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Investigation of Phosphorylated Adjuvants Co-encapsulated with a Model Cancer Peptide Antigen for the Treatment of Colorectal Cancer and Liver Metastasis

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Abstract

The lipid calcium phosphate nanoparticle is a versatile platform capable of encapsulating a wide range of phosphorylated molecules from single nucleotides to pDNA. The use of this platform has shown great success as an immunotherapeutic vaccine carrier, capable of delivering co-encapsulated phosphorylated adjuvants and peptides. Three potent vaccine formulations were investigated for anti-cancer efficacy. The phosphorylated adjuvants, CpG, 2'3'cGAMP, and 5'pppdsRNA were co-encapsulated with a model phosphorylated tumor specific peptide antigen (p-AH1-A5). The anti-cancer efficacy of these adjuvants was assessed using an orthotopic colorectal liver metastasis model based on highly aggressive and metastatic CT-26 FL3 cells implanted into the cecum wall. The results clearly indicate that the RIG-1 ligand, 5'pppdsRNA, co-encapsulated with the p-AH1-A5 peptide antigen greatly reduced the growth rate of the primary colon cancer as well as arrested the establishment of liver metastasis in comparison to the other adjuvant formulations and unvaccinated controls. Further evaluation of the immune cell populations within the primary tumor confirms the ability of the 5'pppdsRNA adjuvant to boost the adaptive CD8⁺ T-cell population, while not inciting increased populations of immune suppressive cell types such as T-regulatory cells or myeloid derived suppressor cells. Furthermore, to our knowledge this is the first study to investigate the anti-cancer efficacy of a specific RIG-1 receptor ligand, 5'pppdsRNA, alongside more established TLR 9 (CpG) and STING (2'3'cGAMP) adjuvants in a cancer vaccine. The 5'pppdsRNA vaccine formulation can be a potent immunotherapy, especially when combined with agents that remodel the immune suppressive microenvironment of the tumor.

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Author contributions

T.G. and L.H. conceived and designed the research. T.G. performed the characterization experiments. T.G. performed the *in vivo* mouse experiments. T.G. prepared, analyzed and quantified the histology and flow cytometry samples. T.G. performed the statistical analysis. T.G. and L.H. analyzed the data and wrote the manuscript.

Competing interests

All data for this study are presented here, and all materials are available.

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Keywords

Lipid Calcium Phosphate Nanoparticles; Colorectal Cancer; Colorectal Liver Metastasis; Phosphorylated Adjuvants; CpG; 2'3'cGAMP; 5'pppdsRNA; Phosphorylated Peptide Antigen; AH1; p-AH1-A5; Vaccine

1. Introduction

Colorectal cancer is one of the major cancers for which new immune-based treatments are currently in development. Colorectal cancer is the third most common type of cancer in the United States, and is the second most deadly. In 2016 over 90,000 new cases of colon cancer, and close to 40,000 new cases of rectal cancer were reported in the US alone. Furthermore, it is estimated that nearly 50,000 patients will succumb to colon cancer in the US, and over 700,000 worldwide this year. Although screening and preventative measures has aided in early treatment to prolong survival rates, only 40% of colorectal cancers are diagnosed at a localized stage. The 5-year survival rate drastically decreases from 90% at the local stage, to only 13% once the cancer has spread to distant organs such as the liver (1).

Cancer immunotherapy is one such strategy to treat colorectal cancer. This strategy is based on manipulating the hosts innate and adaptive immune response in such a way that the host recognizes and resists cancer progression. Numerous published reports have shown that immunotherapies are a promising approach for the treatment of colorectal cancer. One such report from Ohtani et al., demonstrated that the presence and location of CD8+ killer T cells within the tumor microenvironment correlated with better outcomes in colon cancer patients (2). Furthermore, over the decades the development and understanding of unique adjuvants that elicit robust immune responses through a specific mechanism has grown substantially. These adjuvants have seen increased use in cancer vaccine formulations to elicit desired immune responses against cancers. The most reported adjuvants over the past two decades in the field consist of CpG and poly(I:C) which targets toll-like receptors (TLRs) located on the cell membrane or endosome (3). More recently, new generations of adjuvants such as 2'3'cGAMP and 5'pppdsRNA have been studied for targeting the cytoplasmic receptors, stimulator of interferon gene (STING) and retinoic acid-inducible gene 1 (RIG-1) (4–6). The unique mechanism by which these adjuvants elicit an immune response and the type of immune response elicited have been studied in detail (4, 7–11).

