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Accelerated Blood Clearance and Hypersensitivity by PEGylated Liposomes Containing TLR Agonists

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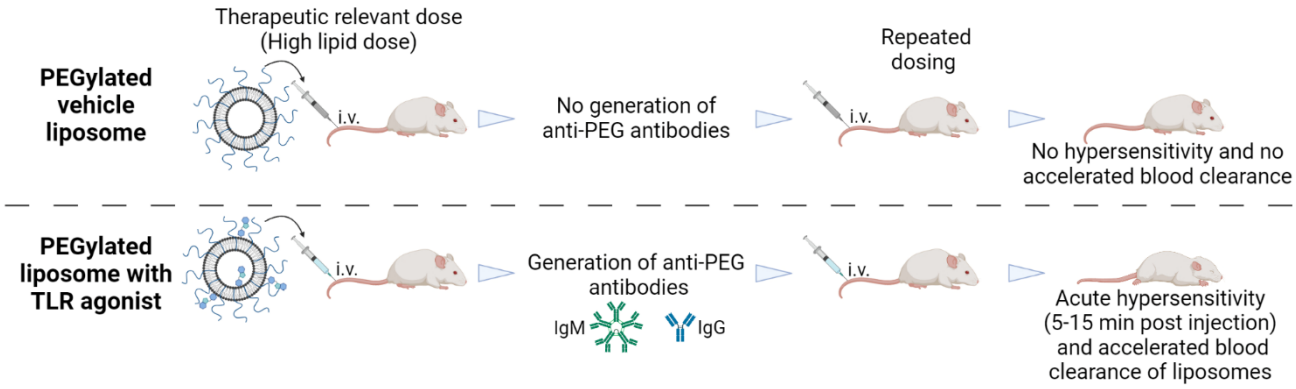
Declarations of interest: none

Highlights

- TLR agonists formulated in PEGylated liposomes induce anti-PEG antibodies
- Repeated dosing leads to accelerated blood clearance and hypersensivity reactions
- The accelerated blood clearance cannot be avoided by increasing liposome dose
- The severe hypersensitivity reactions appear 5-15 min after the third dose
- The hypersensitivity reaction may be caused by anti-PEG IgG antibodies

Graphical abstract



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Abstract

Systemic administration of toll-like receptor (TLR) agonists have demonstrated impressive preclinical results as an anti-cancer therapy due to their potent innate immune-stimulatory properties. The clinical advancement has, however, been hindered by severe adverse effects due to systemic activation of the immune system. Liposomal drug delivery systems may modify biodistribution, cellular uptake, and extend blood circulation, and thus, potentially enable systemic administration of TLR agonists at therapeutic doses.

In this study, we investigated potential barriers for the administration of TLR agonists formulated in polyethylene glycosylated (PEGylated) liposomes with regards to liposome formulation, TLR agonist, administration route, administration schedule, biodistribution, blood clearance, and anti-PEG antibodies. We found that administration of TLR agonists formulated in PEGylated liposomes led to high anti-PEG antibody titers, which upon multiple intravenous administrations, resulted in accelerated blood clearance and acute hypersensitivity reactions. The latter was found to be associated with anti-PEG IgG antibody and not anti-PEG IgM antibody opsonization. This study highlights the need to carefully design and evaluate nanoparticle delivery systems for immunotherapy as anti-nanoparticle immune responses may challenge the therapeutic application.

Keywords

Liposomes, PEG, PEGylated liposomes, anti-PEG antibodies, hypersensitivity, Toll-like receptor agonists, TLRs, systemic dosing

Introduction

The potential of harnessing the body's immune response with cancer immunotherapy has brought new hope for cancer treatment. Immune stimulating drugs can activate the immune system and mount an anti-cancer response. One approach is to stimulate Toll-like receptors (TLR) of the innate immune system. Upon activation, TLRs induce the production of inflammatory cytokines such as type I interferons and tumor necrosis factor α that have anti-viral and anti-cancer effects [1]. Currently, Bacillus Calmette Guérin (BCG), Monophosphoryl lipid A (MPLA), and Imiquimod are the only three TLR agonists approved by the U.S. Food and Drug Administration for anti-cancer therapy. They are all administered locally at the tumor-site or as adjuvants in anti-cancer vaccines [2]. However, local administration can be challenging clinically as the tumor is not always accessible for injection, or the disease may be disseminated and require numerous injections. Thus, there is an unmet need to develop TLR agonists than can be administered systemically. Unfortunately, earlier attempts of systemic TLR agonist treatment in patients resulted in toxicity or limited therapeutic effect at tolerated doses [3,4].

Toxicity associated with systemic administration can be reduced by applying drug delivery systems that alter the biodistribution and pharmacokinetics of the drug. For instance encapsulation of doxorubicin in PEGylated liposomes greatly reduced cardiac toxicity associated with the free drug [5]. PEGylation of liposomes is widely used for increasing circulation time of drugs [6] and may allow accumulate in tumors due to the enhanced permeability and retention effect [7]. However, as shown in mice, rats, rabbits, dogs, minipigs, and monkeys, repeated dosing of PEGylated liposomes can lead to accelerated blood clearance [8–13]. Marginal zone B cells in the spleen recognize the PEG moiety on liposomes as a T cell-independent (TI) type II antigen, leading to the production of anti-PEG IgM, subsequent opsonization, and clearance of liposomes from the circulation [14–16]. Previous studies have shown that increasing the lipid dose of the initial liposomal injection reduces accelerated blood clearance, most likely due to tolerance or anergy induction in marginal zone B cells [17–19]. However, most research regarding accelerated blood clearance of PEGylated liposomes has been performed using low lipid doses (0.001-25 μmol/kg) of vehicle liposomes with limited translational value [20,21].

To potentially overcome systemic side effects and thus improve the therapeutic window for TLR agonists, we formulated TLR agonists in different PEGylated liposomes. For translational purposes, we administered liposomes in high lipid doses to reach relevant therapeutic TLR agonist doses.

However, we observed acute hypersensitivity following repeated administration that did not occur in response to the initial administration. Consequently, we investigated whether this was connected to accelerated blood clearance of liposomes upon repeated dosing and whether the benefit of administering high lipid doses was abrogated when liposomes contained TLR agonists.

Materials and Methods

Materials for liposome preparation

Unless otherwise stated, all chemicals were acquired from Sigma Aldrich. 1-Palmitoyl-2- oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000), and cholesterol were acquired from Lipoid. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]) (DOPE-PEG2000), dioleoyl-3-trimethylammonium-propane (DOTAP), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phopho-(1'-rac-glycerol) (POPG) were acquired from Avanti Polar Lipids. 1V270 (also known as TMX-201) was synthesized by Corden Pharma and Chimete. Gardiquimod was acquired from Invivogen. FITC-5'-CpG-3'-Cholesterol oligodeoxynucleotides (ODN) were purchased from Eurofins Genomics.

Liposome formulations

Liposome formulations investigated in this study; 1) anionic (POPC:POPG:Chol:Drug:mPEG2k), 2) neutral (POPC:Chol:Drug:mPEG2k), 3) cationic (POPC:Chol:DOTAP:Drug:mPEG2k), and 4) stealth (HSPC:Chol:Drug:mPEG2k). Three TLR agonists were incorporated in the liposomal formulations; 1V270, Gardiquimod, and CpG-ODNs. Additional information on the formulation and characterization of liposomes is described in Supplementary Material and Methods and summarized in Supplementary Table 1.

Mice

BALB/cJRj mice (Janvier) aged 8–13 weeks were used. Mice were subjected to at least one week of acclimation upon arrival and were kept under controlled environmental conditions (constant temperature, humidity, and 12:12h light-dark cycle). All experimental procedures were approved by the Institutional Ethical Board at Copenhagen University and the Danish National Animal Experiment Inspectorate.

Liposome treatments

Liposomes were administered intravenously or subcutaneously and administered either in a q1d or q4d (everyone or every fourth day) schedule. Mice were monitored for up to an hour after administration to assess signs of acute hypersensitivity. Signs of hypersensitivity included decreased or lack of activity, piloerection, hunched back and eye squinting. Additional information on the administration of liposomes can be found in Tables 1, and 2 and extended information can be found in Supplementary Table 1.

