Encapsulation of a CpG oligonucleotide in cationic liposomes enhances its local antitumor activity following pulmonary delivery in a murine model of metastatic lung cancer

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Abstract:
Immunotherapy brings new hope to the fight against lung cancer. General immunostimulatory agents represent an immunotherapy strategy that has demonstrated efficacy with limited toxicity when delivered intratumorally. The goal of this study was to enhance the antitumor efficacy of unmethylated oligodeoxynucleotides containing CpG motifs (CpG) and polyinosinic-polycytidylic acid (poly I:C) double-stranded RNA following their local delivery in lung cancer by encapsulating them in liposomes. Liposomes encapsulation of nucleic acids could increase their uptake by lung phagocytes and thereby the activation of toll-like receptors within endosomes. Liposomes were prepared using a cationic lipid, dioleoyltrimethylammoniumpropane (DOTAP), and dipalmitoylphosphatidylcholine (DPPC), the main phospholipid in lung surfactant. The liposomes permanently entrapped CpG but could not efficiently withhold poly I:C. Both poly I:C and CpG delayed tumor growth in the murine B16F10 model of metastatic lung cancer. However, only CpG increased IFN-γ levels in the lungs. Pulmonary administration of CpG was superior to its intraperitoneal injection to slow the growth of lung metastases and to induce the production of granzyme B, a pro-apoptotic protein, and IFNγ, MIG and RANTES, T helper type 1 cytokines and chemokines, in the lungs. These antitumor activities of CpG were strongly enhanced by CpG encapsulation in DOTAP/DPPC liposomes. Delivery of low CpG doses to the lungs induced increased inflammation markers in the airspaces but the inflammation did not reach the systemic compartment in a significant manner. These data support the use of a delivery carrier to strengthen CpG antitumor activity following its pulmonary delivery in lung cancer.

Keywords: lung cancer, toll like receptors, CpG oligodeoxynucleotide, liposomes, pulmonary delivery

Abbreviations: ALK, anaplastic lymphoma kinase; CpG, unmethylated oligodeoxynucleotides containing CpG motifs; CTLs, cytotoxic T lymphocytes; DCs, dendritic cells; DMEM, Dulbecco modified eagle medium; DOPE, dioleoylphosphatidylethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DPPC, 1,2-dipalmitoylphosphatidylcholine; EE, encapsulation efficiency; EGFR, epidermal growth factor inhibitor; EggPC, egg phosphatidylcholine; FBS, Fetal bovine serum; HBSS, Hank's balanced salt solution; IFN-γ, Interferon gamma; IL-6, interleukin 6; IL-10, interleukin 10; IP, Intraperitoneal injection; IT, Intratracheal instillation; MIG, Monokine induced by gamma interferon; NK cells, Natural killer cells; NSCLC, Non-small cell lung cancer; PBS, phosphate buffer saline; PD-L1, programmed death-ligand 1; PD-1, programmed death receptor-1; Poly I:C, polyinosinic-polycytidylic acid double-stranded RNA; PCR, polymerase chain reaction; RANTES, regulated on activation normal T cell expressed and secreted; TLR, toll-like receptor; TNF-α, Tumor necrosis factor alpha.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for 1.8 million deaths in 2018 (WHO, 2018). Non-small cell lung cancer (NSCLC) represents approximately 85% of cases (Planchard et al., 2018). Due to the lack of characteristic clinical symptoms, diagnosis of lung cancer often occurs at an advanced or metastatic stage leading to a poor prognosis. Accordingly, the overall 5-year survival rate is only 18% (Torre LA, 2016).

Treatment approaches for patients with lung cancer have significantly evolved over the last decade and have become more personalized (Economopoulou and Mountzios, 2018; Planchard et al., 2018). Patients positive for sensitizing EGFR mutations, ALK gene rearrangements and ROS1 translocation are treated in first line by specific tyrosine kinase inhibitors, possibly combined with antiangiogenic agents. BRAF mutation should be tested in advanced non-squamous NSCLC for the prescription of BRAF inhibitors. Immune checkpoint inhibitors are front-line treatment in patients with lung cancer and strong programmed death-ligand 1 (PD-L1) positivity. Low PD-L1 expressers receive systemic platinum-based chemotherapy possibly combined with immune checkpoint inhibitors and antagonist antibodies to growth factors. Immune checkpoint inhibitors are recommended as second line therapy in all patients with lung cancer and no driver mutation (Planchard et al., 2018).

Immunotherapy brings new hope to the fight against lung cancer (Dholaria et al., 2016). Immunotherapy stimulates the host immune system to recognize tumor cells as non-self and eliminate them. Immune checkpoint inhibitors (e.g., pembrolizumab, atezolizumab) have been the first immunotherapeutics approved for lung cancer treatment (Reck and Paz-Ares, 2015). Immune checkpoints are inhibitory pathways in the immune system that are crucial for maintaining self-tolerance and preventing excessive and potentially deleterious T-cell activity in peripheral tissues. Immune checkpoint blockade can enhance T cell activation and effector functions resulting in a swift tissue inflammatory response.

Combining immunotherapies could further improve their therapeutic outcomes (Dholaria et al., 2016). In the ideal combination, each component should have single-agent activity, synergy between the components should be proven and the components should not have overlapping safety profiles (Reck and Paz-Ares, 2015). General immunostimulatory agents such as toll-like receptor (TLR) agonists represent an immunotherapy strategy that has demonstrated activity on its own and that has already been combined to immune checkpoint inhibitors in preclinical models and human trials (Ribas et al., 2018). Subcutaneous injection of unmethylated oligodeoxynucleotides containing CpG motifs (CpG), a TLR9 agonist, combined with first-line taxane plus platinum chemotherapy improved survival in patients with advanced NSCLC (Manegold et al., 2008). However, systemic exposure to CpG was associated with sepsis-related serious adverse events and trials were discontinued (Hirsh V, 2011). Intratumoral injection of TLR agonists can preserve their antitumor benefits while avoiding their systemic toxic effects. Accordingly, peritumoral or intratumoral CpG injection resulted in complete regression of subcutaneous tumors in mice (Heckelsmiller et al., 2002; Houot and Levy, 2009). Combining CpG with immune checkpoint inhibitors amplified the antitumor immune response and made it capable of eradicating advanced and systemic tumors in mice (Marabelle et al., 2013). This combination also provided durable tumor responses in patients with unresectable or metastatic malignant melanoma (Ribas et al., 2018). Similarly, local delivery of TLR agonists in the lungs have shown anti-tumor efficacy in murine models of lung metastases (Le Noci et al., 2015; Sfondrini et al., 2013) and combining pulmonary delivery of TLR agonists with systemic administration of immune checkpoint inhibitors led to a durable rejection of both lung tumors and tumor lesions outside the lungs (Gallotta et al., 2018).

Although the efficacy and safety of TLR agonists have been enhanced by their peritumoral or intratumoral administration, their efficacy could further improve by using a delivery carrier targeting intracellular TLRs. Toll-like receptors of nucleic acids are located within the cell endosomal compartment. Therefore, encapsulating TLR agonists within a particulate carrier will directly target TLR agonists to their receptor through phagocytsosis of the carrier and could enhance their activity. Suzuki et al showed that the encapsulation of CpG in cationic liposomes greatly enhanced CpG-induced type 1 innate immunity (Suzuki et al., 2004). CpG uptake by dendritic cells (DCs) was strongly increased following intravenous administration of liposomal CpG as was the activation of DCs, natural killer (NK) cells and NK T cells. Similarly, Bayyurt et al observed enhancement in splenocytes uptake of poly I:C and CpG and in the associated immune stimulation when the TLR agonists were encapsulated in liposomes (Bayyurt et al., 2017).