Cancer vaccines for the treatment of colorectal cancer are promising. Several tumor associated antigens such as carcinoembryonic antigen (CEA), mucin 1 (MUC1), human epidermal growth factor receptor 2 (HER2) and NY-ESO-1 have been identified (12). Many clinical trials using these antigens have reached late phase II and III studies (13). However, the mechanism by which the immune cells are activated, the type of activation, and the response provoked by the adjuvant presented to the immune cells is not entirely clear. In the present study, we co-encapsulated one of three distinct adjuvants, CpG, a TLR9 ligand, 2'3'cGAMP, a STING ligand, or 5'pppdsRNA, a RIG-1 ligand. These distinct adjuvants were co-encapsulated with a model mouse colon cancer peptide antigen (p-AH1-A5) into a lipid calcium phosphate (LCP) nanoparticle (14). Antigen specific interferon gamma (INF- γ) response and cytotoxic T-lymphocyte (CTL) killing were studied. More importantly, we

have identified which adjuvant could help to break the cancer immune tolerance, and reduce tumor growth and liver metastasis. Furthermore, the dependence of the therapeutic effect on the recruitment of CD8⁺ T-cells in the tumor as well as reduced populations of the immune suppressive T-regulatory (T-reg) cells and myeloid derived suppressor cells (MDSC) was also elucidated.

2. Material and Methods

2.1 Reagents

Dioleoylphosphatidic acid (DOPA) and 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000)] ammonium salt (DSPE-PEG) and DSPE-PEG-NHS were purchased from NOF (Tokyo, Japan). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO). DSPE-PEG-mannose was synthesized per the previously established protocol in our lab. Peptides, purity >98%, β -gal, AH1 (SPSYVYHQF), and phosphoserine-modified-AH1-A5 ((pS) (pS)SPSYAYHQF) peptide were synthesized by Peptide 2.0 (Chantilly, VA). CpG ODN 1826 (5'-TCCATGACGTTCCCTGACGTT-3') was ordered from Sigma-Aldrich. 2'3'cGAMP and 5'pppdsRNA were ordered from Invivogen (San Diego, Ca). 4-Dimethylaminopyridine and other chemicals were purchased from Sigma-Aldrich if not noted otherwise.

2.2 Mice and antibodies

Six- to eight-week old female BALB/c mice were obtained from Charles River (Bethesda, MD). Animals were raised in the Center for Experimental Animals (an AAALAC accredited experimental animal facility) in the University of North Carolina (UNC) at Chapel Hill. All animal handling procedures were approved by the UNC at Chapel Hill's Institutional Animal Care and Use Committee. Primary fluorescent antibodies used for immunofluorescent microscopy and flow cytometry analysis include: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 α , FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse FOXP3, FITC-conjugated anti-mouse CD11b, and PE-conjugated anti-mouse Gr were obtained from BD Biosciences (San Jose, CA). Analysis was performed on a FACSCaliber flow cytometer and analyzed using Cell Quest software (BD Biosciences)

2.3 Study design

This was a preclinical study to assess the efficacy and safety of several adjuvant based peptide vaccines delivered via a non-viral vector to elicit a therapeutic immune response. We hypothesized that although adjuvants may stimulate a robust pro-inflammatory immune response, detected via IFN- γ and CTL assays, it is necessary to compare in a side by side efficacy study in an aggressive orthotopic model. This hypothesis was tested through an established orthotopic syngeneic murine colorectal liver metastasis model. The number of mice used for the *in vivo* experiments are outlined in the figure legends. Grouping for tumor experiments was accomplished through measuring the tumor burden via bioluminescence techniques, ranking, and distributing equally. Additional study design details are also included in the statistical analysis and supplemental information section.

2.4 *In vivo* colorectal liver metastasis efficacy study

Mice were inoculated with 2×10^6 CT-26(FL3) RFP/Luc cells into the cecum wall six days prior to vaccination. Vaccination was initiated on days 0, and a boost was administered on day 6 via subcutaneous injection ($n = 6-10$). Progression of tumor mass was followed by administration of 200 μ L luciferin (10 mg/mL) IP. Luciferase bioluminescent imaging was recorded 10 min after administration of luciferin. On day 15, mice were imaged, sacrificed, and organs were then extracted. Images of livers were recorded and metastatic lesions were quantified via counting.

