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.

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

Dioleoylphosphatydic acid (DOPA) and 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, Al). 1,2-Distearoryl-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′-TCCAT GACGTTCCTGACGTT-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 CD8α, 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 μL 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 ± 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 Dunnette'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 Fig. 1A. The core structure can be visualized under transmission electron microscopy (TEM) (Fig. 1B). The DOPA monolayer surrounding the CaP core allows for the addition of the cationic outer leaflet lipids [1,2-dioleoyl-3-trimethylammonium-propane