The goal of this research was to enhance the antitumor efficacy of TLR agonists following their local delivery in lung cancer by encapsulating them in liposomes. Our hypothesis was that liposomes encapsulation of TLR agonists could protect them from rapid degradation extracellularly and increase their uptake by lung phagocytes. Liposomes were selected to encapsulate TLR agonists as they are biocompatible and as they can be prepared with lipids endogenous to the lungs. The antitumor efficacy and the induced immune responses were

analyzed in the B16F10 mouse melanoma lung metastasis model following pulmonary delivery and intraperitoneal injection of CpG and polyinosinic-polycytidylic acid (poly I:C) double-stranded RNA, a TLR3 agonist. Intraperitoneal injection was used as a comparator.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids, Alabaster, AL, USA. 1,2-dipalmitoylphosphatidylcholine (DPPC) was purchased from Lipoid GMBH, Ludwigshafen, Germany. The phosphorothioate-linked CpG oligonucleotides C274 (5'TCGTCGAACGTTCGAGATGAT 3') and B1826 (5'-TCCATGACGTTCCTGACGTT-3') were purchased from Eurogentec, Seraing, Belgium. High molecular weight poly I:C (1.5-8 kb) was purchased from Invivogen, Toulouse, France. Dulbecco modified eagle medium (DMEM) glutamax was purchased from Life Technologies, Merelbeke, Belgium. Fetal bovine serum (FBS), phosphate buffer saline (PBS) and Hank's balanced salt solution (HBSS) were purchased from Gibco, Merelbeke, Belgium. Chloroform (analytical grade) was purchased from VWR International, Leuven, Belgium. RNAlater and Trizol were purchased from ThermoFisher, Merelbeke, Belgium.

2.2. Formulation and characterization of liposomes

Liposomes were prepared by the film hydration method. Briefly, lipids (5.9 mg DOTAP and 4.1 mg DPPC) were dissolved in chloroform (750 µl) in a 3:2 molar ratio (Vanbever et al., 2019). A film was prepared by rotary evaporation at 40°C under vacuum. 1 ml of PBS at room temperature or glucose 5% containing 300 µg poly I:C or 1 ml of water containing 200 µg CpG was added to the film. Hydration of the lipid film with CpG in PBS prevented the lipid film from resuspending and caused the formation of large lipid aggregates. However, this was avoided when CpG was in water. Five cycles of freezing in liquid nitrogen and thawing at 55°C were applied in order to prepare liposomes. Liposomes were downsized by extrusion through polycarbonate membranes of 200 nm pore size (Whatman, Diegem, Belgium) at room temperature using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA). Liposomes were concentrated by ultrafiltration at 3000 g and 4°C for 1h (Vivaspin 2, 100 kDa molecular weight cut-off; Sartorius, Stonehouse, Gloucestershire, UK).

Non-entrapped poly I:C or CpG was measured by UV spectroscopy of the filtrate at 250 and 260 nm, respectively, and compared to a calibration curve. The encapsulation efficiency (EE) was calculated as follows:

$$EE(\%) = \frac{Initial\ amount\ of\ TLR\ agonist - Unentrapped\ TLR\ agonist}{Initial\ amount\ of\ TLR\ agonist}\ x\ 100$$

Liposome size and zeta potential were measured with a Nanosizer NanoZS (Malvern, UK). Release of TLR agonists from liposomes was evaluated as follows: concentrated liposomes were poured into a dialysis bag (Spectra Por, 6-8 kDa molecular weight for CpG and 100 kDa molecular weight for poly I:C, Spectrum labs, Breda, Netherlands). The dialysis bag was kept in PBS at 37°C under stirring. Samples were withdrawn at pre-determined times and analyzed by UV spectroscopy. Liposomes were prepared the same day as *in vitro* or *in vivo* experiments. Previous data have shown that empty liposomes and liposomes encapsulating CpG preserved their physical properties (size, zeta potential and CpG loading) during storage at room temperature over at least two weeks (Vanbever et al., 2019).

Liposome morphology was observed by transmission electron microscopy (Philips Electron Microscope CM12/STEM). Suspension of liposomes encapsulating CpG was diluted 100-times in ultrapure water. A negative staining was made by mixing liposome dispersion with a 2% uranyl acetate (Merck, Darmstadt, Germany) in a 1:1 (v/v) ratio. Stained liposomes were deposited on copper grids (Formvar 300 Mesh Cu grids, Agar Scientific, Essex, UK). The excess liquid was removed and grids were allowed to dry in a Petri box. Microscopic analyses were carried out at a magnification of 60,000.

2.3. In vivo efficacy

Specific-pathogen-free female C57BL/6 Nrj mice, aged between 6 and 8 weeks, were used for the experiments (Janvier elevage, Le Genest-St-Isle, France). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain (Permit number: UCL/MD/2012/006). All studies were performed under anesthesia and all efforts were made to minimize animal suffering.

A murine model of metastatic lung cancer was established by tail vein injection of B16-F10 cells. The B16F10-Red-FLuc Bioware® Brite Cell Line (B16-F10luc cells; Perkin Elmer; Zaventem, Belgium) is a light-producing cell line derived from C57BL/6J mouse melanoma. B16-F10luc cells have been stably transduced with the red-shifted firefly luciferase gene from Luciola Italica (Red-FLuc), for a bright red-shifted signal. B16F10luc cells were cultured in DMEM + glutamax containing 10% FBS and incubated at 37°C and 5% CO_2. The maximum passage for *in vivo* use was 21. On day 0, 3 x10^5 B16F10luc cells suspended in 100 µL of PBS were injected intravenously in the tail vein of the mice. Poly I:C (10 µg per mouse) or CpG oligonucleotide (10 µg or 1 µg per mouse; the C274 sequence was delivered unless otherwise noted) encapsulated in liposomes or in solution were administered intraperitoneally (100 µl) or intratracheally (25 µl) every 5 days starting from day 4 after tumor cells inoculation. Mouse groups included 8 to 10 mice at experiment start. For intratracheal instillation, the mouse was positioned with a 45° angle of tilt and a bended and blunt needle of a 100 µl precision micro-syringe (Hamilton, Bonaduz, Switzerland) was placed into the trachea via the mouth. Following instillation, a 200 µl air bolus was insufflated in the trachea using a 1 ml syringe having an angled 18 gauge needle with a blunt tip to push the solution to the distal tract (Minne et al., 2007). In a first preliminary experiment, the Kaplan–Meier survival rate was performed (Fig. S1). The endpoints of the experiment were determined as ≥20% mouse weight loss, difficulty

in breathing or hindlimb paralysis. At these endpoints, the mice were sacrificed by cervical dislocation. This preliminary experiment allowed to determine that day 15 post-tumor cells inoculation corresponded to the last day without any mouse death (Fig. S1). Mean survival times in mice treated with TLR agonists increased by up to four days compared with non-treated mice (Fig. S1D). In next experiments, mice were sacrificed by cervical dislocation at day 15. Before sacrifice, blood was collected by orbital bleeding. A broncho-alveolar lavage (BAL) was performed on euthanized mice. One ml of HBSS was injected through a cannula into the trachea, left for 10 to 30 s, followed by withdrawal and re-injection of twice 0.5 ml of the fluid. Then, all the BAL liquid was removed from the lungs. The lungs were then removed. The post cava lobe was placed in 500 µl of RNAlater for 24h and then stored at -80°C in RNAse free conditions until analysis. Remaining lung lobes were weighted and then crushed with 1 ml of HBSS using a Polytron PT 1200 E (Kinematica, Lucerne, Switzerland). Sera, BAL and lung homogenates were centrifuged at 2500 g and 4°C for 10 minutes. Supernatants were recovered and stored at -20°C until analysis.

2.4. Quantification of tumor cells in the lungs

The number of tumor cells present in the lungs was assayed in the lung homogenate supernatants with the ONE-Glo luciferase assay system (PROMEGA, Leiden, Netherlands). Briefly, 100 µl of lung homogenate supernatant was mixed with 100 µl of luciferase substrate and incubated for 5 minutes in the dark. Luminescence was measured during one second with a VICTOR 2 multi-function fluorescence, absorbance and luminescence microplate reader (Perkin Elmer). A calibration curve performed with B16F10luc cells was used to calculate the number of tumor cells present in the lungs. The number of cells was normalized to the lung weight and expressed as number of cells/ mg lung.

2.5. Measurement of immune responses

The granzyme B production was assayed in lung homogenate supernatants by ELISA (R&D Systems, Abingdon, UK)) according to the supplier protocol. The production of the cytokines IFNγ, MIG and RANTES was assayed in lung homogenate supernatants by Bio-Plex Multiplex immunoassays (Biorad, USA) according to the supplier protocol. Cytokine levels were measured using Bio-Plex 200 instrument (Biorad, USA). The pro-inflammatory cytokines IL-6 and TNF-α were assayed in sera and BAL by ELISA (DuoSet ELISA kits; R&D Systems).