2.5 Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed via PRISM software using Student t-test when only two value sets were compared, and one-way analysis of variance (ANOVA) with a Dunnett's test for post hoc analysis when the data involved three or more groups. *, **, *** denotes $p < 0.05$, 0.01, and 0.001, respectively, and was considered significant and documented on figure or figure legend. In all statistics, the groups are compared against the PBS control, unless noted otherwise. More detailed methods are provided in the supplemental information.

3. RESULTS

3.1 Characterization of formulated LCP nanoparticles containing phosphorylated adjuvant and antigen

Xu et al. first reported the formulation and delivery of the mannose targeted LCP co-encapsulating a phosphorylated peptide antigen and CpG adjuvant (15). A graphical illustration of the LCP containing the phosphorylated peptide and adjuvant is depicted in Figure 1A. The core structure can be visualized under transmission electron microscopy (TEM) (Figure 1B). The DOPA monolayer surrounding the CaP core allows for the addition of the cationic outer leaflet lipids (1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), helper lipid cholesterol, and mannose conjugated to 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[succinyl(polyethylene glycol)-2000 (DSPE-PEG2000) to assist in dendritic cell uptake, producing the "final" LCP particles (40–60 nm in diameter, shown in Figure 1B/C). In this report the hydrodynamic diameter and the surface charge of the LCP particles were found via dynamic light scattering zetasizer analysis to be approximately 35 nm and 10 mV, respectively (Figure 1C). The p-AH1-A5 peptide encapsulation efficiency was found to be approximately 70 %, while the CpG, 2'3'cGAMP (cGAMP), and 5'ppdsRNA (dsRNA) corresponded to 56 %, 32 %, and 76 % when co-encapsulated with p-AH1-A5. All adjuvants were encapsulated at a 1:10 ratio of adjuvant to peptide by weight. The organ distribution was determined through incorporation of ^{125}I radioisotope onto the tyrosine of the p-AH1-A5. The LCP particles containing the ^{125}I p-AH1-A5 were injected subcutaneously into the left flank of healthy BALB/c mice. The organ distribution profile studies demonstrate that approximately 20–30 % of the ID/g of the mannose-LCP nanoparticles distribute to the proximal lymph nodes, while approximately 5 % of the ID/g is found in distal lymph nodes and spleen (Figure 1D).

3.2 *In vivo* immune response profile of LCP vaccines (IFN- γ ELISPOT/Cytotoxic T-Lymphocyte Assay)

Investigation into the potency in which all three vaccine formulations containing the corresponding adjuvant could elicit a robust immune response was assayed via IFN- γ ELISPOT and CTL. The IFN- γ , which is produced predominantly by cytotoxic T cells, is reported to mediate tumor rejection in colon cancer models. Therefore, investigation into the IFN- γ production by lymphocytes 7 days post vaccination was analyzed using a BD ELISPOT assay system. It was found that 0.125 mg/kg adjuvant and 1.25 mg/kg p-AH1-A5 administered subcutaneously in mannose targeted LCP produced significant antigen-specific IFN- γ within the splenocytes pulsed with AH1 peptide. As shown in Figure 2A, all three vaccine formulations containing adjuvant significant increased IFN- γ production compared to mice vaccinated with a formulation void of any adjuvant or the PBS treated group. These results confirm that the adjuvants are necessary to induce a AH1 specific immune response. To confirm that these vaccines elicit a pro-inflammatory response and direct AH1 specific antigen cytotoxic T-lymphocyte killing, CTL analysis was performed. As shown in Figure 2B, the PBS and p-AH1-A5 vaccine formulations were not able to generate any detectable AH1 peptide-specific CTL response. However, mice that were immunized with the pAH1-A5 LCP containing the corresponding adjuvant provoked high AH1 specific cytotoxic T-cell killing. The data indicates that in all three formulations containing either CpG, cGAMP, or dsRNA that a robust adaptive immunity is elicited through the CD8+T-cytotoxic pathway.

