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Research paper

PLGA nanoparticles modified with a TNFα mimicking peptide, soluble *Leishmania* antigens and MPLA induce T cell priming *in vitro* via dendritic cell functional differentiation

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ABSTRACT

Poly(lactide-co-glycolide) nanoparticles (PLGA NPs) represent a new approach for vaccine delivery due to their ability to be taken up by phagocytes and to activate immune responses. In the present study PLGA NPs were surface-modified with a TNFα mimicking peptide, and encapsulated soluble *Leishmania* antigens (sLiAg) and MPLA adjuvant. The synthesized PLGA NPs exhibited low cytotoxicity levels, while surface-modified NPs were more efficiently taken up by dendritic cells (DCs). The prepared nanoformulations induced maturation and functional differentiation of DCs by elevating co-stimulatory molecule levels and stimulating IL-12 and IL-10 production. Sensitized DCs promoted T cell priming, characterized by the development of mixed T cell subsets differentiation expressing Th lineage-specific transcriptional factors and cytokine genes. Moreover, PLGA NPs were biocompatible, while they were located in lymphoid organs and taken up by phagocytic cells. Our results suggest that surface-modified PLGA NPs encapsulating sLiAg and MPLA could be considered as an effective vaccine candidate against leishmaniasis.

1. Introduction

Polymeric microparticulate and nanoparticulate formulations are considered as ideal vaccine delivery systems. Among them, biodegradable poly(D,L-lactide-*co*-glycolide nanoparticles (PLGA NPs) have attracted considerable attention due to their biocompatibility and have been already approved by the FDA and European Medicine Agency (EMA) as drug carriers [1]. PLGA could act as highly effective vaccine delivery systems, since it possesses advantages such as sustained and controlled antigen release, adjuvant co-encapsulation, protection from degradation by enzymes, as well as the ability of tissue-specific targeting [2,3]. Several studies have demonstrated that these nano-sized delivery systems can induce both humoral and cell-mediated specific immune responses via dendritic cells (DCs) activation in animals, using experimental vaccines against cancer [4,5] or infectious diseases, such as malaria, [6] hepatitis B [7,8] and leishmaniasis [9].

DCs are the most efficient antigen presenting cells (APCs) that are capable of initiating and amplifying adaptive immune responses, important for the treatment of cancer and infectious diseases [10]. DCs take up antigens either by phagocytosis or by receptor-mediated endocytosis, process them through major histocompatibility complex (MHC) class I and II pathways and present antigenic peptides to $CD4^+$ and $CD8^+$ T cells [11,12], leading to antigen-specific T cell expansion. Therefore, targeted delivery of antigens to DCs is a promising approach to enhance vaccine efficacy [13].

Previous studies have shown that DCs targeting with antibodies against specific receptors induce stronger T cell responses [14–16]. PLGA NPs conjugated with target molecules against specific DC receptors, DEC-205, DC-SIGN, CD40 and CD11c, have raised efficient $CD8^+$ T cell responses [17–19]. As an alternative approach, NPs can be surface-modified with peptide ligands that selectively deliver antigen to DCs [20], as well as with synthetic peptides with sequence mimicking docking regions of protein receptors in DCs. In this aspect, a short tumor necrosis factor α (TNFα)-competing peptide, selected by phage selection technique, has been used for

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efficient gene delivery to DCs [21]. Apart from their excellent tissue penetration, synthesis and conjugation of short peptides to NPs is easier compared to larger molecules such as antibodies [22].

Leishmaniasis is a group of diseases that are caused by intracellular parasites of the genus *Leishmania* and displays a spectrum of clinical manifestations from self-healing cutaneous to life-threatening visceral form of disease [23]. The disease is prevalent in large areas of tropical, subtropical and the Mediterranean countries. According to WHO, 310 million people are at risk, nearly 300,000 new cases of visceral leishmaniasis annually occur with 20,000 deaths per year [24]. Available chemotherapy is far from satisfactory, as existing drugs need multiple injections, have many side effects and practically show limited efficacy in some endemic areas due to the development of resistant parasites [25,26]. Hence, there is an urgent need to develop an effective vaccine against leishmaniasis. Different micro- or nano-formulations (i.e. liposomes, chitosan, PLGA) and antigens have been tested for vaccine development against leishmaniasis with encouraging results [27,28]. Protective response against leishmaniasis is associated with the development of IFNγ producing Th1 immunity, as well as activation of $CD8^+$ T cells [29], whereas the absence of strong acquired immunity is associated with immunological dysfunction of DCs [30].

In the present study, nanoformulations based on PLGA NPs or PLGA NPs surface-modified with a TNFα mimicking 8-peptide were synthesized and encapsulated a crude mix of proteins from *Leishmania infantum* promastigotes (sLiAg). In addition, monophosphoryl lipid A (MPLA), a known TLR4 ligand was also co-encapsulated, as adjuvant for immune response enhancement. Physicochemical properties, biomolecule release and NPs' stability were determined. NPs uptake and cytotoxic effect were studied in DCs cultures, while biodistribution and cellular localization were assessed in experimental animals using fluorescein-labeled NPs. The effect of synthesized NPs on DCs maturation and cytokine production, as well as the ability of DCs sensitized with NPs to induce T cell priming and polarization, was analyzed by exploring differential gene expression of Th lineage-specific transcriptional factors and cytokines. The NPs' biocompatibility in terms of systemic production of inflammatory mediators was also estimated. Finally, the perspective of synthesized NPs use as vaccine candidates against experimental visceral leishmaniasis is also discussed.