RNA was extracted from the post cava lung lobe with Trizol. The post cava lobe was crushed with 500 µl of Trizol reagent and extracted according to manufacturer's protocol. cDNA was synthetized from one microgram of isolated RNA using a SuperScript First-Strand Synthesis system (ThermoFisher). Prior to polymerase chain reaction (PCR), cDNA was diluted five times. Quantitative PCR reactions were run in a Thermocycler CFX Real Time System (Biorad, USA) and performed using qPCR Mastermix TaqMan, primer pairs and probes specific for murine IFN-γ, IL-10 and β-actin (Eurogentec S.A, Seraing, Belgium). Reactions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. Results

were analyzed by MIQ software (Biorad, USA). Transcripts were normalized to the levels of the murine β-actin.

2.6. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM) unless otherwise noted. Two-way ANOVA test with Bonferroni post-test was used for the analysis of the data in Figure 1. The Kruskal-Wallis test with Dunns post-test was used to analyze the data in Table 1 and Figures 2 to 7. These tests were performed using the GraphPad Prism software for Windows. P values < 0.05 were considered statistically significant.

3. Results

3.1. Liposome characterization

Liposomes containing the TLR agonists, CpG or poly I:C, were formulated by the hydration film method using dioleoyltrimethylammoniumpropane (DOTAP), a cationic lipid largely used to encapsulate anionic nucleic acids (Lechanteur et al., 2018), and dipalmitoylphosphatidylcholine (DPPC), the most abundant phospholipid in lung surfactant (Vanbever et al., 2019). Liposomes presented a size below 200 nm with a low polydispersity index and were positively charged due to the cationic lipid (Table 1). High encapsulation efficiency was obtained for both TLR agonists. The cationic lipid to DPPC molar ratio was 3 to 2. This high proportion of cationic lipid in the liposomes was needed in order to efficiently encapsulate CpG and poly I:C.

Table 1: Physical characterization of DOTAP:DPPC liposomes

	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Loading (µg/mg lipid)
Empty	134 ± 6	0.091 ± 0.007	+52.9 ± 0.1	-	-
CpG					
in water	161 ± 5*	0.107 ± 0.009	+59 ± 6	98.2 ± 0.7	19.9 ± 0.1
Poly I:C					
in PBS	141 ± 6	0.18 ± 0.03**	+53 ± 2	87 ± 1	26.1 ± 0.8
in glucose 5%	161 ± 1*	0.16 ± 0.05*	+40 ± 4	90 ± 3	27.1 ± 0.8

To prepare liposomes, the lipid film was hydrated with CpG in water or poly I:C in PBS or glucose 5%. The cationic lipid DOTAP to DPPC molar ratio was 3:2 in the liposomes. The data represent the mean values (± SEM) of 3 to 6 liposome preparations. PDI: polydispersity index; * $p<0.05$, ** $p<0.01$ compared to empty liposomes.

When the liposomes were placed in saline at 37°C, different release profiles were observed for the two TLR agonists (Fig. 1). The total amount of CpG encapsulated remained

entrapped within the liposomes and was not released at all for 24h (Fig. 1A). By contrast, liposomes poorly retained poly I:C. When the lipid film was hydrated with poly I:C in PBS, liposomes did not retain poly I:C at all (Fig. 1C). To avoid decreasing electrostatic interactions between poly I:C and DOTAP, the lipid film was then hydrated with poly I:C in glucose 5%, instead of PBS. However, more than 50% of the poly I:C quantity encapsulated in liposomes were still released and the poly I:C release curves were not statistically different between the liposomes and the solution (ANOVA, $p>0.05$; Fig. 1D).

Analysis of liposome morphology by transmission electron microscopy showed that the liposomes encapsulating CpG formed large unilamellar vesicles 90 nm to 200 nm in size (Fig. 1B).

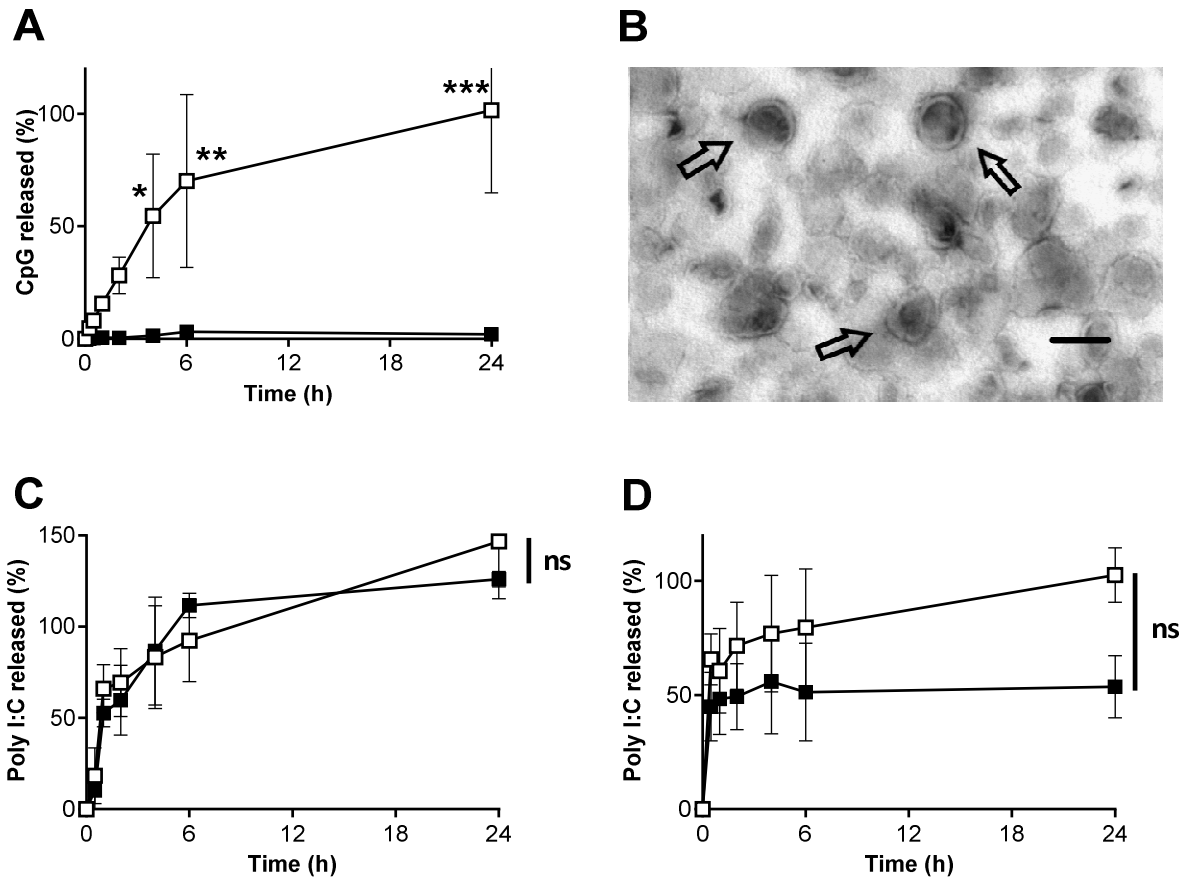

Figure 1: Release of TLR agonists from the liposomes and liposomes morphology. 100 µg of CpG C274 or poly I:C in solution or in liposomes were placed in a dialysis bag and TLR agonists were released in 7.5 ml of PBS pH 7.4 at 37°C. To prepare liposomes, the lipid film was hydrated with CpG C274 in water (**A**) or poly I:C in PBS (**C**) or glucose 5% (**D**). Solution (□), liposomes (■). Two-way ANOVA test with Bonferroni post-test was performed and the liposomes were compared to the solution (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns not significant vs the solution). The data represent the mean values (± SD) of 2 to 3 liposome preparations. **B**. Liposomes encapsulating CpG were imaged by transmission electron microscopy and appeared as large unilamellar vesicles (indicated by arrows in the image). The scale bar is 200 nm.