Efficacy studies

The colorectal cancer cell line CT26 was obtained from ATCC and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM Glutamax, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. For inoculation, mice were anesthetized with 3–5% sevoflurane and inoculated subcutaneously with $3x10^5$ CT26 cells in non-supplemented RPMI 1640 into the right flank. Tumor volume was measured with calibers as length x width^2 / 2 and randomized based on tumor volume. Tumors were allowed to grow to a mean size of 95 mm^3 before treatment. Liposome treatments were given intravenously. Mice receiving radiotherapy (RT), were irradiated under lead-shielding, exposing the right flank at a dose rate of 1.0 Gy/min in an X-RAD 320 (PXi).

Liposome treatments and blood sampling for anti-PEG IgM and IgG determination

Mice were randomized based on weight. All liposome formulations were administered in a q4d schedule for a total of three injections (day 0, 4, and 8). Liposomes were administered intravenously in the tail vein or subcutaneously in the neck of the mouse. Blood samples were collected in hirudin blood tubes (Roche) 1–2 h before the third administration and 5 min after. Samples were centrifuged at 2000 g for 15 min a 4 °C and resulting plasma fractions were collected and stored at −80 °C until ELISA analysis.

ELISA

The ELISA procedure was adapted from [22]. Briefly, 96-well plates were coated with 20 nmol DOPE-PEG in 99.9% ethanol (100 µL) overnight. Plates were washed three times with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% CHAPS). Subsequently, plates were incubated in blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA) for 1 h at room temperature and washed before use. For the anti-PEG IgM assay: Standards were prepared using pooled plasma samples from mice injected with DOPE-PEGylated neutral 1V270 liposomes (formulation #18 in Table 1) on day 0 and 4. Plasma was collected on day 8 and diluted 100-fold with blocking buffer followed by 2-fold serial dilution series to create

a standard curve (256, 128, 64, 32, 16, 8, 4, 2, 1 arbitrary units/mL). For the anti-PEG IgG assay: A standard was prepared from purified mouse IgG1 kappa anti-PEG (clone 5D-3; Abcam) diluted in a 2-fold serial dilution series from 17,920 to 70 pg/mL. Plasma samples were diluted 400-fold. Diluted samples and standards were added to plates and incubated for 1 h. Plates were subsequently washed five times, followed by addition of detection antibodies, and a 1 h incubation. IgM antibody was detected using 100 ng/mL HRP-conjugated goat anti-murine IgM (Bethyl Laboratories) in blocking buffer and IgG was detected using 100 ng/mL HRP-conjugated goat anti-murine IgG (Jackson Immunoresearch Europe) in blocking buffer. Plates were washed and developed using 100 µL SigmaFast OPD (Sigma Aldrich) substrate for 9 min (IgM) or 25 min (IgG). The reaction was stopped by the addition of 100 µL 2 M sulfuric acid. Absorbance was measured at 490 nm (target) and 800 nm (reference) on a FluoStar Omega instrument (BMG Labtech). A non-linear 4 parameter regression was done in Prism software (Graphpad) after subtracting reference measurements.

Biodistribution study

For the biodistribution study, PEGylated neutral 0.75% 1V270 liposomes and PEGylated neutral vehicle liposomes were radiolabeled with ^{64}Cu (^{64}Cu-lip-1V270 and ^{64}Cu-vehicle, respectively). Additional information on the radiolabeling of liposomes can be found in Supplementary Materials and Methods. To investigate biodistribution and blood clearance on the first and third administration of liposomes with or without 1V270, mice were randomized based on weight into four groups. PEGylated neutral liposomes were administered q4d. Groups: 1) 1st injection with ^{64}Cu-lip-1V270, 2) 1st injection with ^{64}Cu-vehicle, 3) 1st and 2nd injection with non-labeled 1V270 PEGylated liposomes (#18) and 3rd injection with ^{64}Cu-lip-1V270, and 4) 1st and 2nd injection with non-labeled PEGylated vehicle liposomes (#17) and 3rd injection with ^{64}Cu-vehicle. PET/CT scans were performed on a small animal PET/CT system (Inveon®, Siemens Medical Systems). Mice were anesthetized using 3–5% sevoflurane. PET images were acquired over 5 min (10 min, 2 h, 24 h post administration) or 10 min (48 h post administration) followed by a CT scan (tube setting: 70 kV, 500 µA, 350 ms exposure time, 0.21x0.21x0.21 mm pixel size). PET data were arranged into sinograms and reconstructed using a maximum posterior reconstruction algorithm (0.388x0.388x0.796 mm pixel size) with attenuation corrected based on the corresponding CT scan. Image analysis was performed in Inveon Software (Siemens Medical System). Regions of interests (ROIs) were manually constructed on co-registered PET/CT images. ROIs were drawn on the heart, spleen, kidney, liver, muscle, and entire mouse. Blood activity was estimated from a ROI covering the heart and subsequently segmented to only include

the voxels above 80% of the maximum activity in the original ROI. ^{64}Cu liposome activity was decay-corrected, subtracted with muscle activity, and reported as % injected dose per gram (%ID/g).

Antibody transfer

Mice were intravenously administered DOPE-PEGylated neutral 0.75% 1V270 liposomes (200 nmol 1V270, #18) or DOPE-PEGylated neutral vehicle liposomes (#17) on day 0 and 4. On day 8, blood (800-1000 µL) was collected by cardiac puncture into hirudin blood tubes. Plasma was obtained by centrifugation at 2000 g for 15 min at 4 °C. Total IgG was purified from plasma with the Nab Protein A/G spin kit (Thermo Fisher) according to the manufacturer's recommendations. The unbound fraction was processed with the NAb protein L spin kit (Thermo Fisher Scientific) to purify the remaining antibodies with kappa chains, including IgMs. Zeba spin desalting columns (Thermo Fisher Scientific) were used to exchange the buffer to sterile PBS. The antibody fractions were analyzed by size exclusion-high performance liquid chromatograph (SEC-HPLC) on a Yarra™ 3 µm SEC-3000 Column 300x7.8 mm (Phenomenex) with 0.1 M phosphate buffer pH 7 as a mobile phase on an isocratic method with a flow of 1 mL/min. The samples were analyzed at 280 nm (Supplementary Fig. S1). Antibody solutions were upconcentrated using Amicon Ultra-4 Centrifugal Devise to obtain a final volume of 50 µL. IgG or IgM from mice receiving 1V270 liposomes or vehicle liposomes were given to naïve mice intravenously, followed by intravenous injections of PEGylated neutral 1V270 liposomes (200 nmol 1V270, #18) 10 min later. Mice were subsequently monitored for hypersensitivity symptoms.

Inhibition of platelet-activating factor

Mice were administered intravenously with DOPE-PEGylated neutral 0.75% 1V270 liposomes (#18) in a q4d schedule for a total of three doses. 5 min before the third administration, mice were given 50 µg CV6209 (platelet-activating factor, PAF, antagonist) by intraperitoneal injection. Mice were subsequently monitored for hypersensitivity symptoms.

Statistical analyses

Statistical analyses were performed using Graphpad Prism 8. The appropriately used statistical test is stated in figure legends. A p-value ≤ 0.05 was considered statistically significant.

Results

PEGylated liposomes containing TLR agonists induce a hypersensitivity reaction upon repeated administration

To investigate if PEGylated liposomes containing a TLR7/8 agonist could be safely administered intravenously, we evaluated repeated administrations of PEGylated anionic, neutral, or cationic formulations (formulation #3, #18, & #25, respectively, Table 1) in mice. All formulations contained 0.75% of the TLR7/8 agonist 1V270. Liposomes were injected intravenously on day 0, 4, and 8 at 1V270 doses of 2 µmol/kg and lipid doses of 230–270 µmol/kg. At the third injection, hypersensitivity symptoms occurred 5–15 min after the administration of neutral (#18) and cationic (#25) liposomes. Symptoms included inactivity, piloerection, eye squinting, and a hunched back. The third administration of the anionic formulation (#3) did not induce hypersensitivity in the initial study; however, hypersensitivity was observed in subsequent studies with the formulation (8 out 11 of liposome batches). In order to determine if therapeutic anti-cancer effects are lost in case hypersensitivity reactions to liposomes we compared treatment setups with or without hypersensitivity. Anionic liposomes (#3) was administered to tumor-bearing mice in combination with radiotherapy. To this end, we saw a partially decreased treatment effect of combining liposomes with radiotherapy when liposomes caused hypersensitivity compared to when they did not (p=0.0596 and p=0.824, respectively, when comparing radiotherapy alone to radiotherapy combined with liposomes; supplementary Fig. S2).Hypersensitivity was not observed for the third injection when injecting anionic vehicle liposomes (#2) on day 0 and 4 and anionic 1V270 liposomes (#3) on day 8.

