Biomaterials 40 (2015) 88–97

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses

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ARTICLE INFO

Article history:
Received 1 August 2014
Accepted 19 October 2014
Available online 26 November 2014

Keywords:
PLGA nanoparticles
Vaccine
DC
Targeting
TLR ligands
CD40

ABSTRACT

Dendritic cells (DC) play a prominent role in the priming of $CD8^+$ T cells. Vaccination is a promising treatment to boost tumor-specific $CD8^+$ T cells which is crucially dependent on adequate delivery of the vaccine to DC. Upon subcutaneous (s.c.) injection, only a small fraction of the vaccine is delivered to DC whereas the majority is cleared by the body or engulfed by other immune cells.

To overcome this, we studied vaccine delivery to DC via CD40-targeting using a multi-compound particulate vaccine with the aim to induce potent $CD8^+$ T cell responses. To this end, biodegradable poly(lactic-co-glycolic acid) nanoparticles (NP) were formulated encapsulating a protein Ag, Pam3CSK4 and Poly(I:C) and coated with an agonistic αCD40-mAb (*NP-CD40*). Targeting NP to CD40 led to very efficient and selective delivery to DC *in vivo* upon s.c. injection and improved priming of $CD8^+$ T cells against two independent tumor associated Ag. Therapeutic application of *NP-CD40* enhanced tumor control and prolonged survival of tumor-bearing mice.

We conclude that CD40-mediated delivery to DC of NP-vaccines, co-encapsulating Ag and adjuvants, efficiently drives specific T cell responses, and therefore, is an attractive method to improve the efficacy of protein based cancer vaccines undergoing clinical testing in the clinic.

1. Introduction

Dendritic Cells (DC) are the main antigen (Ag) presenting cells (APC) of the immune system [1,2] and their ability to orchestrate innate and adaptive immunity is widely being exploited to develop cancer immunotherapies [3]. Immature DC have high endocytic capacity, express various intra- and extracellular pathogen recognition receptors, such as toll-like receptors (TLR), and continuously sample their surroundings for danger signals. TLR-triggering results in phenotypical changes, facilitated Ag processing, MHC presentation and increased cytokine production, a process termed DC maturation [4].

Therapeutic vaccinations against cancer are centered on the delivery of tumor associated Ag (TAA) to DC which then initiate Ag-specific T cell responses [5,6]. However, *in vivo* generation of robust anti-tumor cytotoxic $CD8^+$ T cells (CTL) remains a major challenge. Targeted delivery of TAA to DC using nanoparticle (NP) vaccine carriers formulated with poly(lactic-co-glycolic acid) (PLGA) is an attractive approach to enhance specific T cell responses. PLGA NP can be formulated to encapsulate protein [7] or short- [8] and long-peptide [9] Ag encoding TAA and TLR ligands (TLRL) [10]. Encapsulation of Ag in NP facilitates MHC Ag presentation [8] and *in vivo* anti-tumor T cell responses compared to soluble Ag [11]. Encapsulation of Ag in NP facilitates MHC Ag presentation and *in vivo* anti-tumor T cell responses compared to soluble Ag.

Abbreviations: PLGA, poly-(lactic-co-glycolic-acid); NP, nanoparticles; TLRL, TLR ligands.

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http://dx.doi.org/10.1016/j.biomaterials.2014.10.053
0142-9612/

Due to their physical characteristics, NP are prone to be internalized by scavenger cells, such as macrophages (Mϕ), which offer poor T cell priming capacity compared to DC. Protection from non-specific uptake is achieved by pegylation of NP which also prolongs the *in vivo* half-life [12]. Pegylated NP can be specifically (re-)targeted to DC by additional surface modifications which is suggested to enhance *in vivo* T cell responses. Indeed, C-type lectin specific antibodies coated to PLGA-PEG NP [13,14] but also compounds such as protamine and mannose coated to the PLGA-NP surface core [15,16] have been shown to improve *in vitro* binding and internalization by DC and promote better T cell responses. However, no direct evidence was provided in these studies for selective DC-targeting and improved delivery of the vaccine to DC *in vivo*.

Facilitating *in vivo* delivery of PLGA-NP-vaccines to DC via CD40 and the resulting vaccine induced T cell responses is the subject of this study. CD40 is a tumor necrosis factor-receptor family cell-surface receptor highly expressed on DC. CD40/CD40L ligation plays a crucial role in the maturation of DC into fully competent APC and is a key signal for $CD4^+$ T helper dependent $CD8^+$ T cell priming [17,18]. Moreover, targeting soluble Ag via CD40 using antibody constructs was shown to facilitate the internalization of Ag into early-endosomes [19], intracellular compartments associated with efficient MHC class I Ag cross-presentation, and promotes tumor-specific T cell responses [20].