3.3 Modifying the immune cell populations in colorectal cancer (CRC) mice

The colorectal cancer model was established through injection of 2×10^6 CT-26(FL3) cells into the mouse cecum wall and was first reported by Zhang et al. This model provides an aggressive colorectal liver metastasis platform in immune competent mice to investigate therapies ability to promote anti-cancer immune responses. The formation of large metastatic lesions and termination of the model due to large primary tumors in the intestine was reported 4 weeks post inoculation (16, 17), and corresponded closely to our observations. To more closely model clinical applications in which a therapy is initiated following diagnosis of the tumor, the vaccine treatment was initiated six days post tumor inoculation with a vaccine boost administered six days post initial vaccination. Initial tumor studies were used to investigate and understand the different immune cell populations trafficking to the primary tumor following subcutaneous administration of the different adjuvant formulations. Therefore, on day 21 following tumor inoculation, mice were euthanized and the primary tumor, liver, and spleen were collected. The primary tumors were prepared for immunofluorescent microscopy and flow cytometry analysis. To investigate the morphology and collagen content of the tumors, the paraffin embedded tumor sections were Masson trichrome stained (Figure 3A). It is clear from the trichrome staining that the PBS, p-AH1-A5, and p-AH1-A5 CpG tumors are high in collagen, which has been reported to promote tumor progression. Collagen deposition in the tumor microenvironment also has been directly implicated as a barrier to T-cell entry. Degradation of collagen in an ex vivo model resulted in markedly augmented penetration of T cells in the solid tumor microenvironment, enabling direct contact of T cells with tumor cells (18). To investigate the immune cell recruitment into the primary tumor following treatment, sections were stained for CD8+, CD4+, FOXP3+, CD11b+, and GR-1+ (Figure 3A). It is clear from the

immunofluorescent microscopy analysis that the CD8 recruitment was significantly higher following vaccination of the p-AH1-A5 dsRNA LCP formulation compared to all other treatment groups. Furthermore, analysis showed CD4+FOXP3+ (T-reg) cells and CD11b+GR-1+ (MDSC) cells were significantly decreased in only the p-AH1-A5 dsRNA treatment group compared to the PBS group. Interestingly, all groups besides the p-AH1-A5 dsRNA group had minimal immune cell penetration into the tumor, in which most immune cells were located on the tumor periphery. This may be due to the increased MDSC and T-regs localizing at the periphery which thwarts CD8+ tumor penetration. To confirm the immunofluorescent results single tumor cell suspensions were stained for CD8+, CD4+, FOXP3+, CD11b+, and GR-1+, and analyzed via flow cytometry (Figure 3B). Similarly, as shown in Figure 3B, the percentages of T-reg and MDSC cells were significantly reduced in the p-AH1-A5 dsRNA treatment group compared to the PBS treated group, while the CD8+ population was significantly increased compared to the PBS group. Our results indicated that although all three adjuvant formulations elicited robust IFN- γ production and AH1 antigen specific CTL killing, these results do not directly correlate with tumor recruitment and infiltration of CD8+ cells. This may be due to the higher number of immune suppressive cells being recruited to the tumor periphery. Furthermore, this may shed some insight into the different immune responses downstream following the activation of TLR9, STING, and RIG-1.