3.2. Decrease of tumor growth upon intraperitoneal injection of TLR agonists

To evaluate the ability of TLR agonists to decrease tumor growth, B16F10 luc cells were injected in the tail vein of mice at day 0. Three doses, 10 µg each, of TLR agonists were then injected intraperitoneally at days 4, 9 and 14. Injection of PBS was used as a control. At day 15, mice were sacrificed and the effects of the treatments was assessed by counting tumor cells in the lungs and by measuring the production of granzyme B, a pro-apoptotic protein, IFN-γ and IL-10 mRNA copies in the lungs. Day 15 post-tumor cells inoculation was chosen for sacrifice because it corresponded to the last day without any mouse death (Fig. S1).

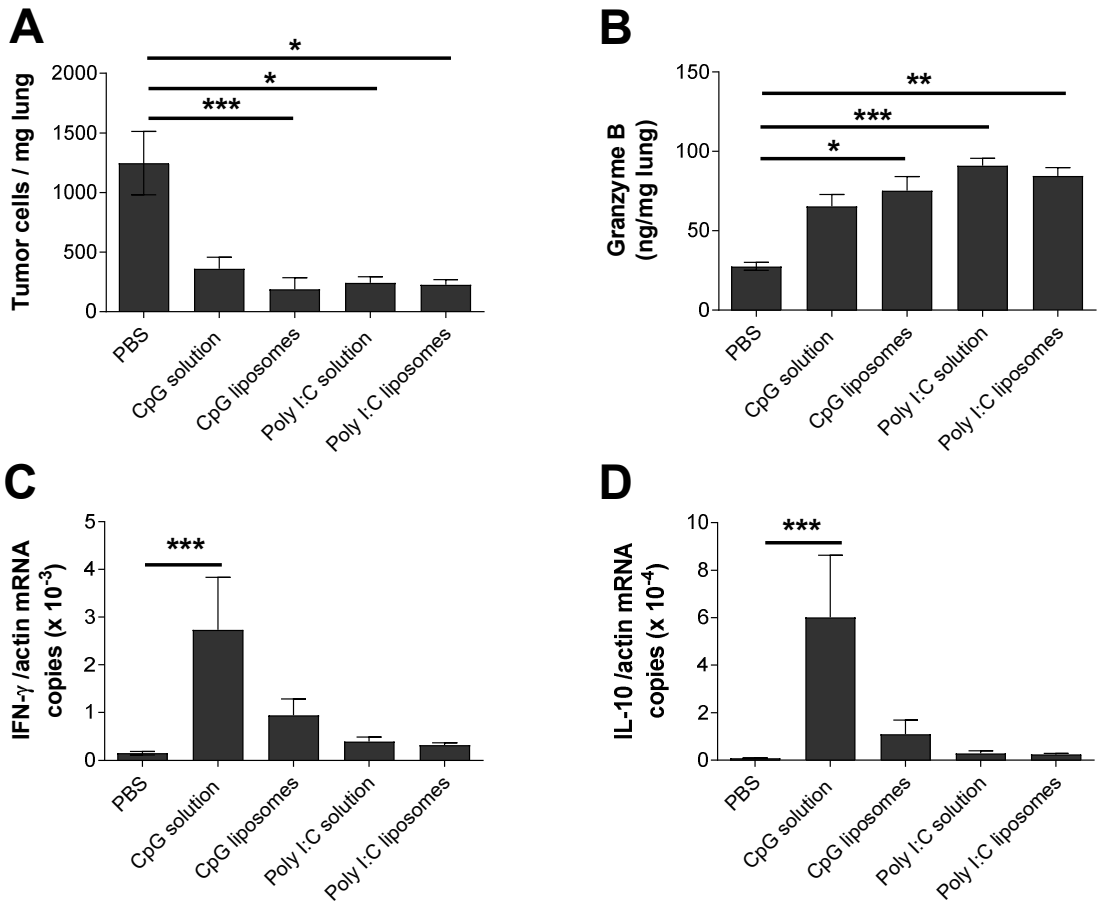

Figure 2: Lung readouts after treatment of B16F10 lung metastases with either CpG C274 or poly I:C injected intraperitoneally. C57BL/6 mice were inoculated with $3x10^5$ B16F10 luc cells by intravenous injection on day 0. TLR agonists (10 µg/mouse) were then delivered in solution or encapsulated in liposomes by intraperitoneal injection on days 4, 9 and 14 post-tumor cells inoculation. In the liposomes preparation, the lipid film was hydrated with CpG in water or poly I:C in PBS. Mice were sacrificed on day 15. The lungs were removed and crushed to analyse the number of tumour cells in the lungs (**A**), the granzyme B production (**B**) and the expression of IFN-γ (**C**) and IL-10 (**D**). The data represent the mean values (± SEM) of 6 to 9 mice. The Kruskal-Wallis test was performed followed by Dunns post-test ($p<0.05$ *; $p< 0.01$ **; $p<0.001$ ***).

The number of tumor cells in the lungs was drastically reduced in treated mice compared with non-treated mice (Fig. 2A). The intraperitoneal injection of CpG in solution and in liposomes decreased the number of tumor cells in the lungs by a factor of 3 and 7, respectively. These values were 5 and 6 in case of poly I:C in solution and in liposomes, respectively. Moreover, the production of granzyme B was increased in all treated mice (Fig. 2B). CpG treatment increased IFN-γ and IL-10 levels in the lungs although the increase was only significant in the CpG solution-treated group. poly I:C did not increase cytokine levels in the lung tissue. Because CpG was effectively retained within liposomes while poly I:C was not and because the efficacy data favour CpG, the TLR agonist CpG was selected to pursue our investigations.

3.3. Decrease of tumor growth upon pulmonary delivery of CpG

The efficacy of CpG for treating locally lung metastases via a pulmonary administration was studied according to the same protocol as above. At day 0, B16F10 luc cells were injected in the tail vein of mice. At days 4, 9 and 14, 10 µg of CpG in solution or in liposomes was delivered by intratracheal instillation. At day 15, mice were sacrificed and the effects of local CpG delivery was assessed. Intratracheal administration of CpG in solution or loaded in liposomes decreased the number of tumor cells in the lungs by a factor of 14 and 30, respectively (Fig. 3A). An increase in granzyme B production was noticed (Fig. 3B). The expression of IFN-γ increased in the lungs of mice treated with CpG and the most in the lungs of mice treated with CpG liposomes (Fig. 3C). At the opposite, the expression of IL-10 was higher in the lungs of mice treated with non-encapsulated CpG than in the lungs of mice treated with CpG liposomes (Fig. 3D). Therefore, this translates into a higher IFN-γ to IL-10 ratio in the mouse group treated by intratracheal instillation of CpG liposomes, indicating an increased anti-tumor effect over an anti-inflammatory effect for CpG liposomes.

Because CpG alone was very effective to limit tumor growth at a dose of 10 µg delivered locally per mouse, we decreased CpG dose to 1µg per mouse in the next experiments in order to better see a potential improvement in efficacy due to CpG encapsulation in liposomes. When a dose of 1µg of CpG per mouse was administered intratracheally after B16F10 luc cells inoculation, the number of tumor cells in the lungs decreased by a factor of 2 and 4 for respectively the CpG solution and the CpG liposomes (Fig. 4A). The intratracheal instillation of empty liposomes did not result in any decrease in the number of tumor cells in the lungs. Moreover, the co-delivery of non-encapsulated CpG and empty liposomes caused a minimal decrease (factor of 1.3) in the number of tumor cells in the lungs, showing that CpG needed to be encapsulated in liposomes for an enhanced effect (Fig. 4A).

