Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Poly-(lactic-co-glycolic-acid)-based particulate vaccines: Particle uptake by dendritic cells is a key parameter for immune activation

A.L. Silva[a,1], R.A. Rosalia[b,c,1], E. Varypataki[a], S. Sibuea[a], F. Ossendorp[c], W. Jiskoot[a,*]

[a] Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands
[b] Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands
[c] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form
19 December 2014
Accepted 22 December 2014
Available online xxx

Keywords:
Protein antigen
PLGA
Microparticles
Nanoparticles
Adjuvants
Immunotherapy
Cellular immune response

ABSTRACT

Poly(lactic-co-glycolic acid) (PLGA) particles have been extensively studied as biodegradable delivery system to improve the potency and safety of protein-based vaccines. In this study we analyzed how the size of PLGA particles, and hence their ability to be engulfed by dendritic cells (DC), affects the type and magnitude of the immune response in comparison to sustained release from a local depot. PLGA microparticles (MP, volume mean diameter ≈ 112 μm) and nanoparticles (NP, *Z*-average diameter ≈ 350 nm) co-encapsulating ovalbumin (OVA) and poly(I:C), with comparable antigen (Ag) release characteristics, were prepared and characterized. The immunogenicity of these two distinct particulate vaccines was evaluated *in vitro* and *in vivo*. NP were efficiently taken up by DC and greatly facilitated MHC I Ag presentation *in vitro*, whereas DC cultured in the presence of MP failed to internalize significant amounts of Ag and hardly showed MHC I Ag presentation. Vaccination of mice with NP resulted in significantly better priming of Ag-specific $CD8^+$ T cells compared to MP and OVA emulsified with incomplete Freund's adjuvant (IFA). Moreover, NP induced a balanced T_H1/T_H2-type antibody response, compared to vaccinations with IFA which stimulated a predominant T_H2-type response, whereas MP failed to increase antibody titers. In conclusion, we postulate that particle internalization is of crucial importance and therefore particulate vaccines should be formulated in the nano- but not micro-size range to achieve efficient uptake, significant MHC class I cross-presentation and effective T and B cell responses.

1. Introduction

In the past few years, extensive efforts in the immunotherapy field have led to the development of several therapeutic vaccine strategies [1–3]. Protein vaccines are popular forms of therapeutic vaccines [3,4] which have been tested successfully in (pre-)clinical studies against various immunological diseases [5,6]. The potency of protein vaccines can be significantly amplified *via* the encapsulation in biodegradable particles. The use of particles facilitates the uptake of the antigen (Ag) by dendritic cells (DC), allows the co-delivery of Ag and Toll-like receptor ligands (TLRL) [7,8] and improves Ag processing, presentation and T cell priming by DC compared to use of soluble Ag. Generally, particulate Ag is better routed into MHC class I cross-presentation pathways and preserved inside intracellular compartments, resulting in sustained and efficient priming of $CD8^+$ T cell responses [9–13]. DC have superior capacity to cross-present exogenous Ag in MHC I molecules and are considered the major target for vaccines aimed at activating a robust $CD8^+$ T cell mediated immunity [3,14].

Most clinical trials for cancer immunotherapy have relied on the use of Montanide, a GMP-grade version of incomplete Freund's adjuvant (IFA), which is a water-in-oil (w/o) emulsion for Ag delivery. The immune-activating properties of Montanide are partially explained through the formation of a local Ag depot and the onset of inflammation, which attracts immune cells toward the site of injection [15] where the Ag is taken up primarily in its soluble form [16]. However, the use of Montanide is associated with significant local adverse effects [5], reason why there is an urgent need for alternatives [17].