The rapid onset of hypersensitivity symptoms after the third injection suggested an anaphylactic reaction to the PEGylated liposomes. To investigate this, we treated mice with a PAF antagonist, PAF being a key mediator of anaphylaxis [23], 5 min prior to the 3rd liposome administration of neutral PEGylated liposomes (#18). This treatment alleviated the hypersensitivity symptoms (n=3), indicating that the hypersensitivity was anaphylactic. To prevent hypersensitivity reactions, liposome formulations were modified on multiple parameters, including 1V270 content (0.25, 0.75, 1.5, and 5 mol%), PEGylation, different lipid anchors for PEG (DOPE, DSPE, DSG, and Cholesterol), and different TLR agonists (1V270, gardiquimod, and CpG). Furthermore, dosing schedule (q1d and q4d) and delivery route (subcutaneous and intravenous) were evaluated. See Table 1 for liposomes administered intravenously and Table 2 for liposomes administered subcutaneously. Varying 1V270 (#4, #5, #9, #10) content did not prevent the hypersensitivity, nor did changing the TLR agonist to CpG (TLR9 agonist; #22) or gardiquimod (TLR7 agonist; #14, #15, & #21). Hypersensitivity was also observed with DSPE (#6, #10, #9, #5, & #14) and DSG (#11 & #15) as lipid anchors, but hypersensitivity was not induced with a cholesterol anchor (#12). Neither anionic nor neutral PEGylated vehicle

liposomes (#2 & #17, respectively) induced hypersensitivity. Additionally, no hypersensitivity was observed for daily administered DOPE-PEGylated anionic 1V270 liposomes (#3 & #6) and non-PEGylated anionic 1V270 liposomes administered every fourth day (#1). However, no anti-cancer effect was observed for these two treatments (Supplementary Fig. S3). Finally, subcutaneous administration of PEGylated liposomes with 1V270 did not induce hypersensitivity.

	PEG anchor	Drug load (molar%)	TLR agonist	Dosing schedule	Hyper-sensitivity	Formulation #
Anionic	Non-PEG	0.75%	1V270	q4d × 5	No	1
	DOPE	-	-	q4d × 3	No	2
	DOPE	0.75%	1V270	q4d × 3 q1d × 10	**Yes** No	3
	DOPE	5%	1V270	q4d x 3	**Yes**	4
	DSPE	0.25%	1V270	q4d × 3	**Yes**	5
	DSPE	0.75%	1V270	q4d × 3 q1d × 5	**Yes** No	6
	DSPE	0.75%	1V270	q4d × 3	**Yes**	7
	DSPE	0.75%	1V270	q4d × 3	**Yes**	8
	DSPE	1.5%	1V270	q4d × 3	**Yes**	9
	DSPE	5%	1V270	q4d × 3	**Yes**	10
	DSG	0.75%	1V270	q4d × 3	**Yes**	11
	Chol	0.75%	1V270	q4d × 3	No	12
	DOPE	10%	Gardiquimod	q4d × 3	No	13
	DSPE	10%	Gardiquimod	q4d × 3	**Yes**	14
	DSG	10%	Gardiquimod	q4d × 3	**Yes**	15
Neutral	Non-PEG	0.75%	1V270	q4d × 3	No	16
	DOPE	-	-	q4d × 3	No	17
	DOPE	0.75%	1V270	q4d × 3	**Yes**	18
	DOPE	5%	1V270	q4d × 3	**Yes**	19
	DSG	0.75%	1V270	q4d × 3	**Yes**	20
Stealth	DSPE	10%	Gardiquimod	q4d × 3	**Yes**	21
	DSPE	0.05%	CpG-ODN	q4d × 3	**Yes**	22
	DSPE	0.05%	Non-CpG-ODN	q4d × 3	No	23

Cationic	Non-PEG	0.75%	1V270	q4d × 3	**Yes**	24
	DOPE	0.75%	1V270	q4d × 3	**Yes**	25

Table 1: Overview of liposomal formulations administered intravenously*. Liposomes were administered intravenously according to the written dosing schedule. Subsequently, mice were monitored for symptoms of acute hypersensitivity for up to an hour after each administration. Anionic, neutral, stealth, and cationic liposomes were formulated as [POPC:Chol:POPG:Drug:PEG], [POPG:Chol:Drug:PEG], [HSPC:Chol:Drug:PEG], and [POPC:Chol:DOTAP:Drug:PEG], respectively. q4d=administration every fourth day, q1d=administration every day. Additional information on liposomal formulations and administered doses can be found in Supplementary Table 1.*

	PEG anchor	Drug load (molar%)	TLR agonist	Dosing schedule	Hyper-sensitivity	Formulation #
Anionic	DOPE	0.75%	1V270	q4d × 3	No	3
Cationic	DOPE	0.75%	1V270	q4d × 3	No	25

Table 2: Overview of liposomal formulations administered subcutaneously in the neck of mice*. Liposomes were administered subcutaneously in the neck of mice following the indicated dosing schedules. Subsequently, mice were monitored for symptoms of acute hypersensitivity. Anionic and cationic liposomes were formulated as [POPC:Chol:POPG:Drug:PEG] and [POPC:Chol:DOTAP:Drug:PEG], respectively. Additional information on liposomal formulations and administered doses can be found in Supplementary Table 1.*

These results show that PEGylated liposomes containing TLR agonists can induce hypersensitivity when administered intravenously every fourth day and that the majority of the formulations tested induced hypersensitivity. In addition, the anti-cancer effect was reduced when hypersensitivity reactions occurred. We wanted to investigate the occurrence of hypersensitivity and decreased treatment effect further to better understand the barriers of using PEGylated liposomes with TLR7/8 agonist for systemic treatment. All further evaluations were conducted using DOPE-PEGylated liposomes containing 0.75% 1V270 or vehicle.

<u>PEGylated liposomes containing 1V270 are subject to accelerated blood clearance</u>

Previous gene therapy studies have reported hypersensitivity reactions following repeated dosing of PEGylated liposomes containing nucleic acids that can be recognized as TLR agonists [23–25]. Additionally, these studies also reported a loss of disease targeting and accelerated blood clearance after repeated dosing. Consequently, we investigated whether our PEGylated TLR liposomes were subject to accelerated blood clearance upon repeated dosing. This was done by PET/CT imaging of the biodistribution after the first and third administration of neutral liposomes with and without 1V270 in a q4d schedule (#18 & #17, respectively). Mice were intravenously administered ^{64}Cu radiolabeled PEGylated neutral liposomes with 1V270 (^{64}Cu-1V270) or without 1V270 (^{64}Cu-vehicle) at a lipid dose of 270 µmol/kg. PET/CT scans was performed 10 min, 2 h, 24 h, and 48 h post injection. Mice imaged on the third administration were pretreated twice with unlabeled PEGylated neutral liposomes with or without 1V270 (#18 & #17, respectively).

Groups receiving ^{64}Cu-vehicle displayed comparable circulation profiles on the first and third administration, thus demonstrating that accelerated blood clearance was not induced by PEGylated vehicle liposomes in accordance with previous studies on high lipid dosing [17,19,21]. ^{64}Cu-1V270 showed a similar circulation profile on the initial administration compared to administrations of ^{64}Cu-vehicle. In contrast, the third administration of ^{64}Cu-1V270 was subject to rapid elimination from the circulation, which was evident as early as 10 min post injection. Similarly, blood activity levels were significantly lower at the 2 h and 24 h PET/CT scans ($p \leq 0.05$, Figure 1A–B). Interestingly, activity was observed in the mouth and front limbs of mice following administration ^{64}Cu-lip-1V270 as a third dose at all imaged time points (Figure 1B). In addition, liver uptake was increased at the 2 h time point for the third administration of ^{64}Cu-1V270 ($p=0.004$). The same tendency was seen for spleen uptake after 2 hours. Altogether, these data demonstrate that administration of high lipid doses of vehicle liposomes did not induce accelerated blood clearance, but incorporation of a TLR7/8 agonist induced accelerated blood clearance at high lipid doses.

