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Hollow microneedle-mediated intradermal delivery of model vaccine antigen-loaded PLGA nanoparticles elicits protective T cell-mediated immunity to an intracellular bacterium

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ABSTRACT

The skin is an attractive organ for immunization due to the presence of a large number of epidermal and dermal antigen-presenting cells. Hollow microneedles allow for precise and non-invasive intradermal delivery of vaccines. In this study, ovalbumin (OVA)-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles with and without TLR3 agonist poly(I:C) were prepared and administered intradermally by hollow microneedles. The capacity of the PLGA nanoparticles to induce a cytotoxic T cell response, contributing to protection against intracellular pathogens, was examined. We show that a single injection of OVA-loaded PLGA nanoparticles, compared to soluble OVA, primed both adoptively transferred antigen-specific naïve transgenic $CD8^+$ and $CD4^+$ T cells with markedly high efficiency. Applying a triple immunization protocol, PLGA nanoparticles primed also endogenous OVA-specific $CD8^+$ T cells. Immune response, following immunization with in particular anionic PLGA nanoparticles *co*-encapsulated with OVA and poly(I:C), provided protection against a recombinant strain of the intracellular bacterium *Listeria monocytogenes*, secreting OVA. Taken together, we show that PLGA nanoparticle formulation is an excellent delivery system for protein antigen into the skin and that protective cellular immune responses can be induced using hollow microneedles for intradermal immunizations.

1. Introduction

The skin is an organ with many immune cells and is considered a potent organ for immunizations [1]. However, the challenge is to deliver high-molecular-weight antigens across the stratum corneum, which is the outermost layer of the skin and acts as an effective natural barrier for penetration of pathogens and allergens into the skin. One of the methods to circumvent the skin barrier is the use of microneedles. Microneedles are miniaturized needles that provide the possibility of minimally invasive vaccination in the dermis and epidermis of the skin. There are other benefits in using microneedles compared to traditional hypodermic needles, like possible painless vaccination, the requirement of less trained personnel and reduced contamination risk [2]. Nowadays a wide variety of these microneedles exist, including solid, coated, dissolving and hollow microneedles [3,4].

Hollow microneedles have multiple benefits, for instance they can be used to inject a wide variety of fluids into the skin at different pressure-driven flow rates [3,5,6] and offer the highest precision in dose delivery among all microneedle types. Furthermore, they offer the possibility to screen formulations without time-consuming design and preparation of microneedles, as in case of coated and dissolving microneedles. Recently, hollow microneedles and an applicator for them were developed in our laboratory to inject formulations in precise manner into the skin. These microneedles were successfully used for formulations with inactivated polio virus vaccine in rats resulting in effective humoral immune responses [7–9]. However, whether hollow microneedle-mediated delivery may also induce $CD8^+$ T cell responses towards vaccine antigens is presently unclear.

Cytotoxic $CD8^+$ T cells play an important role in cellular immune protection against intracellular pathogens or tumor growth. To induce such $CD8^+$ T cell responses, an antigen needs to be processed in the cell and presented by MHC-I molecules on professional antigen-presenting cells (pAPC) to the immune system. Delivery of vaccine protein antigens over the cellular membrane can be achieved using delivery systems and over the past decades different types of them, such as polymeric nanoparticles, emulsions and lipid-based nanoparticles have been

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developed [10–12]. Nano-encapsulation of antigens has several advantages, such as stabilization of antigens in vivo, enhancement of the uptake by pAPC and also reduction of antigen release into systemic circulation [4,13]. The immune outcome can be potentially shaped by using nanoparticles with difference size [14] and surface charge [15], and by co-encapsulating antigen and adjuvant into the nanoparticles [16,17].