Nanoparticles (NP) and microparticles (MP) prepared from biodegradable poly(lactic-co-glycolic acid) (PLGA) have been studied extensively for the sustained delivery of proteins and therapeutic agents and as a potential alternative to w/o emulsions

* Corresponding author at: Division of Drug Delivery Technology, Leiden Academic Center for Drug Research, Leiden University, Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden, The Netherlands. Tel.: +31 71 527 4314; fax: +31 71 527 4565.
E-mail address: w.jiskoot@lacdr.leidenuniv.nl (W. Jiskoot).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.vaccine.2014.12.059
0264-410X/

[18–20]. Plain PLGA particles have sub-optimal adjuvant properties *in vivo* resulting in poor DC maturation [9,21], which can be overcome by the inclusion of TLRL, leading to an efficient induction of T_H1-mediated T cell responses with the capacity to control tumors or protect against a viral challenge [7,22–25].

It is generally assumed that NP, compared to MP, are better for targeted drug delivery due to a better biodistribution [26,27] and ability to cross biological barriers [28]. Still, there is little agreement when it comes to therapeutic vaccines: which size leads to the most efficient MHC class I Ag cross-presentation remains controversial [29–31].

To study the importance of particle uptake for the induction of an immune response, we developed NP and MP containing equivalent amounts of Ag and TLRL with comparable release profiles *in vitro*. Particles co-encapsulating model Ag ovalbumin (OVA) and TLR3L poly(I:C) were formulated to obtain NP that will be efficiently internalized by DC [32], releasing the Ag mostly intracellularly [12], *versus* MP with a size (>20 μm) that is too large to be taken up by DC [31], thus functioning exclusively as a local Ag/TLR3L depot under the skin, similarly to Montanide.

2. Materials and methods

2.1. Reagents

PLGA Resomer RG502H (50:50 MW 5000–15,000 Da) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany); PLGA Resomer RG752H (75:25 MW 4000–15,000 Da), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and HEPES from Sigma–Aldrich (Steinheim, Germany); Ovalbumin (OVA) grade V, 44 kDa from Worthington (New Jersey, USA); Alexa Fluor 488 (AF488) labeled OVA from Invitrogen (Merelbeke, Belgium); Tween 20 from Merck Schuchardt (Hohenbrunn, Germany); polyvinyl alcohol (PVA) 4–88 (31 kDa) from Fluka (Steinheim, Germany); Poly(I:C) LMW and rhodamine labeled poly(I:C) from InvivoGen (San Diego, USA); phosphate-buffered saline (NaCl 8.2 g/l; $Na_2HPO_4{\cdot}12H_2O$ 3.1 g/l; $NaH_2PO_4{\cdot}2H_2O$ 0.3 g/l) (PBS) from B. Braun (Melsungen, Germany); all fluorescently labeled antibodies from BD Pharmingen (San Diego, USA); incomplete Freund's adjuvant (IFA) from Difco Laboratories (Detroit, USA). APC-SIINFEKL/H2-K^b tetramers, SIINFEKL (OVA_8) and ASNENMETM (FLU_9) and carboxyfluorescein succinimidyl ester (CFSE)-labeled synthetic short peptides were produced in house. All other chemicals were of analytical grade and aqueous solutions prepared with Milli-Q water.

2.2. Cells

D1 cells, a murine GM-CSF dependent immature dendritic cell line, were cultured as described previously [33]. Bone-marrow derived DC (BMDC) were freshly isolated from femurs from mice and cultured as published previously [34] and yielded cells which were at least 90% positive for murine DC marker CD11c. B3Z $CD8^+$ T-cell hybridoma cell line, specific for the H-$2K^b$-restricted $OVA_{257–264}$ CTL epitope SIINFEKL was cultured as described before [35].

2.3. Animals

C57BL/6 (Ly5.2/CD45.2; H-2^b) mice were obtained from Charles River Laboratories. Ly5.1/CD45.1 congenic (C57BL/6 background) mice 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.