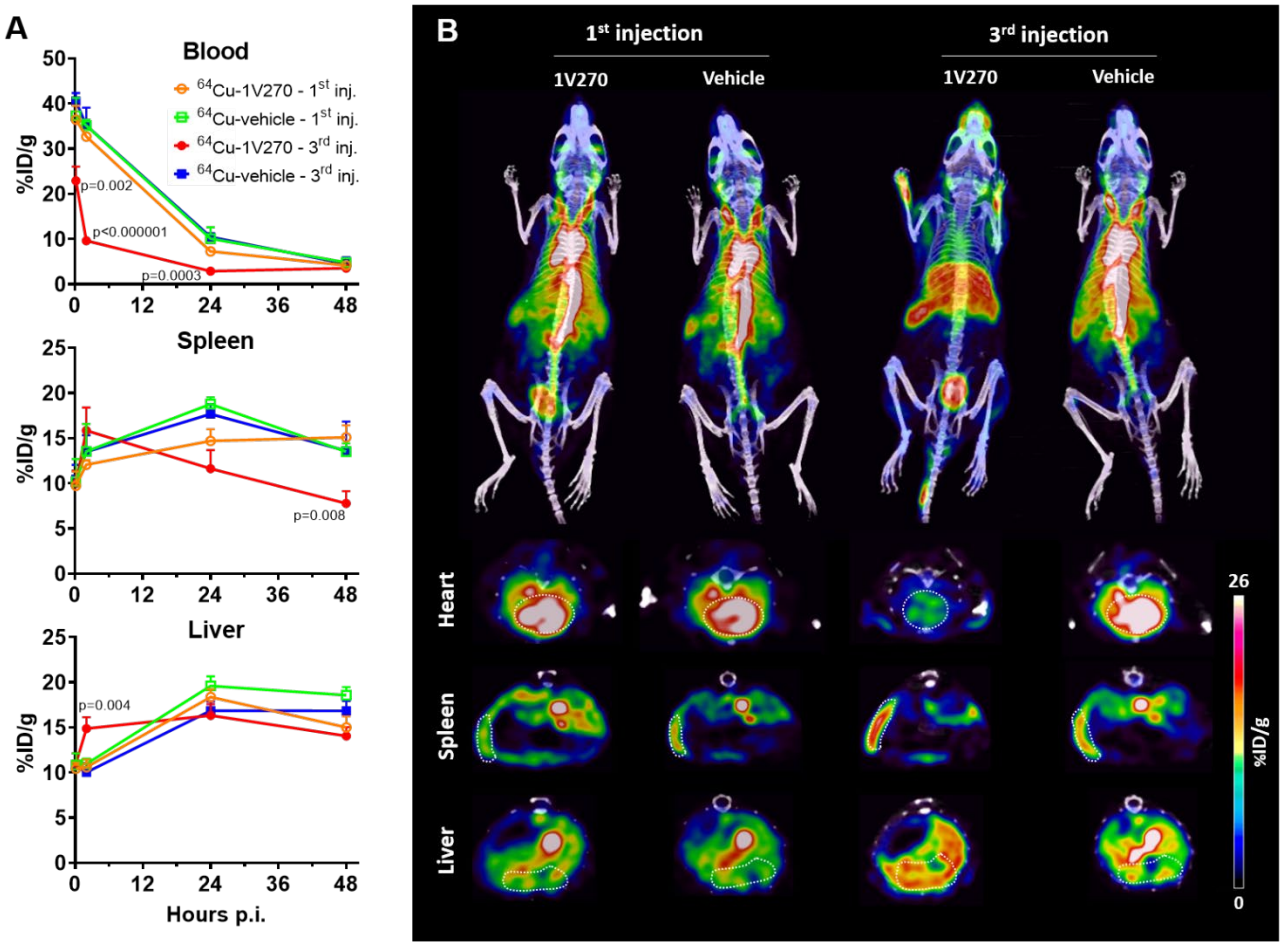

Figure 1: Biodistribution and blood clearance of PEGylated neutral liposomes with and without 1V270 is subject to accelerated blood clearance*. Mice were intravenously administered ^{64}Cu-labelled DOPE-PEGylated neutral liposomes with or without 0.75% 1V270 (formulation #18 or #17, respectively, encapsulated with DOTA and ^{64}Cu) on the first or third injection. The latter having received two unlabeled pretreatments that were administered q4d. The lipid dose administered was 270 µmol/kg for all formulations, and the 1V270 dose was 2.0 µmol/kg for liposomes containing 1V270. PET/CT scans were acquired 10 min, 2 h, 24 h, and 48 h after administration. (**A**) Mean blood, spleen, and liver accumulation presented as %ID/g ± SD (n=4). (**B**) Representative PET/CT images from the 2 h time point. Outline in bottom panels indicates the respective organ. Statistical difference between vehicle at 1st and 3rd injection and 1V270 liposomes at 1st and 3rd injection were determined with multiple t-test with Holm-Sidak correction. p.i. = post injection.*

Rapid clearance of anti-PEG antibodies from circulation following administration of liposomes is associated with hypersensitivity

Accelerated blood clearance of vehicle liposomes is known to be caused by anti-PEG IgM [14,26]. However, a study by Judge et al. demonstrated that both anti-PEG IgM and IgG generated after dosing of PEGylated liposomes containing plasmid DNA (pDNA) [23]. Therefore, we investigated whether anti-PEG IgM or anti-PEG IgG was generated in response to our PEGylated liposomes containing 1V270 and if differences in liposomal formulation affected the anti-PEG antibody levels.

To this end, we investigated intravenously administered PEGylated neutral, cationic, and anionic 1V270 liposomes (#18, #25, & #3, respectively) as well as vehicle liposome. Additionally, subcutaneous injections were included as a control for slower systemic availability and subsequently slower liposomal opsonization. All formulations were administered in a q4d schedule. Anti-PEG antibodies were determined from blood samples collected 1–2 h before the third liposomal administration and five minutes after administration.

Anti-PEG IgM and IgG antibodies were detected for all liposomal formulations containing 1V270 regardless of the investigated composition. In contrast, minimal antibody production was seen for vehicle liposomes. Following intravenous administration of 1V270 liposomes, anti-PEG antibodies were rapidly cleared from the circulation, which corresponded to an observed hypersensitivity reaction (Figure 2A). Although subcutaneously administered liposomes containing 1V270 did induce anti-PEG antibodies, clearance of antibodies upon third administration occurred at a reduced rate and was not associated with hypersensitivity reaction (Figure 2B). These results demonstrate that the addition of a TLR7/8 agonist to PEGylated liposomes induces generation of anti-PEG IgM and IgG and rapid clearance of these antibodies from circulation is associated with hypersensitivity.

