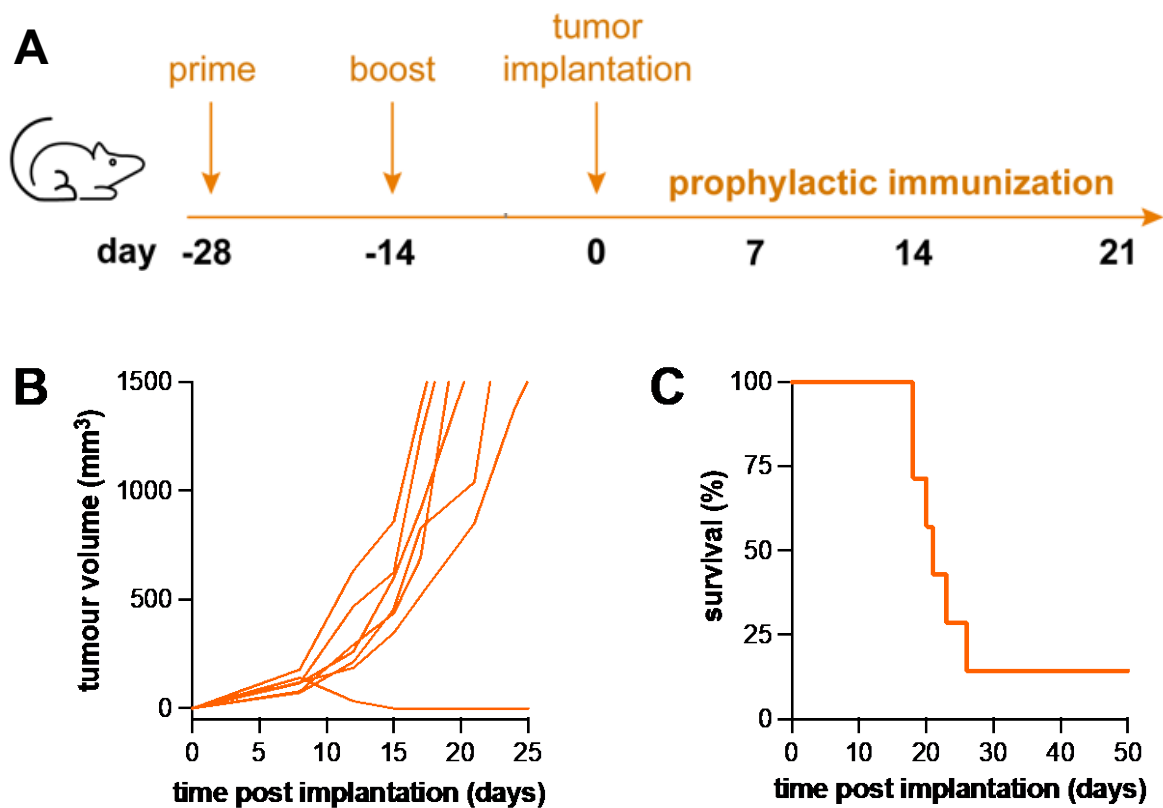


Figure S11. C57BL/6 mice were immunized by prime-boost subcutaneous (SC) injection with LNP containing 10 μg of antigen and 10 μg of IMDQ. The endpoint was reached when the tumor volume reaches than 1500 mm^3 or when the weights lost was $> 20\%$. **(A)** Experimental protocol. **(B)** Individual TC-1 tumor growth curves ($n=6$). **(C)** Survival curves.

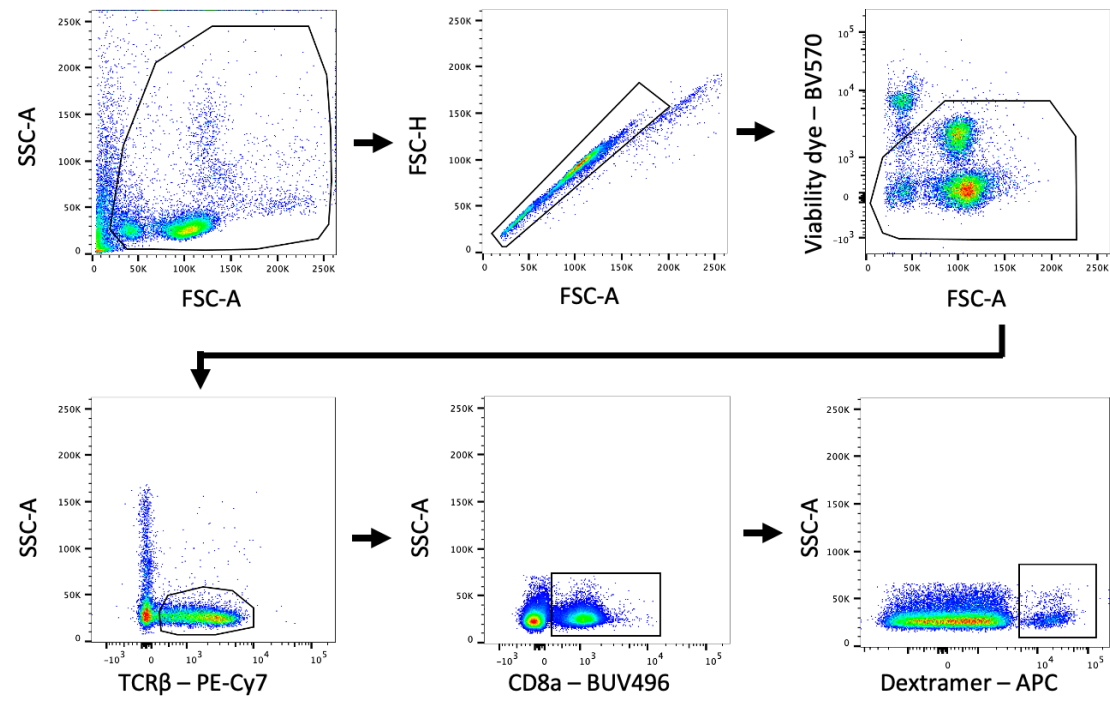


Figure S12. Flow cytometry gating strategy used for Figure 7 and 8.