2.4. Preparation and characterization of OVA- and poly(I:C)-loaded PLGA particles

2.4.1. Preparation of NP and MP and IFA

PLGA 50:50 and PLGA 75:25 NP were prepared as described [36], using 1 mg OVA, 0.25 mg poly(I:C) and 1 μg poly(I:C)-rhodamine dissolved in 85 μl of 25 mM HEPES, pH 7.4, as inner aqueous phase. For NP and MP used in the release and uptake studies, 1% (w/w, total OVA) of OVA-AF488 was added to the inner phase during preparation for detection purposes.

PLGA 50:50 MP were prepared by adding 1 mg OVA, 0.25 mg poly(I:C), and 1 μg poly(I:C)-rhodamine dissolved in 500 μl of 25 mM HEPES pH 7.4 to 1 ml DCM containing 125 mg PLGA 50:50. The mixture was homogenized for 30 s at 25,000 rpm (Heidolph Ultrax 900, Sigma, Germany) and transferred to 10 ml of 2% (w/v) PVA under magnetic stirring for 10 min at 750 rpm at room temperature, followed by 1 h at 500 rpm at 40 °C to allow evaporation of DCM. MP were harvested and washed twice by centrifugation (2000 × *g*, 2 min). To separate particles bigger than 20 μm, MP were diafiltrated with 3 l water under continuous stirring, using a Solvent Resistant Stirred Cell (Millipore, USA) filtration system with a 20-μm stainless steel sieve (Advantech, USA), the retentate collected and particles recovered by centrifugation at 2000 × *g* for 2 min. Particles >200 μm were eliminated by filtration through a 200-μm stainless steel sieve (Advantech, USA). Intactness of MP before and after filtration (see Supplemental Fig. 1) was verified with an Axioskop microscope, equipped with Axiocam ICc 5 (Carl Zeiss, Munich, Germany) and 20× amplification objective. Images were collected with ProgRes CapturePro v2.8.8 software (Jenoptik AG, Jena, Germany). Both NP and MP suspensions were aliquoted and freeze-dried.

Preparation of IFA emulsion was performed by dissolving OVA and poly(I:C) in PBS and mixing with IFA for 30 min in a 1:1 ratio by using a vortex mixer.

2.4.2. Characterization of NP and MP

Size and polydispersity index (PDI) of NP in 5 mM HEPES pH 7.4 were determined by dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern, UK). Zeta potential (ZP) was determined by laser Doppler velocimetry using the same apparatus. Size distribution of MP was determined by light obscuration (LO) using a PAMAS SVSS system (PAMAS GmbH, Rutesheim, Germany) equipped with HCB-LD-25/25 sensor and 1-ml syringe. Each sample was measured three times, each measurement consisting of three runs of 0.2 ml at a flow rate of 10 ml/min.

Encapsulation efficiency (EE) was calculated by fluorescence of OVA-AF488 (excitation 495 nm, emission 520 nm) or poly(I:C)-rhodamine (excitation 546 nm, emission 576 nm) detected in the supernatant with Infinite® M 1000 Pro (Tecan, Switzerland) microplate reader.

To study release kinetics NP/MP containing fluorescently labeled OVA and poly(I:C) were resuspended in PBS, containing 0.01% Tween 20 and 0.01% sodium azide, at 10 mg PLGA/ml, and maintained at 37 °C under tangential shaking at 100 rpm in a GFL 1086 shaking water bath (Burgwedel, Germany) for 30 days. At regular time intervals, 250 μl aliquots were taken and centrifuged for 20 min at 18,000 × *g*. Supernatants were stored at 4 °C until fluorescence intensity was determined (Infinite® M 1000 Pro, Tecan, Switzerland) [37]. OVA concentrations on remaining supernatant samples from the final day were also analyzed by BCA assay (Pierce, Rockford, IL, USA) after dissolving particles in DMSO and 0.5 M NaOH + 0.5% SDS as described [38] to validate fluorescence measurements, with comparable results (Supplemental Table 1).

Please cite this article in press as: Silva AL, et al. Poly-(lactic-co-glycolic-acid)-based particulate vaccines: Particle uptake by dendritic cells is a key parameter for immune activation. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2014.12.059