3.4 Investigation of therapeutic efficacy in an orthotopic syngeneic colorectal liver metastasis model

We examined the effect of the immune response elicited by each individual vaccine and control on the primary colorectal tumor burden as well as on the incidence of liver metastasis found in mice. Five treatment groups were explored, including PBS, p-AH1-A5 LCP containing no adjuvants, LCP containing p-AH1-A5 CpG, p-AH1-A5 cGAMP, and p-AH1-A5 dsRNA. Administration of treatment began six days following cancer cell implantation and a boost vaccination was administered six days following the first treatment. The treatments were administered subcutaneous in the left flank of the mouse (1.25 mg/kg p-AH1-A5/0.125 mg/kg adjuvant). The total tumor burden was followed through intraperitoneal (IP) administration of 200 μ L of luciferin (10 mg/mL) followed by bioluminescent analysis 10 min post luciferin administration. The tumor burden was recorded every 2 to 3 days, and was used to sort mice into treatment groups before treatment was initiated (Figure 4A). At the endpoint, 15 days post treatment initiation, mice were imaged and euthanized due to heavy primary tumor burden in the cecum. The livers were harvested and analyzed for metastatic lesions (Figure 4C). It is clear from the luciferase assay (bioluminescent intensity) that all mice developed large primary tumors by the study endpoint. However, the p-AH1-A5 dsRNA LCP treatment group showed reduced growth rates, in which the primary tumors burden was 3–6 fold lower than all other treatment and control groups (Figure 4A/B). Furthermore, analysis of the metastatic tumor lesions on the liver clearly indicate that the p-AH1-A5 cGAMP LCP and the p-AH1-A5 dsRNA LCP treatment significantly reduced tumor invasion and progression to the liver by 4–10 fold, respectively, compared to PBS control (Figure 4C). Analysis of liver sections further confirmed these results in which increased cirrhosis and fibrotic tissue is more prominent in the PBS, p-AH1-A5 LCP, and p-AH1-A5 CpG LCP treatment groups (Figure 4D). This side

by side comparison is possible due to the versatile LCP vector which is capable of efficiently encapsulating and delivering phosphorylated molecules.

3.5 Effects of vaccine formulations on liver, kidney, and blood (toxicity analysis)

Administration of vaccine formulations showed no significant changes in ALT, AST, creatinine, or BUN levels 14 days post vaccination (Figure 5). Further analysis of blood and immune cell levels showed no signs of change compared to control treated mice one and seven days post vaccination (Figure 5).

4. DISCUSSION

It has been well documented that the success of colorectal cancer vaccines correlates strongly with increased tumor infiltrating CD8⁺ T-cells (2, 19). However, an increased number of tumor infiltrating CD8⁺ T-cells cannot promote an anti-cancer response in the presence of immune suppressive cells due to inactivation by T-regs and MDSCs in the tumor microenvironment (20–22). Therefore, in the study presented here, we aimed to investigate adjuvants which increased CD8⁺ T-cell populations, while not inciting an increase in immune suppressive cell populations. The three adjuvants used in this study were chosen due to their unique and distinct mechanism by which they elicit an immune response. The first adjuvant, CpG, binds to the nucleic acid sensors of the Toll-like receptor (TLR) family, in particular TLR9. Binding to the TLR9 initiates signaling by aggregating the adaptor protein myeloid differentiation primary response protein 88 (MYD88). The MYD88 signaling cascade leads to the activation of nuclear factor- κ B (NF- κ B) and a pro-inflammatory cytokine response (23, 24).

The additional adjuvants formulated, 2'3'cGAMP and 5'pppdsRNA, bind to cytosolic nucleic acid sensors which are expressed in the cytosol of both immune and non-immune cells. The cyclic GMP-AMP, [G(2',5')pA(3',5')p] (2'3'cGAMP), functions as a messenger to activate the stimulator of IFN genes (STING) in the endoplasmic reticulum (ER) (9, 23). The 5'pppdsRNA binds to the DExD/H-box helicases retinoic acid-inducible gene I (RIG-I; also known as DDX58). RIG-I binds to short dsRNA structures and is activated by the 5' triphosphorylated or 5' dephosphorylated end of the dsRNA (23). Complexation to RIG-I in the cytosol produces inflammatory cytokines, chemokines and type I interferons (25). Although all three of these adjuvants have a well-defined and unique mechanism for eliciting a pro-inflammatory response, the investigation of the pathophenotype is necessary to understand which adjuvants have the potential to translate from pre-clinical mouse models into human clinical studies. It has been shown that CpG-ODNs, achieve robust responses in mouse models, but have shown no promising efficacy in human anti-cancer studies. This is due to the varying TLR9 expression patterns between humans and preclinical test species, in which TLR9 has been found to be expressed in only a few human immune cell types. By contrast, RIG-I is broadly expressed in somatic and immune cells of both mice and humans and therefore may be a more promising target in terms of translatability. Interestingly, while all adjuvants elicited a robust IFN- γ response, it was found that the 5'pppdsRNA resulted in a significant increase in CD8 T-cells, while maintaining lower populations of T-reg and MDSC cells within the tumor. This immune response resulted in a significant reduction in

the tumor growth rate and development of liver metastasis compared to other treatment groups.