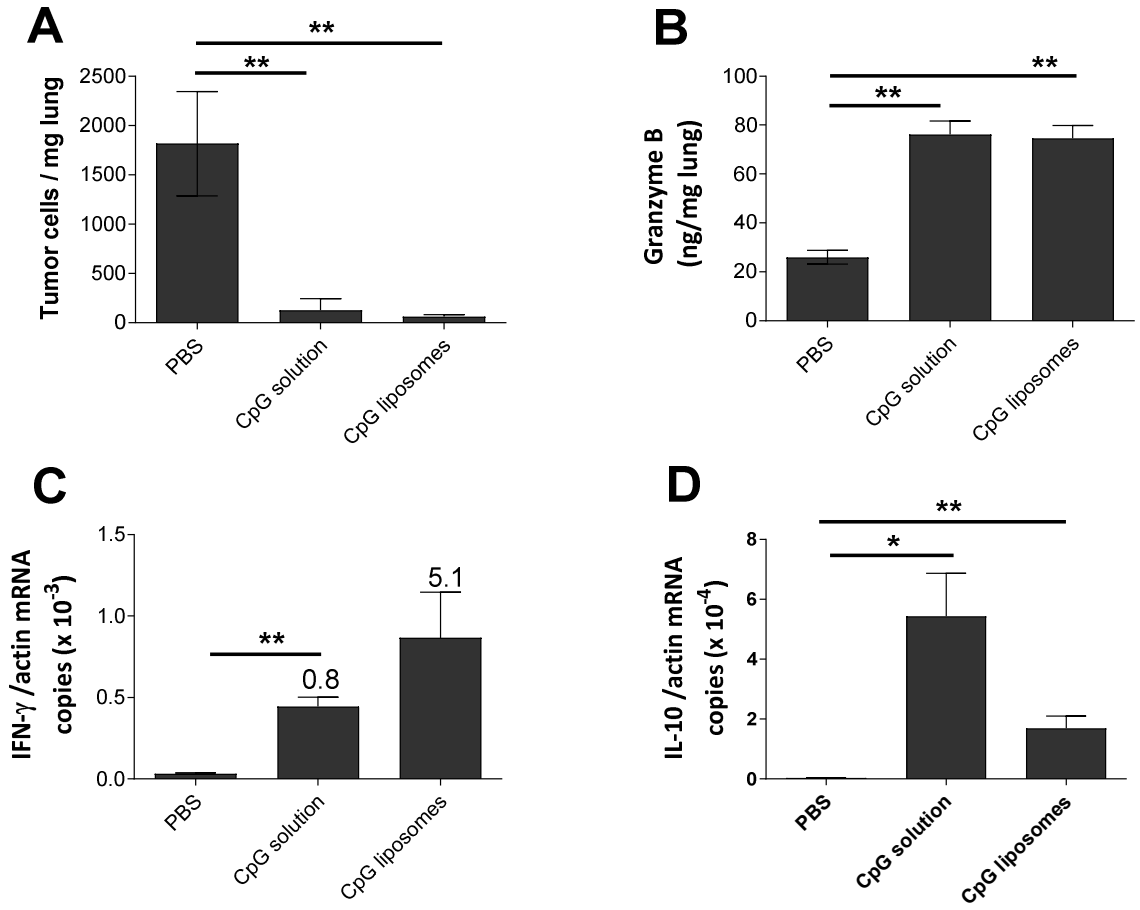

Figure 3: Lung readouts after treatment of B16F10 lung metastases with CpG delivered intratracheally. C57BL/6 mice were inoculated with $3x10^5$ B16F10 luc cells by intravenous injection on day 0. CpG C274 (10 µg/mouse) was then delivered in solution or encapsulated in liposomes by intratracheal instillation on days 4, 9 and 14 post-tumor cells inoculation. Mice were sacrificed on day 15. The lungs were removed and crushed to analyse the number of tumour cells in the lungs (**A**); the granzyme B production (**B**) and the expression of IFN-γ (**C**) and IL-10 (**D**). The numbers above columns in Fig. 3C indicate IFN-γ to IL-10 ratios in case of mouse groups with significant IFN-γ increase. The data represent the mean values (± SEM) of 5 to 8 mice. The Kruskal-Wallis test was performed followed by Dunns post-test (* $p<0.05$; ** $p< 0.01$).

CpG oligonucleotides can be divided in different classes according to their immunostimulatory effects. Class A CpGs trigger plasmacytoid DCs to mature and secrete high levels of IFN-α but have no effect on B cells. Class B CpGs trigger plasmacytoid DCs to differentiate and produce TNF-α and B-cells to proliferate and secrete IgM (Bode et al., 2011). The effects of class C CpG are a combination of class A and B effects. We selected a class C CpG, the C274 sequence, for our work because of its combined stimulatory effects. In particular, the C274 sequence has been shown to induce high levels of IFN-α, IFN-γ, IL-6 and IL-12 secretion in the airways of ovalbumin-sensitized and challenged mice (Marshall et al., 2005). In order to confirm the interest of the C274 sequence, we compared its efficacy to decrease the number of tumor cells in the lungs with a widely used class B CpG, the B1826

sequence (Marabelle et al., 2013). Mice treated locally with the class C CpG in solution showed a decrease in the number of tumor cells in the lungs by a factor of 6 while mice treated with the class B CpG showed a non-significant decrease by a factor of 2 (Fig. 4B). Therefore, this experiment supports the use of this class C CpG. One could notice that the number of tumor cells in the lungs and the effects of CpG could largely vary between experiments (PBS mouse groups in Figs. 4A & B). This might be caused by a different passage number of B16F10 luc cells as well as different mouse batches. Representative images of mouse lungs from the different mouse groups of Figs. 4A and 4B are shown in Figs. 4C and 4D. B16F10 lung metastases appear as black spots on the lung tissue and are less numerous after treatment with the class C CpG in solution and in liposomes.