For the production of polymeric nanoparticles, poly(lactic-co-glycolic acid) (PLGA) is the most commonly used polymer, because of its superior biocompatibility and biodegradability [18–20]. Previous studies have shown that model antigen- and adjuvant-loaded PLGA nanoparticles used for vaccination were able to improve the induction of cell-mediated immune response in mice [17,21–23]. However, relatively little is known about how encapsulation in PLGA nanoparticles modifies T cell responses to antigen/adjuvant combinations that are delivered intradermally by different novel types of microneedles. One recent study reported that PLGA nanoparticles, delivered intradermally using dissolving microneedles arrays [24], induced cellular immune responses and protection against viral infection and tumor growth.

In this study, nanoparticles were prepared and characterized in terms of size, surface charge and antigen/adjuvant release profiles. We investigated the ability of hollow microneedle-delivered protein antigens, encapsulated in either anionic or cationic PLGA nanoparticles without and with co-encapsulated TLR 3 agonist poly(I:C) to induce a protective, cellular immune response towards an intracellular pathogen in a mouse model.

2. Materials and methods

2.1. Materials

PLGA (acid terminated, lactide glycolide 50:50, Mw 24.000–38.000), polyethylenimine (PEI, linear, average M_n 10,000), Roswell Park Memorial Institute medium (RPMI) and Fetal bovine serum (FBS) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). PVA 4–88 (31 kDa) was obtained from Fluka (Steinheim, Germany). Endotoxin-free ovalbumin (OVA), polyinosinic-polycytidylic acid (poly(I:C)) (low molecular weight) and its rhodamine-labelled version were obtained from Invivogen (Toulouse, France). Alexa647 labelled OVA (OVA-Alexa647) was ordered from Thermo-Fischer Scientific (Waltham, MA). Dimethylsulfoxide (DMSO) was obtained from Biosolve BV (Valkenswaard, The Netherlands). Sodium dodecyl sulfate (SDS) was obtained from Merck Millipore (Hohenbrunn, Germany). Ammonium-Chloride-Potassium (ACK) lysis buffer (150 mM NH_4CL, 1 mM $NaHCO_3$; pH 7.40) and 1 mM phosphate buffer (PB; pH 7.4) were prepared in the lab. Milli-Q water (18.2 MΩ/cm, Millipore Co., USA) was used for the preparation of solutions. Sterile phosphate buffered saline (PBS) was obtained from Braun (Oss, The Netherlands). All other chemicals used are of analytical grade.

Purification antibodies used for DynaBeads® selection were all made in house and included the following antibody clones: αCD11b (clone M1/70), αMHC-II (M5/114), αB220 (RA3-6B2), αCD4 (GK1.4), αCD8 (YTS169) and αCD25 (PC61). Purification antibodies for sorting via flow cytometry were αCD8-APC (53-6.7; eBioscience), CD44-FITC (IM7; eBioscience) and CD62L-PE (MEL-14; BD Bioscience) using a BD influx (BD Biosciences). For the detection of the adoptively transferred T cells the antibodies αCD45.2-PerCPCy5.5 (104; eBioscience), αCD4-PE (GK1.5; eBioscience) and αCD8-APC (53-6.7; BD Bioscience) were used. Detection of the endogenous T cells was measured using the antibodies αCD8-APC (53-6.7; eBioscience), αCD4-eFluor450 (GK1.5; eBioscience), αCD62L-Horizon B510 or αCD44-FITC (IM7; eBioscience), αCD16/CD32-unstained (2.4G2; made in house) and αIFNγ-PE (XMG1.2; eBioscience).