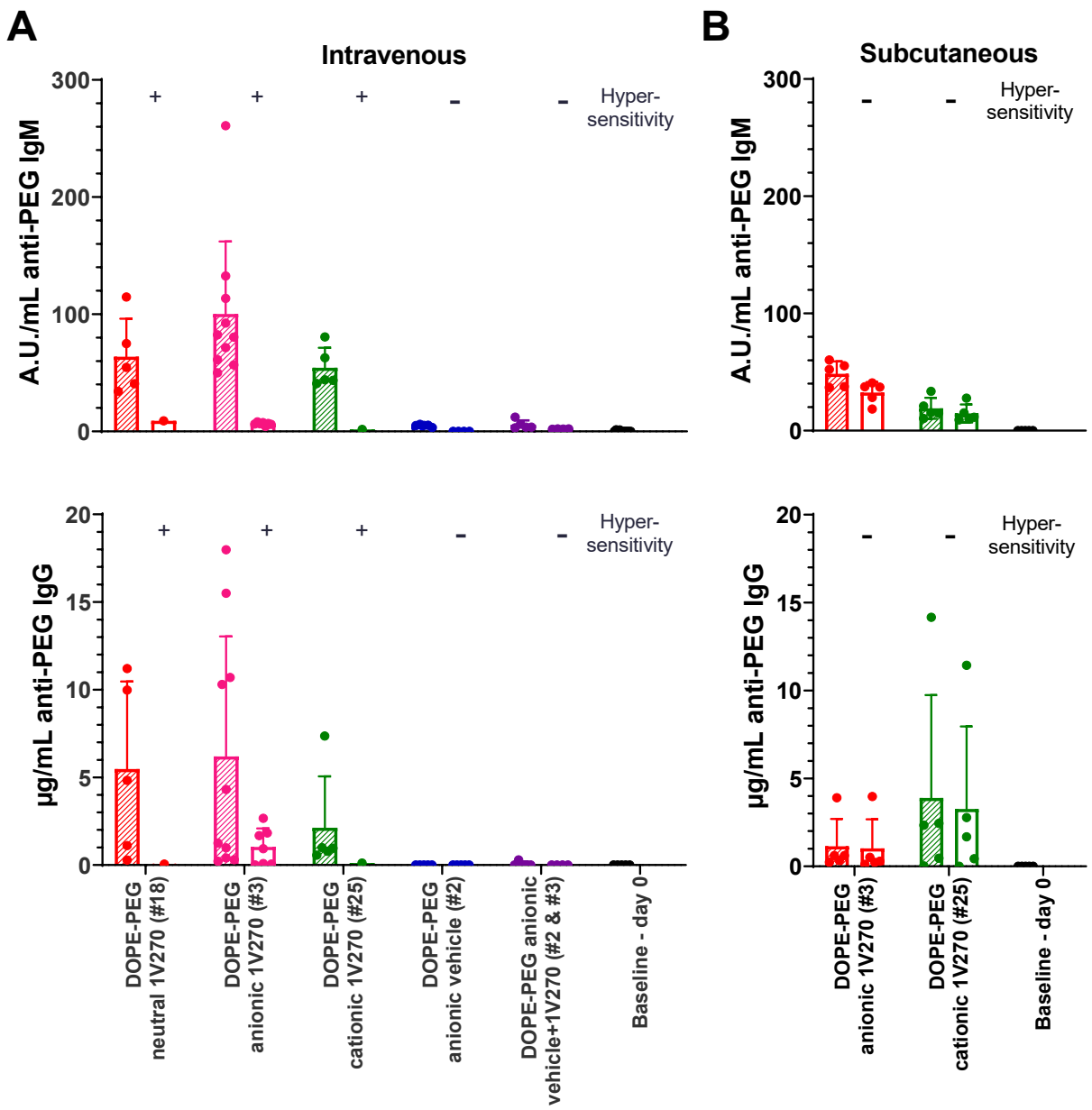

Figure 2: Liposomes containing 1V270 induces anti-PEG IgM and anti-PEG IgG. *Mice were administered intravenously (**A**) or subcutaneously (**B**) with different liposome formulations on day 0 and 4. Formulations further varied in PEGylation and presence of 1V270. For the DOPE-PEG anionic vehicle + 1V270, two doses of DOPE-PEG anionic vehicle liposomes were given on days 0 and 4 and one dose of DOPE-PEG anionic 1V270 on day 8. Formulations containing 1V270 was administered in doses of 2.0 µmol/kg 1V270 and 270 µmol/kg lipid. Plasma was collected on day 8 and anti-PEG IgM and anti-PEG IgG determined by ELISA. Shaded columns = before 3rd administration, open columns = after 3rd administration. Formulations that induced acute hypersensitivity reaction after the third administration on day 8 are marked on the graph by "+" and formulations that did not are marked by "–". # references the formulation, see table 1 and supplementary table 1 for additional information. A.U. = arbitrary units. Shown is mean concentration± SD.*

Since PEGylated 1V270 liposomes resulted in generation of both anti-PEG IgM and anti-PEG IgG, we wanted to investigate further whether anti-PEG IgM or anti-PEG IgG were linked to the observed hypersensitivity reaction following repeated administrations. Consequently, mice were treated with PEGylated neutral liposomes with (#18) or without 1V270 (#17) on day 0 and 4. On day 8, cardiac blood was collected, and total IgG and IgM fractions were isolated using immunoglobulin purification columns. Subsequently, the respective immunoglobulin fractions were administered into naïve mice, followed by the administration of PEGylated liposomes with or without 1V270 10 minutes later. Naïve mice receiving the IgG fraction from mice pretreated with liposomes containing 1V270 developed hypersensitivity 10–20 minutes after liposome administration. In contrast, naïve mice receiving the IgM fraction did not develop any signs of hypersensitivity. Likewise, no symptoms developed in naïve mice receiving the IgG or IgM fraction from mice pretreated with vehicle liposomes, see table 3.

Liposome formulation	Antibody fraction	Hypersensitivity
PEGylated neutral 1V270 liposomes (#18)	IgG	**Yes (5/6)**
	IgM	No (0/6)
PEGylated neutral vehicle liposomes (#17)	IgG	No (0/4)
	IgM	No (0/4)

Table 3: IgG and IgM antibody transfer experiment. *Mice were treated intravenously with PEGylated neutral 1V270 liposomes or vehicle on day 0 and 4. On day 8, blood was collected, and IgG and IgM fractions were purified from the plasma. The antibody fractions were given to naïve mice, followed by an intravenous injection of the PEGylated neutral 1V270 or vehicle liposomes. Mice were monitored for the occurrence of hypersensitivity reactions for up to an hour. Number of mice displaying hypersensitivity is indicated in the parenthesis based on three individual experiments.*

Discussion

In the present study, we showed that repeated systemic administration (q4d) of PEGylated liposomes containing a TLR agonist led to acute hypersensitivity reactions in most batches of various formulations (32 of 40) at high lipid doses (40-540 µmol/kg). We also showed that the hypersensitivity reactions were associated with accelerated blood clearance of liposomes, generation of anti-PEG antibodies and decrease in treatment effect. In accordance with previous studies in mice and rats, we observed minimal anti-PEG antibodies and no accelerated blood clearance when administering PEGylated vehicle liposomes at high lipid doses [19,28], demonstrating that it is the TLR agonist that induce these effects.

The study by Judge et al. (2007) investigated the use of PEGylated liposomes containing pDNA for gene therapy in mice. They observed anti-PEG antibody generation, accelerated blood clearance after the second administration (7 days between) and induction of acute hypersensitivity within 5–10 minutes of administration. Hypersensitivity manifested as lethargy, facial puffing and labored respiration with a fatal outcome at high doses [23]. Other preclinical studies have also reported accelerated blood clearance after repeated administration of PEGylated liposomes containing pDNA [24,25,29]. In addition, accelerated blood clearance, morbidity and mortality have been observed after second dosing (4–6 days between) of PEGylated liposomes containing oligodeoxynucleotides and ribozymes in mice [24].

Including the findings in this study, accelerated blood clearance after repeated administration of high dose PEGylated liposomes has now been shown for liposomes containing 1V270 (TLR7/8 agonist), gardiquimod (TLR7 agonist), CpG oligodeoxynucleotides (TLR9 agonist), non-CpG oligodeoxynucleotides, pDNA (TLR9 agonist), ribozymes, and siRNA (TLR3 agonist) [23–25,29]. Although non-CpG ODNs are not recognized by TLRs, they can still exert adjuvant activity [30]. These observations clearly demonstrate that formulating an immune agonist in PEGylated liposomes often induce anti-PEG antibody generation, leading to accelerated blood clearance and hypersensitivity reactions when dosing repeatedly, ultimately resulting in lack of disease targeting and in severe cases mortality. Several TLR agonists, including TLR7 agonists, can act as B cell mitogens, affect antibody production, and class switching [31–34]. It is possible that binding of liposomal PEG to the BCR on specific B cells enables internalization of the liposomes and subsequently activation of the B cell through TLRs, which in turn induce high production of anti-PEG IgM and IgG. However, immunogenicity studies in mice with defective B cells or with cell-specific deficiencies in TLR signaling would be necessary to confirm this [35,36]. In contrast, PEGylated vehicle liposomes act as a TI type II antigen [16,20,37] where the repetitive PEG structures on the liposome surface crosslink multiple B cell receptors on the surface of B cells, causing activation and production of mainly anti-PEG IgM [20,37,38]. At high antigen concentrations, i.e., when high lipid doses of PEGylated vehicle liposomes are administered, B cells become anergic due to continuous BCR stimulation without co-stimulatory signals, resulting in no anti-PEG IgM production [39,40].