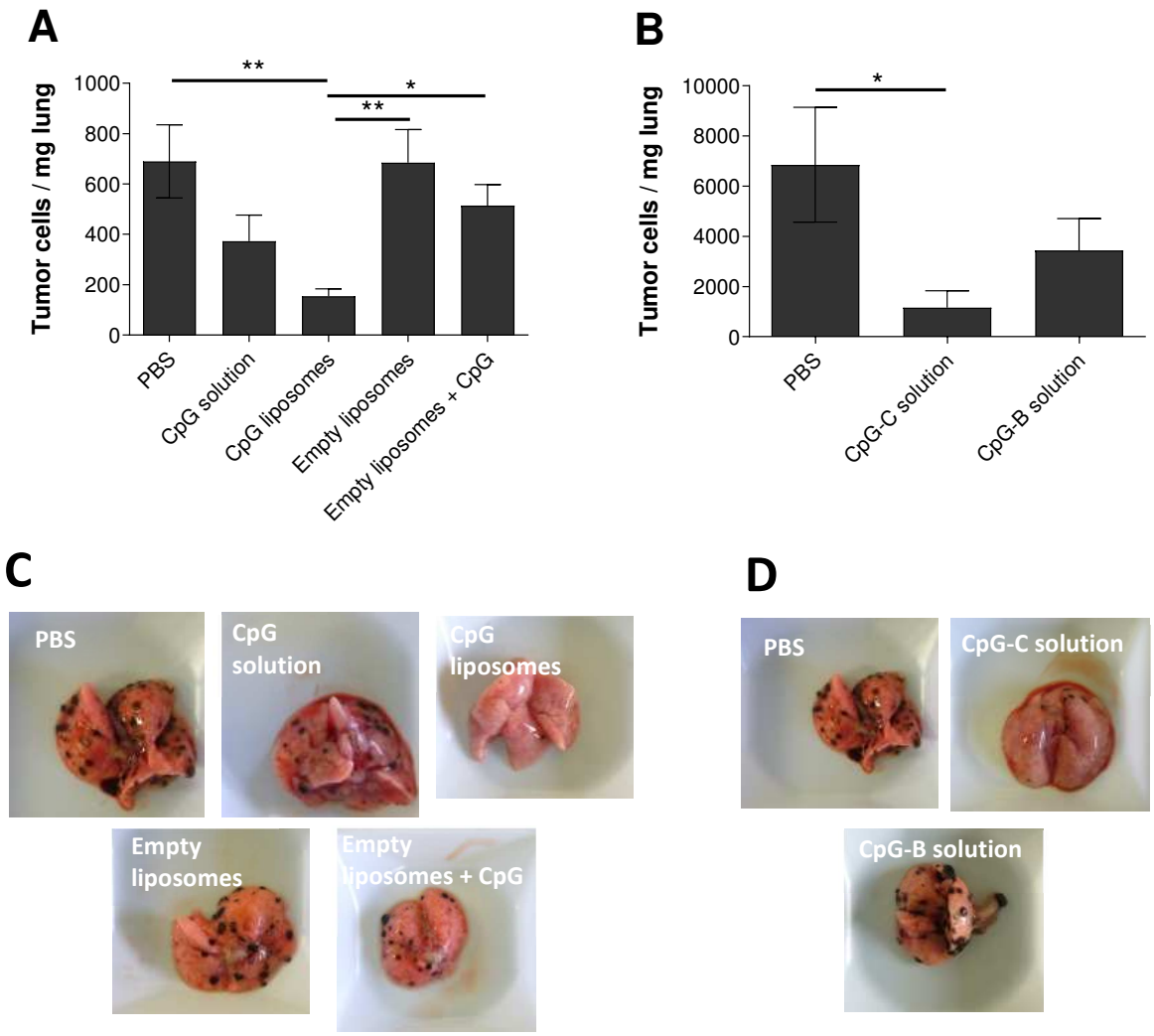

Figure 4: Number of tumour cells in the lungs after treatment of B16F10 lung metastases with CpG delivered intratracheally at a low dose. C57BL/6 mice were inoculated with $3x10^5$ B16F10 luc cells by intravenous injection on day 0. CpG C274 or B1826 (1µg/mouse) was delivered in solution or encapsulated in liposomes by intratracheal instillation on days 4, 9 and 14 post-tumor cell inoculation. Mice were sacrificed on day 15. The lungs were removed and crushed to analyse the number of tumour cells in the lungs (**A**). The effect of CpG classes (C274 and B1826) on tumor cells number in the lungs was compared (**B**). Representative images of mouse lungs from the different mouse groups of Figs. 5A

(**C**) and 5B (**D**). The data represent the mean values (± SEM) of 5 to 8 mice. The Kruskal-Wallis test was performed followed by Dunns post-test ($p<0.05$ *; $p< 0.01$ **).

3.4. Comparison of the pulmonary and intraperitoneal administrations of CpG

As a last step, the intratracheal administration of CpG was compared to its intraperitoneal injection at a CpG dose of 1µg per mouse. Delivery of CpG to the lungs was strongly superior to its intraperitoneal injection to slow down metastases growth in the lungs (Fig. 5A). In addition, CpG encapsulation in liposomes greatly enhanced CpG efficacy by both routes of administration. The number of tumor cells in the lungs decreased 28-times following intratracheal instillation of CpG liposomes and 3-times following their intraperitoneal injection. Granzyme B production was increased after local treatment with CpG solution and liposomes as well as after the systemic treatment with CpG liposomes (Fig. 5B). Moreover, the highest increase in INF-γ production was obtained with local treatment with CpG-loaded liposomes (Fig. 5C). Although an increase of IL-10 production was observed in mice treated locally with CpG liposomes, the ratio of IFN-γ to IL-10 was in favour of the IFN-γ production (Figs. 5C and D).

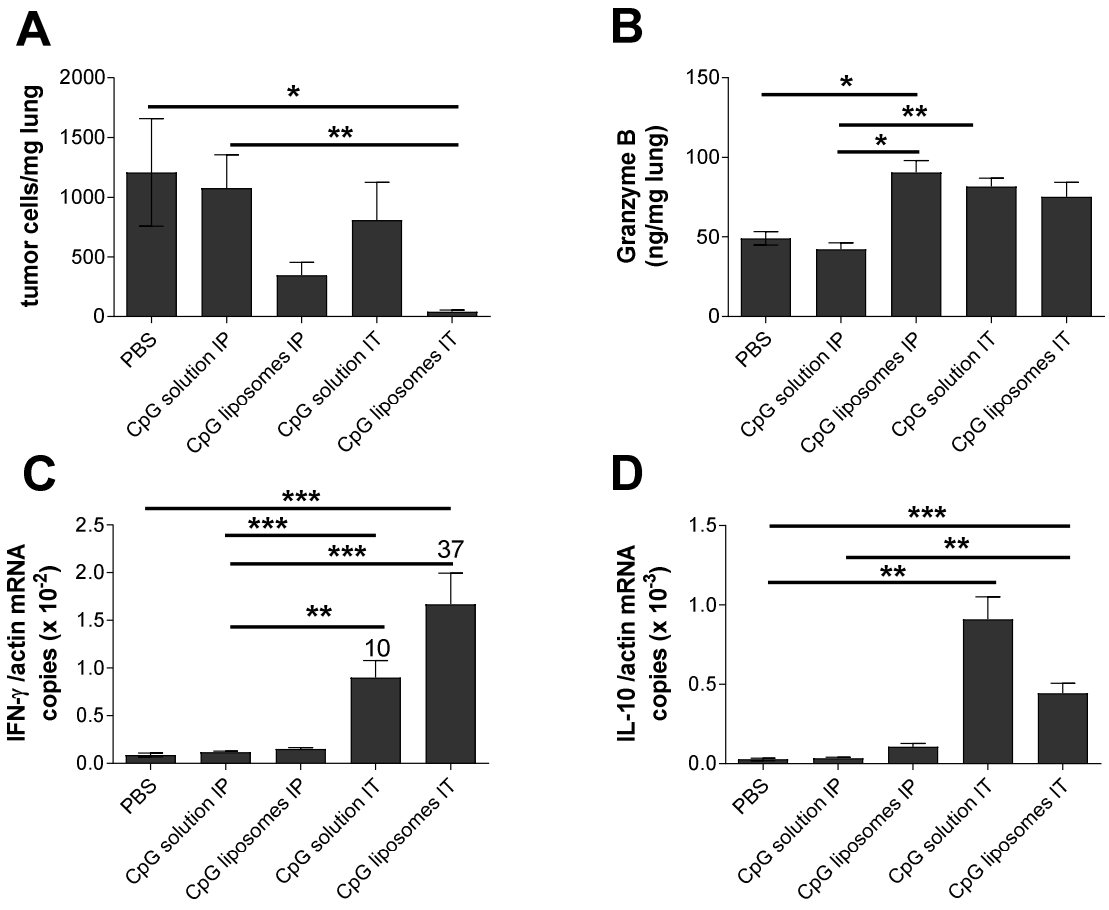

Figure 5: Lung readouts after treatment of B16F10 lung metastases with CpG delivered either intratracheally or intraperitoneally. C57BL/6 mice were inoculated with $3x10^5$ B16F10 luc cells by intravenous injection on day 0. CpG C274 (1 µg/mouse) was delivered in solution or encapsulated in

liposomes by either intraperitoneal injection (IP) or intratracheal instillation (IT) on days 4, 9 and 14 post-tumor cells inoculation. Mice were sacrificed on day 15. The lungs were removed and crushed to analyse the number of tumour cells in the lungs (**A**); the granzyme B production (**B**) and the expression of IFN-γ (**C**) and IL-10 (**D**). The numbers above columns in Fig. 6C indicate IFN-γ to IL-10 ratios in case of mouse groups with significant IFN-γ increase. The data represent the mean values (± SEM) of 8 mice. The Kruskal-Wallis test was performed followed by Dunns post-test ($p<0.05$ *; $p< 0.01$ **; $p<0.001$ ***).

The production of different cytokines in the mouse lungs of this experiment was then evaluated using a multiplex array (Fig. 6). The production of IFN-γ, MIG and RANTES are involved in the cytotoxic responses against tumor cells. Only the local treatment with CpG increased the production of IFN-γ and RANTES (Figs. 6A & 6C). MIG production was induced by both intratracheal and intraperitoneal administrations of CpG but intratracheal administration was superior to induce MIG production in the lungs. CpG in liposomes better induced cytokines production in the lungs than CpG in solution following both routes of administration.

