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In vivo delivery of peptides and Toll-like receptor ligands by mannose-functionalized polymeric nanoparticles induces prophylactic and therapeutic anti-tumor immune responses in a melanoma model

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ABSTRACT

We hypothesized that the co-entrapment of melanoma-associated antigens and the Toll-like receptor (TLR) ligands Poly(I:C) and CpG, known to be Th1-immunopotentiators, in mannose-functionalized aliphatic polyester-based nanoparticles (NPs) could be targeted to mannose receptors on antigen-presenting cells and induce anti-tumor immune responses. High entrapment efficiencies of antigens and immunopotentiators in 150 nm NPs were obtained. The co-entrapment of the model antigen ovalbumin and the TLR ligands was crucial to induce high IgG2c/IgG1 ratios and high levels of IFN-γ and IL-2. Mannose-functionalization of NPs potentiated the Th1 immune response. The nanoparticulate vaccines decreased the growth rate of murine B16F10 melanoma tumors in therapeutic and prophylatic settings. The combination of mannose-functionalized NPs containing MHC class I- or class II-restricted melanoma antigens and the TLR ligands induced the highest tumor growth delay. Overall, we demonstrate that the multifunctional properties of NPs in terms of targeting and antigen/adjuvant delivery have high cancer immunotherapeutic potential.

1. Introduction

There is increasing evidence that the immune system is able to mount responses against tumors that can be enhanced using several strategies [1]. Cancer immunotherapy must envision the activation of cell-mediated immunity, particularly $CD8^+$ cytotoxic T lymphocytes (CTLs). Activated $CD8^+$ T-cells are able to directly kill malignant cells and also acquire a long-lasting memory phenotype, important in the prevention of cancer relapses [2]. $CD4^+$ T helper (Th) 1 cells are also essential to obtain an effective anti-tumor response by enhancing clonal expansion of CTLs at the tumor site and promoting the generation and maintenance of a memory phenotype [3]. To activate both $CD8^+$ and $CD4^+$ T-cells against tumors, tumor-associated antigens (TAAs) must be presented through major histocompatibility complex (MHC) class I and II molecules in an immunostimulatory context [4].

Improved cancer vaccination depends on the targeted delivery of TAAs to antigen presenting cells (APCs) – dendritic cells (DCs) by excellence but also macrophages and B cells – which present antigens to T-cells [5,6]. Notwithstanding, their capacity to properly stimulate T-cells depends on the presentation of antigens in an immunostimulatory environment, which can be achieved by using molecules with known adjuvant potential, such as the Toll-like receptor (TLR) ligands. The stimulation of TLRs in APCs activates intracellular signaling pathways that culminate in the induction of inflammatory cytokines, chemokines, interferons and upregulation of co-stimulatory molecules, which provide the proper environment for T-cell stimulation [7]. Several vaccination modalities have been tested as whole tumor cells, cell lysates, proteins or specific peptide fragments, leading to the generation of tumor-specific T-cell responses [8]. Innovative strategies based on nucleic acid delivery, such as the use of mRNA, siRNA or viral-vectors, such as lentivirus, envisioned to transduce DCs and induce TAA presentation and persistent TLR signaling are also under focus for cancer immunotherapy [9,10]. However, these strategies might impose several drawbacks such as limited stability of the transgene, poor nuclear import and safety issues related

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to the use of viral vectors, making the use of peptide antigens and TLR ligands an appealing strategy.

Some studies have demonstrated that the combined administration of TLR ligands and antigens enhances specific immune responses [11,12]. However, additional strategies are needed in order to (i) prevent their prompt *in vivo* degradation; (ii) simultaneously target antigens and adjuvants to an APC and (iii) promote their sustained and modulated release in those cells. This can be accomplished by the use of biodegradable nanoparticles (NPs), which have been shown to improve immune responses by enhancing the uptake of antigens and adjuvants by APCs [13,14,15]. NPs are able to target the immune system and co-deliver antigens and adjuvants simultaneously to the same APC, enabling its coordinated activation [16]. The large surface area of NPs potentiates electrostatic and/or receptor-interactions to a greater extent compared to soluble antigens [17]. By providing direct intracellular access, the engagement of intracellular receptors is facilitated, which improves the efficacy of vaccine adjuvants [18]. Also, by using NPs, immediate degradation of the key vaccine components *i.e.* proteins, peptides, nucleic acids and oligonucleotides can be avoided [19,20]. Furthermore, biodegradable polymeric NPs can provide different release profiles to the entrapped molecules according to polymer's physicochemical properties [21]. Finally, NP functionalization further allows the active targeting of APCs potentiating the delivery of their cargo at the intracellular level and thus improving vaccine outcome [22]. APCs possess a multitude of surface receptors known to be involved in the recognition and internalization of pathogens. One example of such receptor is the mannose receptor (MR/CD206) expressed on DCs and macrophages [23]. All these advantageous properties confer intrinsic adjuvanticity to NPs and make them promising vaccine delivery systems [24].