Judge et al. also showed generation of both IgM and IgG upon repeated administration with PEGylated liposomes containing pDNA. They demonstrated that PAF antagonists could alleviate the acute hypersensitivity and hypothesized that the hypersensitivity was mediated by PAF and IgG

opsonization [23]. In mice, particles opsonized by IgG can activate FcγRIII expressing macrophages and platelets that, in response, release PAF and cause an anaphylactic reaction [27]. In support of this, we found that transfer of IgG but not IgM from mice pretreated with PEGylated TLR7 agonist liposomes induced hypersensitivity in naïve mice following liposome administration. Thus, indicating a key role of anti-PEG IgG in mediating acute hypersensitivity upon repeated dosing of PEGylated liposomes with a TLR agonist. Further studies are needed to determine whether the hypersensitivity are caused by IgG opsonization, Fc receptors and PAF release or by liposome-Ig complex activation of complement resulting in hypersensitivity as in complement activation-related pseudoallergy [18]. Anti-PEG IgG was demonstrated in a clinical study on PEGylated RNA aptamers, where pre-existing anti-PEG IgG in patients caused severe allergic responses upon administration of the aptamers [41]. They also found that high anti-PEG IgG levels correlated with severity of the allergic responses. Furthermore, anti-PEG antibodies have been reported in healthy individuals and are associated with diminished treatment response [42,43], which further complicates the usage of PEGylated liposomes.

In summary, the present study demonstrated that PEGylated liposomes containing a TLR7/8 agonist are subject to accelerated blood clearance even at high lipid doses, which does not occur for PEGylated vehicle liposomes. Additionally, the liposomes induced high anti-PEG antibody titers and acute hypersensitivity upon repeated administrations, the latter of which was found to be associated with anti-PEG IgG. Ultimately, this study highlights the need to carefully consider the immunogenicity of nanoparticle formulations and comprehensively evaluate antibody responses generated against nanoparticles.

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Supplementary Information

1V270 Liposomes formulation

Lipids were dissolved in tert-butanol:milliQ water (9:1), mixed to the desired lipid compositions in glass vials, snap-frozen in liquid nitrogen and freeze-dried overnight in a Scanvac Coolsafe lyophilizer (Labogene). Dry lipids were re-hydrated in PBS buffer (pH 7.4) to a concentration of 50 mM total lipid and placed stirring for 1 hour at 65 °C. Size of liposomes was controlled by extruding one time through three stacked Whatman filters (400 nm, 200 nm and 100 nm, GE Healthcare), followed by seven extrusions through two stacked 100 nm Whatman filters. Extrusion was done at 20 bar nitrogen pressure using a 10 mL LIPEX thermobarrel pressure extruder on a heating block at 65 °C.

Gardiquimod liposome formulation

Liposomes with encapsulated gardiquimod were formulated following as described above, with the exception that dry lipids were rehydrated in an ammonium sulfate (150 mM) buffer (pH 5.75) instead of PBS. After extrusion, the liposomes were left at 4 °C overnight before proceeding with the remote loading protocol. The liposomes were transferred to Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) and dialyzed for 86 h against a dialysis buffer (10% Sucrose, 25 mM HEPES, pH 7.4), with buffer exchanges after 18 and 26 h. After dialysis, the phosphorus concentration was measured using ICP-MS, as described below. The liposomes were transferred to new glass vials with gardiquimod in powder form, weighed out to achieve a final drug/lipid ratio of 1:10. The solution was left stirring at 55 °C for 4 h. Due to the high loading efficiency (>95%), the liposomes were used with no further purification.

Determination of gardiquimod loading efficiency

For measuring the encapsulation efficiency of gardiquimod, liposome-encapsulated gardiquimod was separated from free gardiquimod using PD10 Desalting Columns containing Sephadex G-25 resin (GE Healthcare), equilibrated in the dialysis buffer. The loading percentage was calculated by comparing the gardiquimod concentration in the void volume from the column to the total gardiquimod concentration in a liposome before separation.

CpG liposome formulation

Liposomes were prepared using a stealth lipid mix (HSPC/DSPE-mPEG2k/Cholesterol: 3:1:1 w/w/w) as a scaffold. Dry lipid mix was hydrated with a mixture of FITC-CpG-Chol and 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. Hydration was carried out at 70 °C for 1 h under vigorous mixing of the sample. Liposomes were obtained by extruding the lipids 21 times through a 100 nm

nucleopore membrane (Whatman) at 70 °C using a mini extruder set (Avanti Polar Lipids). 500 µL of the crude Liposomes were purified using a self-packed Sephadex G100 superfine (GE Healthcare) column (250 mg, h 45 mm x d 10 mm) using 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl as eluent.

Determination of phospholipid concentration

The total lipid concentration of liposome stocks was determined by measuring the phosphorus concentration using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Samples were first diluted 10,000 times in an ICP-MS diluent (2% HCl, 10 ppb Ga) to fall within a standard range of 25-100 ppb phosphorus. The phosphorus content was then measured on an ICAP-Q from Thermo Fisher Scientific. The total lipid concentration was calculated based on the assumption that 70% of the lipids in our formulations contain a phosphorus atom (50% for DOTAP liposomes). For 1V270 formulations, the total lipid concentration was estimated based on the TMX201 concentration measured by HPLC, which was in agreement with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) measurements.

Determination of 1V270, gardiquimod, and CpG concentration

The concentration of 1V270 and gardiquimod was measured using analytical reversed-phase (RP)-HPLC on a LC-20AD liquid chromatograph (Shimadzu Corporation) equipped with a DGU-20A SR degassing unit and a SIL-20AC HT autosampler. The HPLC Eluent A consisted of a 5% CH_3CN aqueous solution with 0.1% trifluoroacetic acid (TFA); HPLC Eluent B consisted of 0.1% TFA in CH_3CN. 1V270 liposomes were diluted 5 times in PBS and compared to a 1V270 standard (50-200µM diluted in DMSO). An XBridge C8 (5 µm, 4.6x150 mm) column (Waters) was used, and quantification of 1V270 was done using UV detection at 280 nm with an SPD-M20A Photodiode Array Detector fitted with a Deuterium Tungsten lamp (Shimadzu Corporation). A gradient from 40% to 100% B over 15 minutes was applied (flow rate 1 mL/min). Gardiquimod liposomes were diluted four times in dialysis buffer and compared to a gardiquimod standard (25-100 µg/mL diluted in MQ). A Waters XTerra C18 (5 µm, 4.6x150 mm) column (Waters) was used for measuring Gardiquimod and quantification was done using the same detector as for 1V270 at 320 nm. Quantification was done with a gradient from 0% of 100% B over 15 minutes (flow rate 1 mL/min). The ODN content of CpG liposomes was determined by measuring the fluorescence (ex: λ = 490 nm; em: λ = 525 nm) in comparison to free FITC-CpG-Chol (+/−) in triplets using a Spark® (Tecan) plate reader. A 20-fold dilution of purified ODN-bearing liposomes with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl was used.

^{64}Cu labeling of liposomes for biodistribution study

DOPE-PEGylated neutral vehicle (formulation #17) and DOPE-PEGylated neutral containing 1V270 (formulation #18) liposomes for biodistribution studies were encapsulated with DOTA. Liposomes were formulated following the same procedure as described above, with the exception that the dry lipids were rehydrated in a buffer containing 10 mM DOTA, 10 mM HEPES, and 150 mM NaCl (pH 7.4). After extrusion, liposomes were left at 4 °C overnight before proceeding with dialysis. Liposomes were transferred to Slide-A-Lyzer dialysis and dialyzed for 8 days (buffer exchanges on day 2 and 5) against dialysis buffer (10 mM HEPES, and 150 mM NaCl (pH 7.4).