However, to achieve full regression of the tumor, numerous manipulations in the tumor immune microenvironment must be established. The vaccines investigated here are only one piece of achieving full regression of the tumor burden. Along with increasing the tumor infiltrating CTLs, we must also block the factors that play a fundamental role in cancer progression. In our previous work we demonstrated that the targeting of immune modulating pathways can reduce cancer progression and the recruitment of immunosuppressive cell types to the tumor microenvironment (17). Therefore, future combination of these therapies with our model p-AH1-A5 dsRNA LCP vaccine will allow us to further investigate which manipulations in the tumor immune microenvironment drives cancer regression in pre-clinical mouse models.

In conclusion, all three adjuvant formulations incited similar pro-inflammatory responses measured by IFN- γ production and in vivo CTL. However, only the 5' pppdsRNA was able to promote a significant anti-cancer response. This result is most likely due to maintaining low populations of immune suppressive T-reg and MDSCs present in the tumor microenvironment. Most importantly, this approach has allowed us to determine which adjuvant/vaccine formulation can achieve an enhanced antitumor response by increasing tumor infiltrating CD8 T-cells, while not inciting increased populations of immune suppressive cells. In future studies, we will use this model adjuvant/vaccine formulation in combination with immune modulating therapies that are capable of further reducing the tumors ability to recruit immune suppressive cell types and promote tumor specific T-cell killing. Investigating the synergistic anti-cancer responses of this model vaccine in combination with immune modulating therapies will allow us to understand the necessary immune manipulations needed to elicit robust and safe anti-cancer responses. Ultimately, this approach will guide our future investigation of more clinically relevant vaccines in combination with the most promising immune modulating therapies to elicit a robust anti-cancer response for the treatment of colorectal liver metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALT

Alanine Aminotransferase

AST	Aspartate Aminotransferase
BLI	Bio-Layer Interferometry
BUN	Blood Urea Nitrogen
CpG	5'-tccatgacgttcctgacgtt-3'
2'3' cGAMP	cyclic [G(2',5')pA(3',5')p]
CRC	Colorectal Cancer
DLS	Dynamic Light Scattering
DAPI	4',6-diamidino-2-phenylindole
DOPA	1,2-dioleoyl-sn-glycero-3-phosphate
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DSPE	1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine
5'pppdsRNA	5'-pppGCAUGCGACCUCUGUUUGA-3' 3'-CGUACGCUGGAGACAAACU-5'
ELISPOT	Enzyme Linked Immunospot Assay
LCP	Lipid Calcium Phosphate
NHS	N-Hydroxysuccinimide
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
RFP	Red Fluorescent Protein
TEM	Transmission Electron Microscopy

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Highlights

- LCP formulation allows for co-encapsulation of phosphorylated peptides/ adjuvants
- Co-encapsulated p-AH1-A5 and 5' pppdsRNA breaks cancer immune tolerance
- p-AH1-A5+5' pppdsRNA and p-AH1-A5+2'3' cGAMP reduce establishment of liver metastasis

Figure 1. Characterization of formulated LCP nanoparticles containing phosphorylated adjuvant and antigen

A) Graphical illustration of the LCP nanoparticle containing co-encapsulated 5'pppdsRNA and p-AH1-A5, the inner core consists of an amorphous calcium phosphate which aids in encapsulation of phosphorylated peptide and adjuvant, the inner lipid layer consists of DOPA, the outer lipid layer consists of cationic DOTAP, cholesterol helper lipid, DSPE-PEG, and DSPE-PEG-Mannose. **B)** The transmission electron microscopy (TEM) image and **C)** dynamic light scattering (DLS) zeta potential and size (number and volume average) of final LCP formulation with 5'pppdsRNA and p-AH1-A5. **D)** Biodistribution of mannose targeted LCP containing 5'pppdsRNA and ¹²⁵I labelled p-AH1-A5 following subcutaneous injection at 4, 16, and 48 h.