In this study, we evaluated CD40-targeting of a particulate Ag, by formulating PLGA-NP co-encapsulating ovalbumin protein, the adjuvants Pam3Csk4 (TLR2L) and Poly(I:C) (TLR3L), as well as the murine αCD40-mAb FGK45 [17] coupled to the NP-surface, PLGA(-Ag/TLR2 + 3L)-αCD40 (*NP-CD40*).

We report here, that *NP-CD40* administered as a vaccine displays selective and improved capacity to deliver Ag to DC *in vivo*, over other APC, and better DC maturation in comparison to non-targeted NP-vaccines. Vaccinations with *NP-CD40* resulted in the priming of robust Ag-specific $CD8^+$ T cells with the capacity to control tumor growth and prolong survival of tumor-bearing animals.

2. Materials and methods

2.1. Animals

C57BL/6 (CD45.2/Thy1.2; H-2^b) mice were obtained from Charles River Laboratories. Ly5.1/CD45.1 (C57BL/6 background), CD40 KO (C57BL/6 background), transgenic OT-I/Thy1.1/CD45.2 (specific for the $OVA_{257–264}$ CTL epitope presented by H2-K^b) and transgenic OT-II/Ly5.1/CD45.1 mice (specific for the $OVA_{323–339}$ Th epitope presented by I-A^b) were bred in the specific pathogen-free animal facility of the Leiden University Medical Center. All animal experiments were approved by the animal experimental committee of Leiden University.

2.2. DC and cell lines

Mouse BMDC were cultured published previously [18]. In brief, Freshly isolated mouse bone marrow (BM) cells from WT C57BL/6 mice or CD40 KO femurs were and cultured for 10 days in medium supplemented with GM-CSF (50 ng/mL). After 10 days of culture, large numbers of typical DC were obtained which were at least 90% positive for murine DC marker CD11c (data not shown). D1 cells, a GM-CSF dependent immature dendritic cell line were cultured as described before [21]. OVA-transfected B16 tumor cell line (B16-OVA), syngeneic to the C57BL/6 strain, was cultured as described previously [22].

2.3. Preparation and characterization of targeted PLGA-NP

PLGA-NP (Ag/TLRL)-mAb were formulated encapsulating a model protein Ag and in combination with TLR2L (Pam3Csk4) and/or TLR3L (Poly(I:C)) using double-emulsion and solvent evaporation technique as previously described [20]. PLGA-NP was coated with mAbs, murine agonistic αCD40 mAb, FGK45 and mouse IgG2a Isotype control respectively, essentially as described before [23]. In brief, 200 μL OVA antigen free from endotoxin (50 mg/mL in PBS) and Poly(I:C) (InvivoGen) (25 mg/mL in H_2O) and/or Pam3CSK4 (InvivoGen) (5 mg/mL in H_2O) were emulsified in 2 mL PLGA (50 mg/mL in ethyl acetate) under sonification (Branson, sonofier 250) during 60 s. This first emulsion was rapidly added to 1 mL of 1% polyvinyl alcohol/7% ethyl acetate in distillated water during 15 s. A combination of pegylated lipids (DSPE-PEG(2000) succinic acid (6 mg) and mPEG 2000 PE (6 mg)) were dissolved in chloroform and added to the vial. The chloroform was removed by a stream of nitrogen gas. Subsequently, the emulsion was rapidly added to the vial containing the lipids and the solution was homogenized during 30 s using a sonicator. This solution was added to 100 mL of 0.3% PVA/7% of ethyl acetate in distillated water and stirred overnight to evaporate ethyl acetate. The PLGA-NP were collected by centrifugation at 12,000 × *g* for 10 min, washed four times with distilled water and lyophilized. Next, mAbs was covalently coupled to 10 mg of PLGA-NP by activating surface carboxyl groups in isotonic 0.1 M MES buffer pH 5.5 containing 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (10 equiv.) and N-hydroxysuccinimide (10 equiv.) for 1 h. Mouse IgG2a Isotype control (Clone:C1.18.4 Catalog #:BE0085) was purchased from Bio X Cell Antibody Production and Purification. The activated carboxyl-PLGA-NP was washed one time with MES buffer by centrifugation. Subsequently, mAbs (200 μg per mg NP) were added and the suspension was stirred during 3 h at room temperature and later overnight at 4 °C. Unbound antibodies were removed by centrifugation (12,000 × *g*, during 10 min) and the PLGA-NPs-mAbs was washed four times with PBS. The presence of Abs on the particle surface was determined by Coomassie dye protein assay. Physicochemical characteristics of formulated NP are summarized in Table 1.