Radiolabelling of liposomes was conducted as previously described[44]. Briefly, 1.4 mL of each set of liposomes (DOPE-PEGylated neutral vehicle liposomes, DOPE-PEGylated neutral 1V270 liposomes, 36 mM) was added to 180 MBq dry [^{64}Cu]$CuCl_2$ and stirred at 55 °C for 75 min. The samples were equilibrated at room temperature, 2 µL was spotted for Radio-TLC, and 50 µL was pipetted onto pre-equilibrated PD10 columns. Radio-TLC were performed on silica gel 60 F254 plates (Merck) with 5% (w/v) NH_4OAc in H_2O-MeOH (1:1) as eluent. The PD10 columns were eluted and equilibrated by ISO-HEPES (150 mM NaCl, 10 mM HEPES, pH 7.4). Both the vehicle liposome and the 1V270 liposome formulation displayed >95% loading on PD10. Radio-TLC further showed that the vehicle liposomes (^{64}Cu-lip) and 1V270 liposomes (^{64}Cu-lip-1V270) contained 0.6% and 3.1% unloaded $^{64}Cu^{2+}$ respectively. ^{64}Cu-lip had an activity concentration of 94.4 MBq/mL and ^{64}Cu-lip-1V270 an activity concentration of 98.4 MBq/mL.

Sterile filtering

Before usage, the liposomes were diluted to a final drug concentration, so the desired dose could be reached by injection 150 µL. 1V270 liposomes were diluted in PBS buffer, gardiquimod liposomes were diluted in the dialysis buffer. Finally, the liposomes were sterile filtered through 0.45 µm syringe filters (Frisenette) and transferred to sealed sterile vials (Sterillab A/S)

Liposome characterization

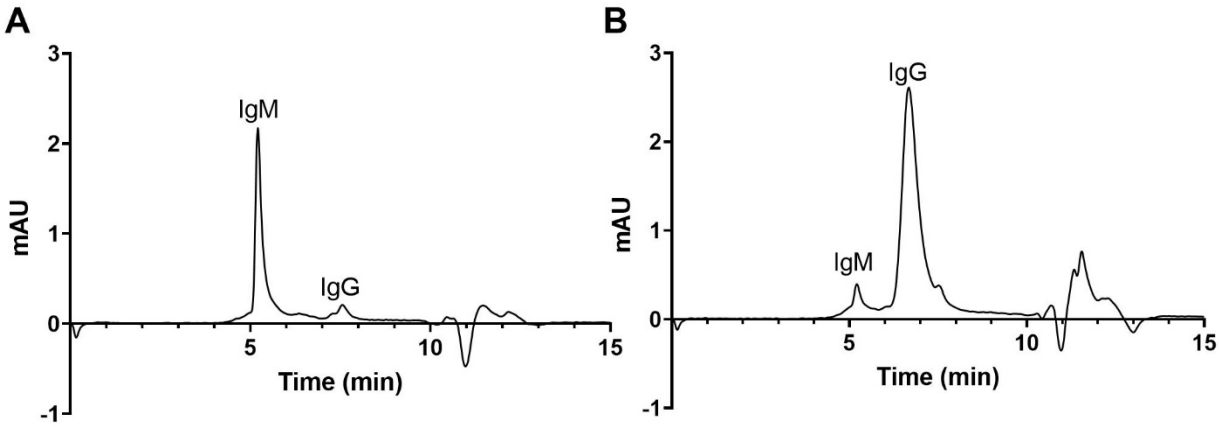
The hydrodynamic diameter and polydispersity index of the liposomes were measured by dynamic light scattering using a ZetaSizer Nano ZS (Malvern Instruments), equipped with a 633 nm laser. The liposomes were diluted to approximately 120 µM total lipid in the buffer the liposomes was formulated in (PBS buffer for 1V270 liposomes, dialysis buffer for the Gardiquimod liposomes) and the size measured as the average from 3 runs of 15 cycles each. The zeta potential of the liposomes was measured using the same instrument by Mixed Measurement Mode Phase Analysis Light

Scattering in glucose buffer (300 mM glucose, 10 mM HEPES, 1 mM $CaCl_2$ at pH 7.4) at 120 µM total lipid. Each measurement consisted of 3 individual runs in automatic mode (10-100 cycles).

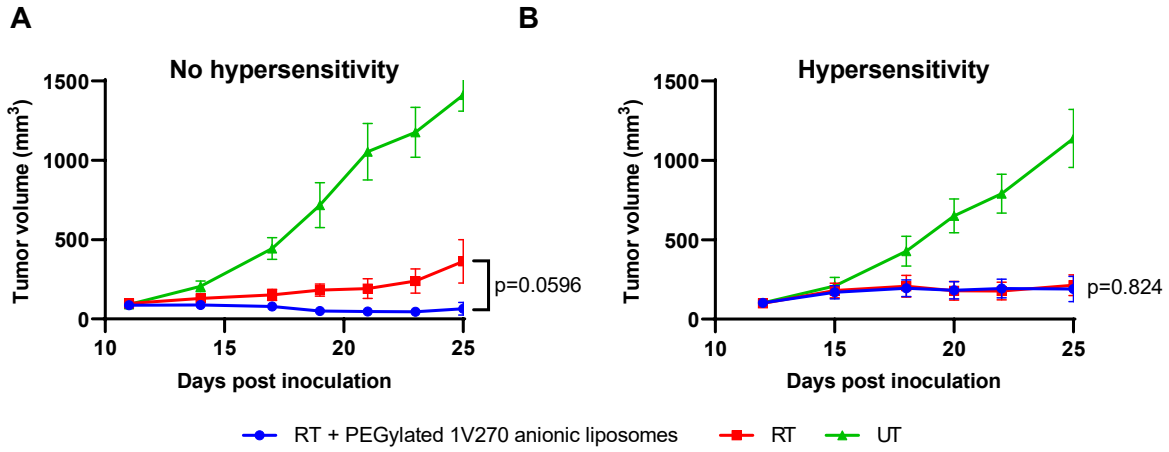
#	Name	Formulation	Batches	Size (nm)	PDI	Zeta potential (mV)	Drug dose (µmol/kg)	Lipid dose (µmol/kg)	Dosing schedule	Hyper-sensitivity
Anionic										
1	non-PEG 0.75% 1V270	POPC:Chol:POPG:1V270:(49.25:30:20:0.75)	3	128 (105-141)	0.062 (0.055-0.07)	-21.3 (-20.4 to -22.1)	1.1-2.0	130-270	q4d × 5	No
2	DOPE-PEG vehicle	POPC:Chol:POPG:DOPE-mPEG2k (45:30:20:5)	1	86	0.047	-6.0	0	270	q4d × 3	No
3	DOPE-PEG 0.75% 1V270	POPC:Chol:POPG:1V270:DOPE-mPEG2k (44.25:30:20:0.75:5)	10	101 (90-122)	0.04 (0.023-0.079)	-7.8 (-5.6 to -10.2)	1.4-2.2 0.7	90-270 90	q4d × 3 q1d × 10	Yes No
4	DOPE-PEG 5% 1V270	POPC:Chol:POPG:1V270:DOPE-mPEG2k (40:30:20:5:5)	1	149	0.017	-14.5	2.0	41	q4d × 3	Yes
5	DSPE-PEG 0.25% 1V270	POPC:Chol:POPG:1V270:DSPE-mPEG2k (44.75:30:20:0.25:5)	1	107	0.037	-9.1	0.7	270	q4d × 3	Yes
6	DSPE-PEG 0.75% 1V270	POPC:Chol:POPG:1V270:DSPE-mPEG2k (44.25:30:20:0.75:5)	6	112 (93-128)	0.04 (0.037-0.052)	-8.3 (-5.4 to -13.3)	1.0-4.1 0.7	90-540 90	q4d × 3 q1d × 5	Yes No
7	DSPE-PEG 0.75% 1V270 (10% POPG)	POPC:Chol:POPG:1V270:DSPE-mPEG2k (54.25:30:10:0.75:5)	1	106	0.042	-9.5	2.0	270	q4d × 3	Yes
8	DSPE-PEG 0.75% 1V270 (30% POPG)	POPC:Chol:POPG:1V270:DSPE-mPEG2k (34.25:30:30:0.75:5)	1	103	0.028	-9.3	2.0	270	q4d × 3	Yes
9	DSPE-PEG 1.5% 1V270	POPC:Chol:POPG:1V270:DSPE-mPEG2k (43.5:30:20:1.5:5)	1	104	0.029	-9.1	4.1	270	q4d × 3	Yes
10	DSPE-PEG 5% 1V270	POPC:Chol:POPG:1V270:DSPE-mPEG2k (40:30:20:5:5)	1	101	0.036	-9.6	13.3	270	q4d × 3	Yes
11	DSG-PEG 0.75% 1V270	POPC:Chol:POPG:1V270:DSG-mPEG2k (44.25:30:20:0.75:5)	2	98 (97-99)	0.029 (0.028-0.029)	-6.9 (-6.6 to -7.2)	2.0	270	q4d × 3	Yes
12	Chol-PEG 0.75% 1V270	POPC:Chol:POPG:1V270:Chol-mPEG2k (44.25:30:20:0.75:5)	1	91	0.037	-8.4	2.0	270	q4d × 3	No
13	DOPE-PEG Gardiquimod	POPC:Chol:POPG: DOPE-mPEG2k (45:30:20:5) + Gardiquimod (1:10 drug:lipid)	1	116	0.029	-7.1	9.6	86	q4d × 3	No
14	DSPE-PEG Gardiquimod	DSPC:Chol:DSPG: DSPE-mPEG2k (45:30:20:5) + Gardiquimod (1:10 drug:lipid)	1	131	0.033	-6.2	9.6	94	q4d × 3	Yes
15	DSG-PEG Gardiquimod	POPC:Chol:POPG:DSG-mPEG2k (45:30:20:5) + Gardiquimod (1:10 drug:lipid)	1	116	0.056	-7.3	9.6	70	q4d × 3	Yes
Neutral										
16	non-PEG 0.75% 1V270	POPC:Chol:1V270 (69.25:10;0.75)	1	112	0.05	-3.1	2.0	270	q4d × 3	No
17	DOPE-PEG vehicle	POPC:Chol:DOPE-mPEG2k (65:30:5)	1	158	0.201	-3.9	0	270	q4d × 3	No
18	DOPE-PEG 0.75% 1V270	POPC:Chol:1V270:DOPE-mPEG2k (64.25:30:0.75:5)	4	101 (95-112)	0.089 (0.042-0.146)	-5.4 (-4.3 to -7.3)	2.0	270	q4d × 3	Yes
19	DOPE-PEG 5% 1V270	POPC:Chol:1V270:DOPE-mPEG2k (60:30:5:5)	1	105	0.082	-12.8	2.0	41	q4d × 3	Yes
20	DSG-PEG 0.75% 1V270	POPC:Chol:1V270:DSG-mPEG2k (64.25:30:0.75:5)	1	84	0.076	-8.4	2.0	270	q4d × 3	Yes
Stealth										
21	DSPE-PEG Gardiquimod	HSPC:Cholesterol:DSPE-PEG2000 (56.6:38.2:5) + Gardiquimod (1:10 drug:lipid)	1	113	0.041	-3.9	9.6	120	q4d × 3	Yes