2.2. Preparation of PLGA nanoparticles

OVA-loaded PLGA nanoparticles were prepared by double emulsion with solvent evaporation method as previously reported with modifications [25]. Briefly, 75 μl OVA (20 mg/ml) in PBS was dispersed in 1 ml PLGA (25 mg/ml) in ethyl acetate by a Branson sonifier 250 (Danbury, USA) for 15 s with a power of 20 W. To prepare anionic OVA-loaded PLGA nanoparticles (anPLGA-OVA), the obtained water-in-oil emulsion was emulsified with 2 ml 2% (w/v) PVA with the sonifier (15 s, 20 W) to get a water-in-oil-in-water double emulsion. In case of cationic OVA-loaded PLGA nanoparticles (catPLGA-OVA), the single emulsion was emulsified with 2 ml 2% (w/v) PVA and 4% (w/v) PEI solution. The double emulsion was added dropwise into 25 ml 0.3% (w/v) PVA (40 °C) under stirring. The ethyl acetate was evaporated by a rotary evaporator (Buchi rotavapor R210, Switzerland) for 3 h (150 mbar, 40 °C). The nanoparticle suspension was centrifugated (Avanti™ J-20XP centrifuge, Beckman Coulter, Brea, CA) at 35000 g for 10 min, washed twice with 1 mM PB to remove the excess OVA and PVA, and dried in a Alpha1-2 freeze dryer (Osterode, Germany, − 49 °C, 90 mbar) overnight. To prepare OVA and poly(I:C) co-encapsulated PLGA nanoparticles (anPLGA-OVA-PIC), 18.75 μl OVA (40 mg/ml) and 75 μl poly(I:C) (46.7 mg/ml, including 0.03% fluorescently labelled equivalent) were emulsified with 1 ml PLGA (25 mg/ml) in ethyl acetate to obtain the water-in-oil emulsion. The remaining of the procedure was identical to that of anPLGA-OVA. The obtained nanoparticles were stored at 4 °C for analysis and further use. To prepare the PLGA nanoparticles for release study, 10% of total OVA was replaced with OVA-Alexa647 the preparation.

2.3. Characterization of PLGA nanoparticles

The size (*Z*-average) and polydispersity index (PDI) of nanoparticles were measured by dynamic light scattering and the zeta potential of nanoparticles was measured by laser doppler velocimetry using a Nano ZS® zetasizer (Malvern Instruments, Worcestershire, U.K.). The samples were diluted with 1 mM PB buffer to a nanoparticle concentration of 25 μg/ml before each measurement. To determine the loading efficiency of OVA and poly(I:C) in PLGA nanoparticles, approximately 1 mg of nanoparticles were dissolved in a mixture of 15% (v/v) DMSO and 85% (v/v) 0.05 M NaOH and 0.5% SDS. The amount of OVA was determined by MicroBCA method following the manufacturer's instructions. The amount of poly(I:C) was quantified by the fluorescence intensity of rhodamine labelled poly(I:C) (λ_{ex} 545 nm/λ_{em} 576 nm). The encapsulation efficiency (EE) and loading capacity (LC) of OVA and poly(I:C) in the nanoparticles were calculated as below:

$$EE\% = \frac{M_{\text{loaded OVA/poly(I:C)}}}{M_{\text{totoal ova/poly(I:C)}}} \times 100\% \quad (1)$$

$$LC\% = \frac{M_{\text{loaded OVA/poly(I:C)}}}{M_{\text{nanoparticles}}} \times 100\% \quad (2)$$

where $M_{\text{loaded OVA/poly(I:C)}}$ represents the mass of loaded OVA or poly(I:C), $M_{\text{total OVA/poly (I:C)}}$ is the total amount of OVA or poly(I:C) added to the formulation and $M_{\text{nanoparticles}}$ is the weight of nanoparticles.

2.4. Release of OVA and poly(I:C) from PLGA nanoparticles

Nanoparticles were prepared in triplicate as described above. To study the release of OVA and poly(I:C) from PLGA nanoparticles, 3 mg anPLGA-OVA, catPLGA-OVA or anPLGA-OVA-PIC were dispersed into 1 ml RPMI supplemented with 10% FBS and incubated at 37 °C with a shaking speed of 350 rpm. At different time points, the suspensions were centrifugated (9000*g*, 5 min) with Sigma 1–15 centrifuge (Osterode, Germany). A release sample of 600 μl of the supernatant was collected and replaced by fresh medium. The released amount of OVA and poly(I:C) was determined by fluorescence intensity of OVA-