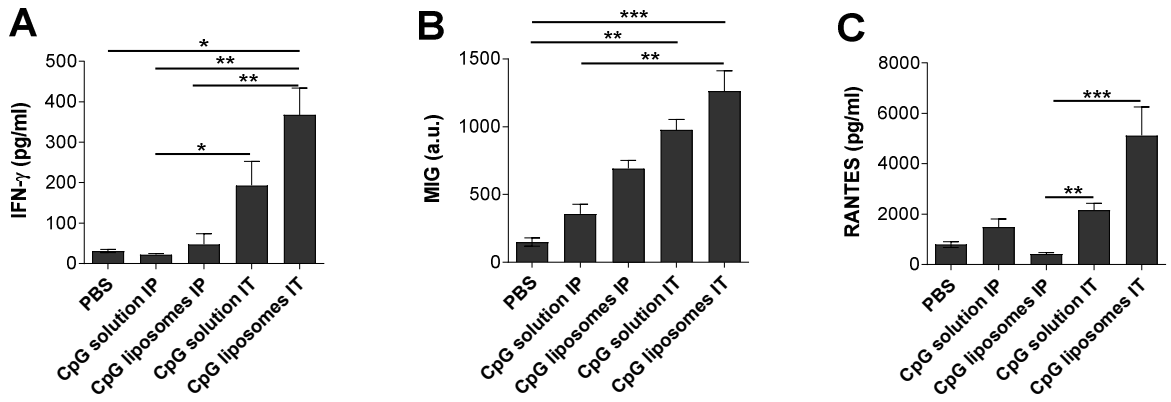

Figure 6: Cytokines production in the lungs following treatment of B16F10 lung metastases with CpG delivered either intratracheally or intraperitoneally. C57BL/6 mice were inoculated with $3x10^5$ B16F10 luc cells by intravenous injection on day 0. CpG C274 (1 µg/mouse) was then delivered in solution or encapsulated in liposomes by either intraperitoneal injection (IP) or intratracheal instillation (IT) on days 4, 9 and 14 post-tumor cell inoculation. Mice were sacrificed on day 15. The lungs were removed and crushed to analyse cytokines using a multiplex assay: IFN-γ (**A**); MIG (**B**) and RANTES (**C**). The data represent the mean values (± SEM) of 4 to 7 mice. The Kruskal-Wallis test was performed followed by Dunns post-test (* $p<0.05$; ** $p< 0.01$; *** $p<0.001$).

Finally, the systemic and local toxicity possibly caused by CpG was assessed in the mice by measuring the pro-inflammatory cytokines IL-6 and TNF-α in serum and broncho-alveolar lavage (BAL) fluid (Fig. 7). CpG delivered as a solution either intratracheally or intraperitoneally did not induce any increase in IL-6 and TNF-α levels in sera. A slight, often non-significant, increase in systemic IL-6 and TNF-α levels could be observed after intratracheal instillation and intraperitoneal injection of liposomal CpG. However, intratracheal instillation of CpG in solution or liposomes caused a large increase in IL-6 and TNF-α levels in BAL fluid. Soluble CpG induced a sixteen-fold increase in BAL IL-6 concentrations, compared with the PBS group, and

liposomal CpG further increased these IL-6 concentrations by a factor of 1.8. Similarly, soluble CpG induced a 388-fold increase in BAL TNF-α concentrations, compared with the PBS group, and liposomal CpG further increased these TNF-α concentrations by a factor of 1.4.

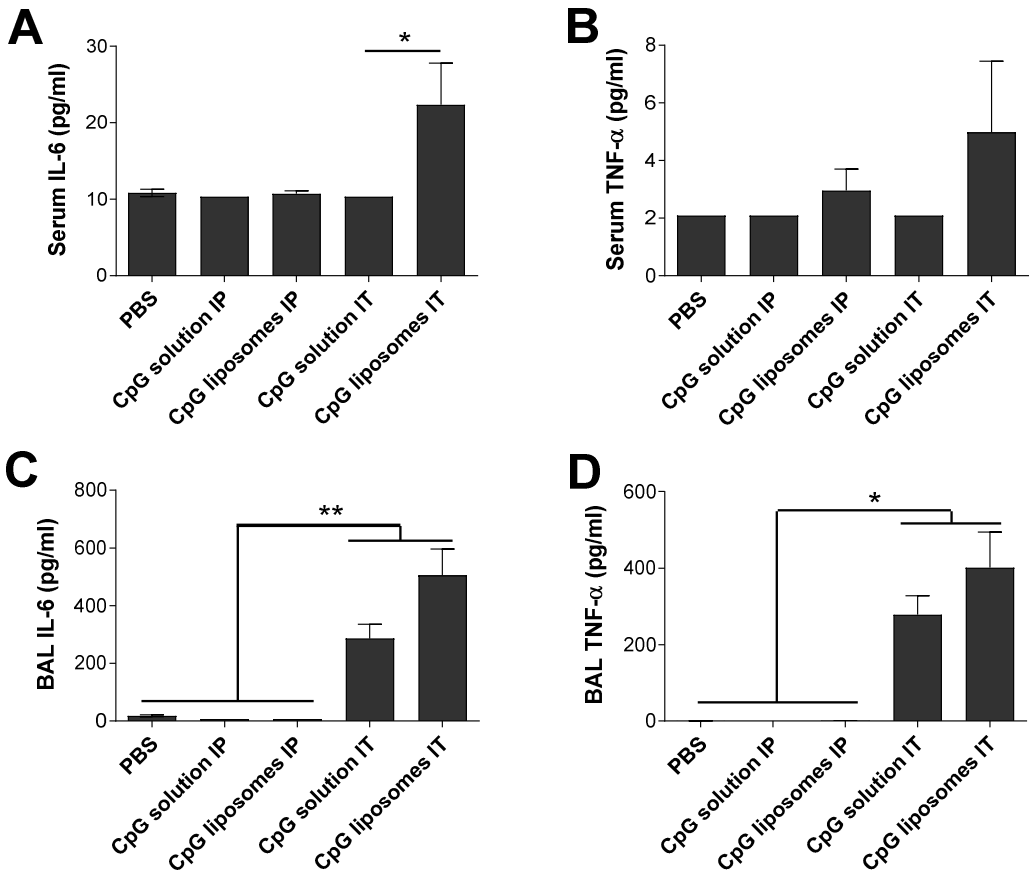

Figure 7: Measurement of inflammation markers in sera and bronchoalveolar lavage fluid following treatment of B16F10 lung metastases with CpG delivered either intratracheally or intraperitoneally. C57BL/6 mice were inoculated with $3x10^5$ B16F10 luc cells by intravenous injection on day 0. CpG C274 (1 µg/mouse) was delivered in solution or encapsulated in liposomes by either intraperitoneal injection (IP) or intratracheal instillation (IT) on days 4, 9 and 14 post-tumor cells inoculation. On day 15, blood was taken and mice were then sacrificed. Bronchoalveolar lavage (BAL) was carried out and the pro-inflammatory cytokines IL-6 (**A**, **C**) and TNF-α (**B**, **D**) were measured in sera (**A**, **B**) and BAL fluid (**C**, **D**). The data represent the mean values (± SEM) of 4 to 8 mice. The Kruskal-Wallis test was performed followed by Dunns post-test (* $p<0.05$; ** $p< 0.01$).

4. Discussion

This study emphasizes the enhancement in antitumor activity of TLR agonists in lung cancer that can result from their local delivery to the lungs and encapsulation in liposomes. Both poly I:C and CpG delayed tumor growth in the murine B16F10 model of metastatic lung cancer. However, only CpG increased IFN-γ levels in the lungs and was effectively entrapped within cationic liposomes. Pulmonary administration of CpG was superior to its intraperitoneal injection to slow the growth of lung metastases and to induce the production of granzyme B, a pro-apoptotic protein, and T helper type 1 cytokines and chemokines in the lungs. In addition, CpG encapsulation in liposomes greatly enhanced these effects.

Liposomes have frequently been used to co-encapsulate TLR agonists and tumor antigens and improve anti-tumor immunity of cancer vaccines following their systemic administration by injection (Bayyurt et al., 2017; Suzuki et al., 2004). However, no studies have investigated the interest of liposomes to increase the anticancer potency of TLR agonists delivered alone by the pulmonary route. A few scientific articles have reported the immunotherapeutic activity of non-encapsulated TLR agonists following their pulmonary administration. Accordingly, pulmonary delivery of CpG with or without poly I:C to lung metastases-bearing mice prolonged mice survival (Gallotta et al., 2018; Le Noci et al., 2015). This effect appeared mediated by an influx of activated T cells in the lung tumors and by the formation of tertiary lymphoid structures adjacent to the lung tumors. Sato et al formulated CpG-adsorbed polyketal microparticles and showed that the microparticles increased CpG accumulation and persistence in lung tumor nests and promoted intratumoral immunity while reducing immunosuppression (Sato et al., 2015). Yet, no studies have investigated the interest of liposomes to enhance the potency of TLR agonists while liposomes are a particularly suitable drug carrier for pulmonary administration.

