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Research paper

Skin vaccination using microneedles coated with a plasmid DNA cocktail encoding nucleosomal histones of *Leishmania* spp.

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ABSTRACT

Vaccine delivery using microneedles (MNs) represents a safe, easily disposable and painless alternative to traditional needle immunizations. The MN delivery of DNA vaccines to the dermis may result in a superior immune response and/or an equivalent immune response at a lower vaccine dose (dose-sparing). This could be of special interest for immunization programs against neglected tropical diseases such as leishmaniasis. In this work, we loaded a MN device with 60 μg of a plasmid DNA cocktail encoding the *Leishmania infantum* nucleosomal histones H2A, H2B, H3 and H4 and compared its immunogenicity and protective capacity against conventional s.c. or i.d. injection of the plasmid. Mice immunized with MNs showed increased ratios of IFN-γ/IL-10, IFN-γ/IL-13, IFN-γ/IL-4, and IFN-γ/TGF-β in the spleens and lymph nodes compared with mice immunized by s.c. and i.d. routes. Furthermore, CCXCL9, CXCL10 and CCL2 levels were also higher. These data suggest that the nucleic acid immunization using MNs produced a better bias towards a Th1 response. However, none of the immunizations strategies were able to control *Leishmania major* infection in BALB/c mice, as illustrated by an increase in lesion size and parasite burden.

1. Introduction

Leishmaniasis is an infectious disease caused by protozoan parasite species of the genus *Leishmania*. This disease is one of the six major tropical diseases classified by the World Health Organization. Approximately, one billion people are at risk of infection worldwide and more than 1.3 million new infections occur each year (Alvar et al., 2012). Depending on the species, *Leishmania* can cause visceral leishmaniasis (VL), which is fatal if untreated, or cutaneous leishmaniasis (CL). Current treatments are expensive, toxic and do not prevent disease relapse. Drug resistance is also increasing (Akbari et al., 2017). However, people previously infected with *Leishmania* can gain resistive immunity to reinfection and therefore the development and delivery of anti-leishmanial vaccines could be an effective means of controlling or eliminating CL or VL (Gillespie et al., 2016). Although great efforts have been made in this area, there is still no effective vaccine against human leishmaniasis (Costa et al., 2011).

In recent decades, potential candidate antigens such as killed and lived attenuated parasites, crude parasites, pure or recombinant *Leishmania* spp. proteins, DNA encoding leishmanial proteins or immunomodulators from sand fly saliva have been tested in animal models, but very few candidate vaccines have progressed beyond the experimental stage (Badiee et al., 2013; Jain and Jain, 2015; Khamesipour et al., 2006; Srivastava et al., 2016). DNA vaccines have numerous advantages over other vaccine strategies: (i) simple and cheap to produce, (ii) enhanced temperature stability, (iii) concurrent expression of multiple proteins *in vivo,* which are folded and modified in a comparable manner to their corresponding native proteins, and (iv) ability to elicit Th1 responses and induce both $CD4^+$ and $CD8^+$ T cells, which is necessary for parasite eradication (Cui, 2005; Kumar and Samant, 2016). A variety of antigens have been studied as DNA vaccine expression products against *Leishmania* spp. including gp63 (Mazumder

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et al., 2011), LACK (Ramos et al., 2008), CP-Ldccys1 (Ferreira et al., 2008), ORF (Sukumaran et al., 2003), KMP-11 (Guha et al., 2013), LiPABPs (Soto et al., 2015), LiP0 (Pereira et al., 2015) and LEISHD-NAVAX (Das et al., 2014). Whilst varying degrees of efficacy have been demonstrated in animal models, the level of protection elicited by DNA vaccines improves when multiple antigens are co-administered.

There are five main classes of histones and four of them, H2A, H2B, H3 and H4, form the nucleosomal unit core of chromatin. Several studies suggest that *Leishmania* spp. histones are immunologically important during leishmaniasis (Baharia et al., 2014; Chenik et al., 2006; de Carvalho et al., 2003), making them attractive targets in the development of *Leishmania* spp. vaccines. Moreover, it is also known that *Leishmania* spp. histones are highly conserved between different species (Iborra et al., 2004). Studies carried out with these histones of *L. infantum* concluded that the combination of the four nucleosomal histones, expressed *via* a DNA vaccine, provided stronger immunity than separate or paired histone immunizations. This study also revealed that the DNA cocktail maintained protection against *L. major* reinfection (Carrion et al., 2008b). This plasmid DNA cocktail resulted in a specific Th1 response with enhanced IFN-γ production and low IL-4 levels, which contributed to control *L. major* infection in immunized mice (Iborra et al., 2004). Carneiro et al. also determined that BALB/c mice immunized with the plasmid DNA mixture expressing four different *L. infantum chagasi* nucleosomal histones (homologous immunization, DNA/DNA) was as effective as the combination of the DNA cocktail followed by the corresponding recombinant proteins and CpG as an adjuvant (heterologous vaccination, DNA/rprotein + CpG) (Carneiro et al., 2012). Furthermore, HISA70, a DNA vaccine composed of the *Leishmania* spp. histones (H2A, H2B, H3 and H4), A2 and HSP70, was able to shift the response away from the more undesirable Th2 pathway, producing high levels of IL-17 and IFN-γ in vaccinated mice. These observations correlated with a complete absence of parasites in spleens after *L. major* infection (Dominguez-Bernal et al., 2012).