2.4. Dynamic light scattering and zeta-potential measurements

Dynamic light scattering (DLS) measurements were taken on different PLGA-NP using an ALV light-scattering instrument equipped with an ALV5000/60X0 Multiple Tau Correlator and an Oxxius SLIM-532,150 mW DPSS laser operating at a wavelength of 532 nm. A refractive index matching bath of filtered cis-decalin surrounded the cylindrical scattering cell, and the temperature was controlled at 21.5 ± 0.3 °C using a Haake F3-K thermostat. In each sample, the $g2(\tau)$ autocorrelation function was recorded ten times at a detection angle of 90°. For each measurement, the diffusion coefficient (D) was determined by using the second-order cumulant, and the corresponding PLGA-NP diameter was calculated assuming that the PLGA-NP were spherical in shape. Zeta potential measurements were performed on PLGA-NP using a Malvern ZetaSizer 2000 (UK).

2.5. Quantifying encapsulated OVA in NPs

OVA-protein encapsulating efficiency was determined after hydrolyzing 5 mg PLGA-NPs in 0.5 mL 0.8 M NaOH overnight at 37 °C. The OVA-protein content was then measured using Coomassie Plus Protein Assay Reagent (Pierce) according to the manufacturer's protocol. OVA encapsulation efficiency was calculated by dividing the measured amount of encapsulated Ag by the theoretical amount assuming all was encapsulated.

2.6. Quantifying encapsulated TLR ligands

Biodegradable PLGA-NP was hydrolyzed with 0.8 M NaOH overnight at 37 °C. The encapsulation efficiency of Poly(I:C) was determined by reversed-phase high-performance liquid chromatography (RP_HPLC) and was also determined by UV spectrometry using a Nanodrop system (Thermo Scientific). Poly(I:C) was assayed by RP-HPLC at room temperature using a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with a reversed-phase Symmetric C18 column (250 mm × 4.6 mm). The flow rate was fixed at 1 mL/min and detection was obtained by UV detection at 254 nm. A linear gradient of 0–80% of acetonitrile (containing 0.036% trifluoroacetic acid) in water (containing 0.045% trifluoroacetic acid) was used for Poly(I:C). The retention time of the Poly(I:C) was approximately 20 min. The regression analysis was constructed by plotting the peak–area ratio of Poly(I:C) versus concentration. The calibration curves were linear within the range of 2.5–150 μg for Poly(I:C). The correlation coefficient (R^2) was greater than 0.99.

2.7. Analysis of in vitro NP-association with DC

WT C57BL/6 or CD40 KO BMDC (100,000/well) were plated into a 96-well flat bottom plate and incubated for 1 h at either 4 °C (binding analysis) or 37 °C (uptake analysis) with titrated amounts of CD40-targeted or non-targeted (PLGA-Ag/TLR2 + 3L)-PEG-mAb formulations labeled with the near infrared dye (near-IR dyes, CW800). Cells were washed twice to remove residual non-bound NP. Binding and uptake of PLGA-NP by DC was determined based on near-IR fluorescence using odyssey equipment (LI-COR) at 800 nm. Data analyses were corrected for the number of amount cells per measurement via co-staining with TO-PRO® (Invitrogen) at 700 nm.

2.8. Analysis of in vivo NP-uptake by immune cells

Animals were vaccinated with the CD40-targeted or non-targeted (PLGA-Ag/TLR2 + 3L)-PEG-mAb formulations containing OVA-Alexa647 (Invitrogen) by subcutaneous (s.c.) injection into the right flank. *In vivo* NP-uptake by cells was analyzed 24 or 48 h after vaccination by sacrificing the animals and isolating the inguinal lymph nodes. Single cell suspensions were prepared and flow cytometry was used to determine the fluorescence intensity of OVA-Alexa647 in F4/80$^-$CD11b$^+$CD11c$^+$ DC and CD19$^+$B220$^+$ B cells as a measure for NP-uptake. All fluorescent-mAb used for staining were purchased from BD Pharmingen. Flow cytometry was performed using an LSRII (BD Pharmingen) and data analyzed with FlowJo software (Treestar).