2. Materials and methods

2.1. Materials

RPMI-1640 medium was purchased from Biochrom AG (Berlin Germany), while $NaHCO_3$, penicillin, streptomycin and 10 mM HEPES from Gibco (Invitrogen, Paisley, UK), L-glutamine from Sigma–Aldrich (Vienna, Austria) and fetal bovine serum (FBS) from Applichem (Darmstadt, Germany) were also used for cell cultures. Protease inhibitors cocktail and collagenase D were obtained from Roche (Mannheim, Germany). rmGM-CSF was purchased from Peprotech (London, UK). [^{3}H]-thymidine from PerkinElmer (Massachusetts, USA) was employed to lymphoproliferation assays. Ultracell® Ultra-15, 30 K Amicon filter devices from Merck Millipore (Darmstadt, Germany) were used for antigen concentration. All culture plates were obtained from Greiner (Berlin, Germany). The p8 peptide was chemically synthesized by JPT (Berlin, Germany). Poly(lactide-co-glycolide) (PLGA) (Resomer RG752H, MW: 4–15 kDa), polyvinyl alcohol (PVA) (MW: 30–70 kDa, 87–90% hydrolyzed), fluorescein isothiocyanate labeled bovine serum albumin (BSA-FITC, extent of labeling ⩾ 7 mol FITC per mol albumin), monophosphoryl lipid A (MPLA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide 98% (NHS), propidium iodide (PI), paraformaldeyhyde, Mowiol 40–88, concanavalin A (ConA), Ficoll-Hypaque and lipopolysaccharide (LPS) were obtained from Sigma–Aldrich (Vienna, Austria). Micro BCA Protein Assay Kit was from Pierce (Rockford, IL, USA) and Limulus Amebocyte Lysate kit was from Lonza (Basel, Switzerland). RNeasy mini kit, RT^2 HT First Strand Kit and RT^2 Profiler™ PCR Array were purchased from Qiagen (Germany). Milliplex 4-Plex mouse cytokine detection system from Millipore Corporation (Billerica, MA, USA) was employed for cytokine levels determination. All antibodies used were purchased from BD Biosciences or BD Pharmingen (Erembodegem, Belgium). All other reagents were of analytical grade and commercially available.

2.2. Experimental animals, parasite maintenance and sLiAg preparation

Female BALB/c mice (6- to 8-week old) used in the present study were obtained from the breeding unit of the Hellenic Pasteur Institute (HPI, Athens, Greece) and reared in institutional facilities under specific pathogen-free environmental conditions at ambient temperatures of 25 °C. Mice were provided with sterile food and water *ad libitum* and all animal studies were performed following protocols approved by the Animal Bioethics Committee of the HPI regulating according to the EC Directive 2010/63 and the National Law 1992/2015.

The *Leishmania infantum* strain GH8 (MHOM/GR/2001/GH8) [31], used for sLiAg preparation, was kept in a virulent state by continuous passage in BALB/c mice. Tissue amastigotes were obtained after homogenization of a spleen tissue sample from an infected BALB/c mouse and promastigote transformation was achieved during culture in RPMI-1640 medium supplemented with 24 mM $NaHCO_3$, 2 mM L-glutamine, 100 u/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES and 10% (v/v) FBS (complete culture medium) at 25 °C. Stationary phase promastigotes were harvested by centrifugation at 650*g* for 10 min at 4 °C. The pellet was washed by phosphate buffered saline (PBS; 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl) and re-suspended at a concentration of 2×10^8 cells/mL. A volume of 100 μL of this preparation was injected intravenously in the lateral tail vein of each mouse.

sLiAg was prepared in order to be encapsulated in PLGA NPs. Preparation of sLiAg was carried out as previously described, with slight modifications [32]. Late log phase promastigotes ($\sim 7 \times 10^9$) were harvested in ice-cold PBS containing protease inhibitors cocktail. The suspension was subjected to 3 cycles of sonication, each step for 30 s, with 30 s intervals and centrifugation at 8000*g* for 30 min at 4 °C. The supernatant containing sLiAg was collected and concentrated using appropriate filter devices (Ultracell® Ultra-15, 30 K Amicon), after centrifugation at 7500*g* for 20 min at 4 °C (Kubota, Tokyo, Japan). The concentrate was dialyzed against excess PBS and total protein concentration was determined using the Micro BCA Protein Assay Kit. sLiAg was stored at −70 °C in aliquots until use.

2.3. Preparation and characterization of PLGA NPs

PLGA NPs were prepared with poly(D,L-lactide-co-glycolide) 75:25 (Resomer RG752H, MW: 4–15 kDa) using the double emulsion method [33]. Initially, 2.9 mL of a PLGA chloroform solution (31 mg/mL) was mixed with 0.1 mL of an MPLA solution (10 mg/mL) in methanol:chloroform (1:4 v/v). A water-in-oil (w/o) emulsion was then formed by adding the antigen solution in PBS (i.e., BSA-FITC solution: 0.3 mL, 16.66 mg/mL; sLiAg solution: 0.3 mL, 22.50 mg/mL) into the PLGA/MPLA solution [34]. The emulsification was performed in an ice bath with the aid of a microtip sonicator (Sonicator Sonics Vibra Cell VC-505, Sonics, Newtown, CT,