The liposomes made of DOTAP and DPPC we prepared permanently entrapped CpG but could not effectively withhold poly I:C. Both CpG and poly I:C are highly negatively charged and can bind the cationic lipid DOTAP in the liposomes by electrostatic interactions. However, while CpG was made of 21 bases, poly I:C was made of an average of 5 thousands of base pairs and was more cumbersome to encapsulate than CpG. Therefore, while CpG was probably well encapsulated within the internal water cavity of the large unilamellar liposomes, part of the poly I:C likely remained at the liposome surface. Similarly, Du et al prepared eggPC/DOPE/DOTAP liposomes and encapsulated poly I:C within these liposomes (Du et al., 2018). In line with our data, they observed a burst release of poly I:C of about 20% from the liposomes. After 3 days, approximately 40% of the loaded poly I:C was released and no additional release was noted afterwards. Because CpG was effectively entrapped within liposomes while poly I:C was not, we pursued our investigations using CpG.

CpG activates the innate immune system by binding to TLR9 localized within the endosomal compartment of plasmacytoid and conventional DCs, B cells and macrophages in mice (Bode et al., 2011). TLR9 engagement translates into increased antigen-presenting cell function, increased expression of costimulatory molecules at the cell membrane and production of Th1 and proinflammatory cytokines and chemokines. These effects indirectly

stimulate the maturation, differentiation and proliferation of NK cells and T cells into T helper type 1 cells and cytotoxic T lymphocytes (CTLs) (Bode et al., 2011). The antitumor immune response initiated by CpG was witnessed by the delay in tumor growth and the production of granzyme B, IFNγ, MIG and RANTES in our study. Cytotoxic granules containing granzyme B and the pore-forming protein perforin are released by NK cells and CTLs onto the surface of target cells, penetrate the cell membrane and induce programmed cell death (Murphy and Weaver, 2017). IFNγ can be produced by innate lymphoid cells. It increases the expression of MHC molecules on all cell types, which facilitates recognition of tumor cells by CTLs via the display of tumor antigens complexed to MHC class I molecules on the tumor cell surface. IFNγ also activates populations of innate immune cells such as NK cells and it stimulates production of the chemokine, MIG (CXCL9). MIG recruits NK cells and Th1 cells. RANTES (CCL5) is a chemokine that recruits T cells and induces the proliferation and activation of NK cells (Murphy and Weaver, 2017). These antitumor activities of CpG were strongly enhanced by CpG encapsulation in liposomes. However, liposomes did not increase these effects when they were co-delivered with unencapsulated CpG. Liposomes have been shown, in our previous study, to increase the uptake of encapsulated compounds by alveolar macrophages (AMs) and DCs in culture *in vitro* as well as *in vivo* following pulmonary administration in mice (Vanbever et al., 2019). In addition, liposomal CpG was able to stimulate alveolar macrophages *in vitro*, while CpG in solution was not. Accordingly, liposomes likely increased the uptake of the encapsulated CpG by AMs and pulmonary DCs, hence CpG transport to the endosomes and CpG activation of TLR9 within the endosomes (Magalhaes et al., 2014; Vanbever et al., 2019; Yasuda et al., 2005).

Pulmonary administration of CpG was superior to its intraperitoneal injection to induce antitumor immunity and to slow the growth of lung metastases. This result implies that a direct encounter between CpG and cells of the lung tissue or CpG and tumor cells within lung metastases was beneficial for the induction of an antitumor immunity. Similarly, Li et al showed that CpG injection directly into subcutaneous lymphoma tumor nodules combined with systemic chemotherapy resulted in complete and permanent regression of the local subcutaneous tumors and metastases and cure of the animals (Li et al., 2007). However, when CpG was injected into a distant subcutaneous site or in a peritumoral site, the antitumor effects were not greater than that seen with systemic chemotherapy alone. This suggests that CpG interaction with tumor cells or with DCs at the tumor site was critical for the induction of a local and systemic CTL response in this model. It should be noted that the pulmonary administration of CpG was efficacious at very low CpG doses. In fact, a tremendous antitumoral effect was observed at CpG doses of 10 µg per mouse by Gallotta et al and at 10 µg and 1 µg per mouse in our study (Gallotta et al., 2018). For comparison, 50 µg to 100 µg of CpG have been commonly injected in subcutaneous tumor nodules (Li et al., 2007; Sagiv-Barfi et al., 2018).

Of note, pulmonary administration of low CpG doses induced the production of the inflammation markers IL-6 and TNF-α in the airspaces. This inflammation remained localized within the lungs and did not reach the systemic compartment in a significant manner. TLR9

engagement triggers the transcription factor nuclear factor (NF)- κB and activation of this pathway is behind the expression of several inflammatory mediators (Bode et al., 2011). IL-6 and TNF-α levels in the respiratory tract further increased after pulmonary administration of liposomal CpG, compared with pulmonary delivery of CpG in solution, because liposomes enabled improved CpG targeting to TLR9 in endosomes (Vanbever et al., 2019). Kell et al recently investigated the local toxicity induced by the delivery of DV281, a C class CpG, to the lungs in mice and monkeys (Kell et al., 2019). Delivery of DV281 to the murine lungs led to substantial but transient cytokine responses -including IL-6 and TNF-α- in the lungs. Murine lung responses to repeated delivery of DV281 were partially to fully reversible 2 weeks after the final dose. In the repeat-dose safety study in monkeys, inhaled DV281 was well tolerated and findings were mechanism of action-related and non-adverse as well. In our studies, intraperitoneal injection of low CpG doses did not result in any significant inflammation either in the systemic compartment or in the lungs. However, intraperitoneal injection of low CpG doses was also less effective to induce antitumor immunity.

The C274 sequence, a class C CpG, was superior to the widely used B1826 sequence, a class B CpG, to limit the number of B16F10 cancer cells in the lungs. The chemical structure of CpG oligonucleotides is different according to CpG class and is at the origin of differences in immunostimulatory effects (Bode et al., 2011). Class A CpGs are made of a mixed phosphodiester/phosphorothioate backbone, contain a single CpG motif flanked by palindromic sequences and have poly G tails at the 3’ and 5’ ends. Phosphorothioate nucleotides are more resistant to nuclease digestion than native phosphodiester nucleotides, resulting in longer *in vivo* half-lives. Class A CpGs trigger plasmacytoid DCs to mature and secrete high levels of IFN-α but have no effect on B cells. Class B CpGs include multiple CpG motifs on a phosphorothioate backbone. They trigger plasmacytoid DCs to differentiate and produce TNF-α and B-cells to proliferate and secrete IgM. Class C CpGs are entirely composed of phosphorothioate nucleotides and contain palindromic CpG motifs. Their immunostimulatory effects are a combination of class A and B effects and class C CpGs are very effective to induce strong Th1 responses *in vivo*. Class B CpGs were the first to be characterized and have been the most extensively studied in pre-clinical (Li et al., 2007; Marabelle et al., 2013) and clinical trials (Hyer and Janssen, 2019). Although class C CpGs have been the last characterized, their antitumoral effectiveness is nowadays exploited in preclinical studies (Gallotta et al., 2018; Kell et al., 2019; Sagiv-Barfi et al., 2018) as well as in human trials (Ribas et al., 2018).

To sum up, CpG encapsulation in liposomes greatly increased CpG effectiveness to slow down tumor growth in the murine lungs. A similar conclusion could not be drawn for poly I:C since the liposomes could not effectively retain it. Delivery of TLR agonists to the lungs using inhalation aerosols is non-invasive and very practical because it could be applied to many types of lung cancer without prior knowledge of their unique tumor antigens. However, inhaled liposomal CpG will not be sufficiently effective alone and will require its combination with other cancer therapies. In particular, it might be worthwhile to assess the combined administration of different immunotherapies to the lungs, including CpG, in future studies.

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