DNA vaccines provide promising candidates in the pursuit of new vaccines against *Leishmania* spp. In most cases these vaccines have been administered by the intramuscular (i.m.) or subcutaneous (s.c.) route. However, intradermal (i.d.) vaccination may provide an opportunity to increase the immunogenicity of DNA vaccines (Engelke et al., 2015). Muscle and subcutaneous tissue contains relatively few dendritic cells (DCs) compared to the dermis and epidermis of skin, which are densely populated by different subsets of DCs. These competent antigen presenting cells (APCs) play a central role in developing adaptive immune responses (Banchereau and Steinman, 1998), undoubtedly important in *Leishmania* spp. infection control. The delivery of DNA vaccines to the dermis may therefore result in a superior immune response and/or an equivalent immune response at a lower vaccine dose (dose-sparing) (Kenney et al., 2004; Van Damme et al., 2009). Dose-sparing and the resulting reduction in unit costs could be of special interest for immunization programs in resource-poor areas.

Currently, the Mantoux method is the conventional technique for i.d. vaccination. This technique, used for tuberculosis and rabies immunization, involves insertion of a hypodermic needle into the skin at an acute angle. It is painful and administration requires trained healthcare personnel (Scheiblhofer et al., 2013). Furthermore, the reuse of contaminated needles is a risk, particularly where their supply is short. The introduction of minimally-invasive microneedle (MN) devices may confer a number of advantages for i.d. injection: (i) ease of use and disposal, (ii) painless administration, (iii) reduced risk of infection through minimal blood contact, and (iv) potential for self-administration by patients (Larraneta et al., 2016). In addition, simultaneous delivery of antigen at multiple skin sites using MN arrays could result in effective distribution of vaccines, leading to superior immune responses (Fehres et al., 2013). The coating of therapeutic antigen onto the outer surface MNs has been successfully used for vaccination purposes (Larraneta et al., 2016). Antigen delivery is achieved by diffusion of the antigen from the MN surface following their insertion into the skin, depositing the payload to a depth determined by the MN length and application process (Bal et al., 2010). Silicon MNs coated with a vaccine against malaria showed an efficacy comparable to conventional i.d. injection after challenge with live *Plasmodium berghei* sporozoites (Pearson et al., 2015). Kim et al. revealed that a single dose of an influenza vaccine coated onto stainless steel MNs produces superior immune response compared to i.m. vaccination (Kim et al., 2010). More recently, a solid MN patch coated with influenza virus induced higher Th1 responses, such as IgG2a isotype antibodies and IFN-γ, compared to those induced by i.m. injection (Kim et al., 2015). In another study, stainless steel coated MNs were used to vaccinate against human papillomavirus and stimulated strong protective immune responses (Kines et al., 2015).

This study aims to develop and test, for the first time, a minimally-invasive method of vaccination against *Leishmania* spp. by coating a solid MN device with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania infantum*. The study compares the immunogenicity of the MN approach with conventional s.c. and i.d. injection and evaluates the protective capacity in BALB/c mice that have been challenged with *L. major* parasites.

2. Material and methods

2.1. Animals and parasites

Female BALB/c mice (Harlan, Spain) weighing approximately 20 g and aged 8 weeks were kept under conventional conditions, with unrestricted access to food and water. Animals were housed in groups of 5, in controlled environmental conditions according to ethical standards approved by the Animal Ethics Committee of the University of Navarra and in strict accordance with the relevant European legislation.

The appropriate virulency of *Leishmania major* parasites (clone VI, MHOM/IL/80/Friedlin) was maintained by serial passages in BALB/c mice. Promastigotes were cultured in flasks at 26 °C in continuous stirred Schneider's modified medium (Sigma, USA), supplemented with 20% FBS and 40 mg/mL of gentamicin (Sigma, USA). *L. major* amastigotes were obtained from popliteal lymph nodes or lesions and, after transformation to the promastigote form, parasites were grown to the stationary phase (5–6 days) before they were used for subcutaneous murine inoculation at the base of the tail.

2.2. Preparation of DNA plasmids and recombinant proteins

The recombinant plasmids used in these experiments (Iborra et al., 2004) (pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 and pcDNA3-LiH4) were obtained using the endotoxin-free Giga-preparation Kit (Qiagen, Germany) following the manufacturer's instructions. Purified DNA was re-suspended in phosphate buffered saline (PBS, Gibco, USA) and stored at − 20 °C until use. Expression and purification of the His-tagged recombinant proteins (pQEH2A, pQE-H2B, pQE-H3 and pQE-H4) were performed as previously described (Iborra et al., 2004). After binding to a Ni-NTA agarose column (Qiagen, Germany), recombinant proteins were gradually refolded on the affinity column as described (Shi et al., 1997). Recombinant proteins were eluted with 0.3 or 0.5 M imidazole (Sigma) and dialyzed against PBS. Finally, proteins were passed through a polymyxin-agarose column (Sigma) in order to eliminate endotoxins (endotoxin-free, less than 30 ng/mg of recombinant protein) and kept at − 80 °C until use.

2.3. Microneedle fabrication

Solid MNs were fabricated at Cardiff School of Engineering by cutting needle structures from stainless steel sheets using wire electrical discharge machining (wire-EDM). The steel needles were then electro-polished to debur MN edges and sharpen the tips. The electro-polishing was carried out using a method described previously (Gill and