22	DSPE-PEG CpG ODN	HSPC:DSPE-mPEG2k:Cholesterol (57:38:5) + FITC-tccatgacgttcctgacgtt-Chol	1	119	0.019	-15.1	0.07	138	q4d × 3	Yes
23	DSPE-PEG non-CpG ODN	HSPC:DSPE-mPEG2k:Cholesterol (57:38:5) + FITC-tccatgagcttcctgagctt-Chol	1	125	0.041	-13.3	0.06	123	q4d × 3	No
Cationic										
24	non-PEG 0.75% 1V270	POPC:Chol:DOTAP:1V270 (61:30:8.25:0.75)	1	100	0.08	38.3	2.0	270	q4d × 3	Yes
25	DOPE-PEG 0.75% 1V270	POPC:Chol:DOTAP:1V270:DOPE-mPEG2k (44.25:30:20:0.75:5)	2	135 (117-153)	0.101 (0.026-0.175)	+16.3 (14.2 to 18.4)	2.0	270	q4d × 3	Yes

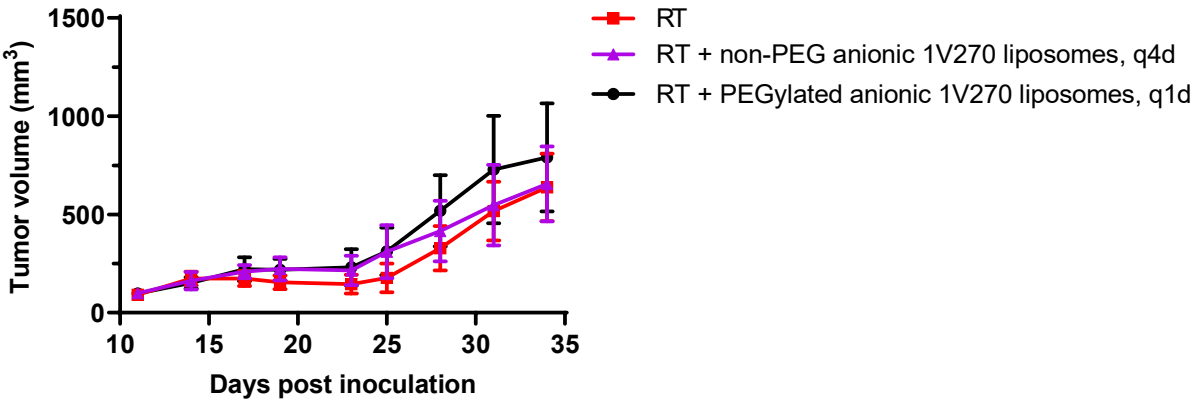
Supplementary Table 1: Overview of liposomal formulation given throughout the study*. N.D. = not determined. PDI = polydispersity index. For size and zeta potential, mean value (range) are displayed.*



Supplementary Figure S1: *Mice were treated with DOPE-PEGylated neutral 0.75% 1V270 liposomes (2.0 µmol/kg 1V270 per administration, formulation #18) on day 0 and 4. Blood was collected on day 8, and IgG and IgM antibody fractions were purified from plasma using protein A/G and protein L spin columns. HPLC chromatograms for IgM* ***(A)*** *and IgG* ***(B)*** *antibody.*



Supplementary Figure S2: *CT26 tumor-bearing mice were treated with 1V270 liposomes. All groups received 2 Gy RT on 5 consecutive days from the day of the first liposome treatment. RT was administered to the tumor-bearing flank.* ***A)*** *Mice were randomized on day 11 post inoculation (mean tumor volume: 90 mm^3) and treated with RT in combination with DOPE-PEGylated anionic 0.75% 1V270 liposomes (formulation #3) q4d for a total of 5 treatments. Mice were administered 2.0 µmol/kg 1V270 and 266 µmol/kg lipid per liposome administration. No acute hypersensitivity reactions were observed.* ***B)*** *Mice were randomized on day 12 post inoculation (mean tumor volume ≈100 mm^3) and treated with RT in combination with DOPE-PEGylated anionic 0.75% 1V270 liposomes (formulation #3) q4d for a total of 3 treatments. Acute hypersensitivity reaction was observed after the third administration. Mice were administered 2.0 µmol/kg 1V270 and 263 µmol/kg lipid per liposome administration. Graph show mean tumor volume±SEM. Unpaired t-test was used to compare RT and RT+1V270 liposomes on day 25 in both A and B.*



Supplementary Figure S3: *CT26 tumor-bearing mice were treated with 1V270 liposomes. All groups received 2 Gy RT on 5 consecutive days from the day of the first liposome treatment. RT was administered to the tumor-bearing flank. Mice were randomized on day 11 post inoculation (mean tumor volume: 95 mm^3) and treated with RT in combination with non-PEGylated anionic 0.75% 1V270 liposomes (formulation #1) given q4d for a total of 5 treatments (2.0 µmol/kg 1V270 and 270 µmol/kg lipid per administration) or DOPE-PEGylated anionic 0.75% 1V270 liposomes (formulation #3) given q1d for 10 treatments (0.7 µmol/kg 1V270 and 74 µmol/kg lipid per administration). Graph show mean tumor volume±SEM.*