We hypothesized that the co-entrapment of several melanoma-associated antigens (MAAs) along with TLR ligands in mannose-functionalized aliphatic polyester-based NPs could be an effective anti-tumor strategy. The developed nanoparticulate vaccines were able to induce anti-tumor immune responses in a murine melanoma model, when used in therapeutic and prophylactic approaches. We investigated multiple factors that have an impact on the therapeutic effect of a cancer vaccine, as the importance of using an active targeted delivery system, the synergy between TLR ligands and the relevance of combining multiple TAAs, including MHC class I- and class II-restricted peptides.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolide) (PLGA) Resomer® RG 502 (lactide: glycolide 50:50) (average Mw = 7000–17,000 g/mol), sodium cholate hydrate, dichloromethane (DCM), Tris, fluorescamine, ovalbumin (OVA), lipopolysaccharide (LPS) from *Escherichia coli* and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO,USA). Poly(D,L-lactic-co-glycolide-b-ethylene glycol) (PEG-b-PLGA) (average Mw(PEG) = 4600 g/mol and Mw(PLGA) = 16,500 g/mol) and poly(ε-caprolactone-b-ethylene glycol) (PEG-b-PCL) (average Mw(PEG) = 6000 g/mol and Mw(PCL) = 12,000 g/mol) were synthesized by ring opening polymerization followed by a "clip" and "click" reaction of α-methoxy-ω-alkyne poly(ethylene glycol) (PEG) to N-hydroxy-succinimidyl activated esters [25,26]. Mannose-grafted poly(ε-caprolactone-b-ethylene glycol) (man-PEG-b-PCL) (average Mw(PCL) = 17,000 g/mol and Mw(PEG) = 2000 g/mol) was synthesized by the Huisgen 1,3-dipolar cycloaddition, where each PCL long chain was grafted with three PEG chains, with 30% of PEG chains containing one mannose residue [27].

Dimethylsulfoxide (DMSO) and hydrochloric acid were purchased from Merck (Darmstadt, Germany). The MicroBCA™ Protein assay kit and the Oligreen® ssDNA quantitation kit were supplied by Thermo Fisher Scientific Inc. (Rockford, IL USA) and Life Technologies (Carlsbad, CA, USA), respectively.

Melan-A:26-35(L27) (hereafter Melan-A:26 or M), ELAGIGILTV, gp100:209-217(2 M) (hereafter gp100:209 or G_{209}), IMDQVPFSV and gp100:44-59 (hereafter gp100:44 or G_{44}), WNRQLYPEWTEAQRLD, were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). CpG ODN 1826 Vaccigrade™ (hereafter CpG or C), 5′-TCCATGACGTTCCTGA CGTT-3′, and Poly(I:C) (HMW) (average size 1.5–8 kb) Vaccigrade™ (hereafter Poly(I:C) or P) were purchased from InvivoGen (San Diego, CA, USA).

Dulbecco's Phosphate Buffered Saline (DPBS) (1×), RPMI + Glutamax® medium, heat inactivated fetal bovine serum (FBS), trypsin EDTA 0.05%, penicillin/streptomycin (PEST) 10,000 Unit/ml/10,000 μg/ml, sodium pyruvate 100 mM, HEPES 1 M, 2-mercaptoethanol 50 mM, ACK lysing buffer and AlamarBlue® reagent were purchase from Life Technologies (Carlsbad, CA, USA).

Rat anti-mouse PE-conjugated anti-CD4 for flow cytometry was purchased from Miltenyi Biotec (Koln Germany). Rat anti-mouse APC-conjugated anti-CD8α and PerCP-Cy5.5-conjugated anti-CD3 monoclonal antibodies for flow cytometry were purchased from Biolegend (San Diego, CA, USA). Horseradish peroxidase (HRP) conjugate goat anti-mouse IgG (1:2000), IgG1 (1:6000) and IgG2c (1:10,000) for ELISA were purchased from AbD Serotech (Raleigh, NC, USA). TMB One Component HRP substrate was acquired from Tebu-bio (Le Perray-en-Yvelines, France). DuoSet® ELISA Development kits for IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10 and Granzyme B (Grz B) were purchased from R&D Systems Europe (Abingdon, UK). Anti-mouse ELISA sets for IL-2, IL-4, IL-6 and IL-10 were also acquired from Southern biotech (Birmingham, AL, USA).

2.2. Mice

Female transgenic OT-II mice were bred in-house at Instituto Gulbenkian de Ciência (Oeiras, Portugal), under conventional, non-specific pathogen-free conditions.

Male C57Bl6 mice 8–10 weeks old were purchased from Charles River (Wilmington, MA, USA). This strain was chosen because it is the strain in which the melanoma cell line B16F10 was created [28].

Procedures were reviewed and ethically approved by the local ethics committee and Portuguese competent authority for animal protection, Direcção Geral de Alimentação e Veterinária, Lisbon, Portugal.

2.3. Preparation of NPs

NPs were aseptically prepared at room temperature by the "double emulsion solvent evaporation" method, as previously reported with modifications [29]. Two different types of NPs were formulated: non-functionalized NPs (hereafter NPs), constituted by a blend of PLGA, PEG-b-PLGA and PEG-b-PCL in a 70:15:15 w/w ratio; and functionalized (mannose-grafted) NPs (hereafter man-NPs) using PLGA, PEG-b-PLGA and man-PEG-b-PCL (70:15:15 w/w). Polymers were dissolved in DCM or DCM:DMSO 70:30 v/v to prepare NPs or man-NPs, respectively. Aqueous solutions of antigens (Melan-A:26, gp100:209 or gp100:44) at 5 mg/ml and adjuvants CpG (5 mg/ml) and Poly(I:C) (2.5 mg/ml), were added to the polymer solutions (50 mg/ml) and mixtures were emulsified using an ultrasonic processor for 15 s at 70 W. OVA (10 mg/ml) was also used as a model antigen. A second emulsion was performed with 1 % (w/v) sodium cholate aqueous solution under the same conditions. The double emulsion was then poured drip into a 0.3 % (w/v) sodium cholate aqueous solution, and stirred at 37 °C for 1 h. The NP suspension was then washed twice with ultrapure water by centrifugation at 22,000 ×*g* for 45 min, 4 °C (Beckman Coulter Avanti® J-E Centrifuge JA-20) and finally resuspended in DPBS. The initial amount of polymer was considered to determine the final concentration of NP suspensions.