Figure 2. *In vivo* immune response profile of LCP vaccines

A) Interferon- γ (IFN- γ) production from the treated mice was measured through IFN- γ ELISPOT analysis. Spleens were collected from which single-cell suspensions were prepared 7 days after a single subcutaneous vaccination. The splenocytes were co-cultured with either β -gal or AH1 peptide at 37 °C for 18 h. The production of IFN- γ was calculated with a BD ELISPOT assay set. Two of the representative results are shown above. $n = 6$. **B)** *In vivo* CTL killing was measured through CFSE high/low staining of splenocytes collected from naive mice which were pulsed with either β -gal (low stain) or AH1 (high stain) peptide. The splenocytes were stained with 0.5 or 5 μ M carboxyfluorescein succinimidyl ester (CFSE), respectively. The mix of both pulsed cells at a ratio of 1:1 were injected into the vaccinated mice via tail vein. 16 h after injection, mice were euthanized and splenocytes were collected, washed, and analyzed via flow cytometry. The specific lysis activity of CTL was calculated using the equation mentioned in the Materials and Methods section. $n = 6$. A representative graph from each treatment is shown. Data was expressed as mean $\pm$ s.d. N.S. denotes no significance, N.D. denotes under detection limit, *, **, or *** denotes $p < 0.05$, 0.01, or 0.001, respectively.

Figure 3. Immune cell (MDSC, T-reg, CD4+ and CD8+ T-cell) recruitment within primary colorectal cancer tumors

A) Changes of tumor-infiltrating immune cells in tumor microenvironment. The CT-26FL3 colorectal cancer mice were divided into five treatment groups, including PBS control, p-AH1-A5 LCP, p-AH1-A5 CpG LCP, p-AH1-A5 cGAMP LCP, and p-AH1-A5 dsRNA vaccines. On day 21, mice were euthanized and tumor tissues were collected for **A)** Immunofluorescence microscopy and **B)** Flow cytometry analysis. The statistical analyses were calculated by comparison with the control group if not specifically mentioned. Data was expressed as mean $\pm$ s.d., calculated from samples ran in triplicate. N.S. denotes no significance, N.D. denotes under detection limit, *, **, ***, **** denotes $p < 0.05$, 0.01, 0.001, or 0.0001, respectively, compared to corresponding PBS control. Scale bar: 200 μ m.

Figure 4. Reduced tumor growth and liver metastasis following p-AH1-A5 dsRNA LCP treatment

A) Inoculation and treatment schedule/dose, tumor mass on days 3, 9 and 15 post first treatment administration. Mice were inoculated with 2×10^6 CT-26(FL3) RFP/Luc cells into the cecum wall. Treatment of 1.25 mg/kg p-AH1-A5 and 0.125 mg/kg adjuvant on days 0 and 6 was administered through subcutaneous injection. Treatment groups included PBS (n=10), p-AH1-A5 LCP (n=7), p-AH1-A5 CpG LCP (n=6), p-AH1-A5 cGAMP LCP (n=6), and p-AH1-A5 dsRNA LCP (n=7). Progression of overall tumor mass was followed by administration of 200 μ l luciferin (10mg/mL) IP. Luciferase bioluminescent imaging was recorded 10 min after administration of luciferin. **B)** Quantification of tumor burden was quantified using image IVIS/Kodak software and recorded as fold change over day 0 tumor burden. **C)** Liver metastasis tumor burden was quantified through visual analysis of the livers on day 15, endpoint. Number of metastatic lesions were counted and recorded. Data was expressed as mean $\pm$ s.d., calculated from samples. N.S. denotes no significance, N.D. denotes under detection limit, *, **, or *** denotes $p < 0.05$, 0.01, or 0.001, respectively, compared to corresponding PBS control.

Figure 5. Toxicological analysis

A) ALT, AST, creatinine, and BUN enzyme levels, blood cell levels, after treatment of PBS, p- β -gal LCP, p-AH1-A5 LCP, p-AH1-A5 CpG LCP, p-AH1-A5 cGAMP LCP, p-AH1-A5 dsRNA LCP, treatment dose was 1.25 mg/kg p-AH1-A5, and 0.125 mg/kg adjuvant, administered subcutaneously on days 0 and 6. Mouse blood was collected via tail vein nick on days 1 and 7 following treatment and analyzed for blood cell levels. Mice were sacrificed 14 days post final injection and blood serum was collected and analyzed for liver and kidney biomarkers. All LCP treatments fall within normal range. Data was expressed as mean $\pm$ s.d., calculated from samples ran in triplicate, *, **, or *** denotes $p < 0.05$, 0.01, or 0.001, respectively, compared to corresponding untreated control.