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Induction of protective cellular immune responses against experimental visceral leishmaniasis mediated by dendritic cells pulsed with the N-terminal domain of *Leishmania infantum* elongation factor-2 and CpG oligodeoxynucleotides

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ABSTRACT

Leishmania elongation factor 2 (EF-2) has been previously identified as a T_H1-stimulatory protein. In this study, we assayed the protective potential of the N-terminal domain of EF-2 (N-*Li*EF-2, 1–357 aa) that has been predicted to contain several overlapping MHC class I and II-restricted epitopes injected in the form of dendritic cell (DC)-based vaccine. Ex vivo pulsing of DCs with the recombinant N-*Li*EF-2 domain along with CpG oligodeoxynucleotides (ODNs) resulted in their functional differentiation. BALB/c vaccinated with CpG-triggered DCs pulsed with N-*Li*EF-2 were found to be the most immune-reactive in terms of induction of DTH responses, increased T cell proliferation and IL-2 production. Moreover, vaccination induced antigen-specific T_H1 type immune response as evidenced by increased IFN-γ and TNFα levels followed by a significant increase of nitrite (NO) and reactive oxygen species (ROS) in splenocyte cultures. Vaccinated mice showed a pronounced decrease in parasite load in spleen and liver when challenged with *L. infantum*, increased expression of Stat1 and Tbx21 mRNA transcripts versus reduced expression of Foxp3 transcripts and were able to produce significantly elevated levels of IL-2, IFN-γ and TNFα but not IL-10 compared to non-vaccinated mice. Both antigen and parasite-specific $CD4^+$ T and $CD8^+$ T cells contributed to the IFN-γ production indicating that both subtypes contribute to the resistance to infection and correlated with robust nitrite generation, critical in controlling *Leishmania* infection. Together, these findings demonstrated the immunogenic as well as protective potential of the N-terminal domain of *Leishmania* EF-2 when given with CpG-triggered DCs representing a basis for the development of rationalized vaccine against leishmaniasis.

1. Introduction

The leishmaniases are vector-borne protozoal diseases with different clinical manifestations in humans ranging from self-healing cutaneous leishmaniasis (CL), disfiguring mucocutaneous leishmaniasis (MCL) to fatal visceral leishmaniasis (VL) (Alvar et al., 2012). The causative agents are kinetoplastid parasites of the genus *Leishmania* that are transmitted by female sand flies to mammalian hosts, where they survive and multiply inside macrophages of skin, mucosa or visceral organs, such as spleen and liver, depending on *Leishmania* species and the immune status of the infected individual host (Kaye and Scott, 2011). Current treatment is based on chemotherapy, relying on a handful of drugs with serious limitations such as high cost and toxicity, difficult route of administration and lack of efficacy in some areas (Croft and Olliaro, 2011). Thus, vaccines could be the most important tools for prevention and control of leishmaniasis. Unfortunately, despite the enormous progress that has been made in vaccines design there is currently no acceptable vaccine against human leishmaniasis (Iborra et al., 2018). In contrast, there are several licensed veterinary anti-*Leishmania* vaccines in Brazil and Europe i.e. Leishtec® and Leishmune® - the second one was available till 2014 - and CaniLeish® and LetiFend®, respectively, that could provide the basis for the development of an

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efficient vaccine against human leishmaniasis (Ribeiro et al., 2018).

Active VL, the most severe form of the disease, is associated with impaired $CD4^+$ T_H1 response characterized by down-regulation of IL-12 and IFN-γ production along with up-regulation of T_H2 cytokines, such as IL-4 and IL-10, and strong humoral responses against parasites resulting in immune complexes formation and tissue destruction (Bhattacharya et al., 2016; Nylen and Gautam, 2010; Silva-Barrios et al., 2017). By contrast, immunity against VL requires co-operated actions between parasite-specific $CD4^+$ T_H1 and $CD8^+$ effector T cells, resulting to macrophage stimulation (Kaye and Scott, 2011). In particular, $CD8^+$ T cells along with $CD4^+$ T_H1 cells are involved in the clearance of primary infection through IFN-γ production that boosts the leishmanicidal capacity of macrophages via nitric oxide (NO) and reactive oxygen species (ROS) production (von Stebut and Tenzer, 2017).

Dendritic cells (DCs) also play a crucial role in the resistance against leishmaniasis through naive T cells priming. Moreover, DCs along with activated macrophages are able to restimulate and recruit effector and memory T cells making them dispensable for resistance to reinfection (von Stebut and Tenzer, 2017). This is achieved by efficiently presentation of various leishmanial antigens through enhanced expression of MHC class I and II molecules, as well as of co-stimulatory molecules i.e. CD40, CD80 and CD86, on their surface along with secretion of high amounts of IL-12. However, DCs require additional danger signals to become fully activated in addition to antigen uptake and T-cell interactions. It has been shown that DCs maturation into efficient antigen-presenting cells is achieved after pathogen-derived antigens or other molecules recognition through toll-like receptors (TLRs) (Takeda and Akira, 2004). Considering these features, manipulation of DCs offers an interesting approach in the fight against various pathogens including viruses, bacteria and protozoans as well as cancer (Bagirova et al., 2016). Regarding leishmaniasis, various antigens including live or attenuated *Leishmania* parasites, soluble *Leishmania* antigens, proteins and genes encoding for different *Leishmania* proteins along with different TLR agonists, such as CpG oligodeoxynucleotides (ODNs), have been used for in vitro stimulation of DCs (Bagirova et al., 2016). These ex vivo pulsed DCs were given as preventive vaccines against different forms of leishmaniasis. Their anti-*Leishmania* activity was attributed to the promotion of a protective T_H1-mediated cellular immune response in the presence or not of IgG2a-mediated humoral immune response (Bagirova et al., 2016). Moreover, it has been shown that co-administration of DCs with antimonial therapy or therapeutic injections of *L. donovani* antigen-pulsed DCs or DCs engineered to secrete IL-12 can lead to significant reduction of parasites in both liver and spleen in terms of immunotherapy (Ahuja et al., 1999; Ghosh et al., 2003).

Identifying effective immunogenic antigens is one of the biggest challenges in DC-based vaccine development. Ideally, any antigen able to induce the activation of effective immune responses against infection could be considered as a candidate vaccine molecule. Among different proteins tested as candidate vaccine antigens against leishmaniasis, elongation factor 2 (EF-2) has shown great promise since it is a cytoplasmic protein abundantly expressed in both promastigote and amastigote life stages of the parasite. It codifies a member of the GTP-binding translation elongation factor family and it is a relevant factor in protein synthesis (Hizli et al., 2013). Additionally, it was found with higher abundance in antimonials-resistant *L. donovani*, *L. panamensis*, *L. infantum* and *L. braziliensis* isolates and thus can serve also as a potential drug target (Biyani et al., 2011; Matrangolo et al., 2013; Moreira and Murta, 2016; Walker et al., 2012). Its immunogenic potential was shown for the first time using an amastigote cDNA expression library with peripheral blood monocytes (PBMCs) as well as in short-term T cell lines obtained from CL patients (Probst et al., 2001). In later studies restricted to isolated PBMCs and hamster lymphocytes, EF-2 has been identified through proteomics analysis as one of the soluble leishmanial proteins in a *L. donovani* sub-fraction ranging from 89.9 to 97.1 kDa that could induce T_H1 responses (Kumari et al., 2008a,b; Kushawaha et al., 2011). These results were confirmed by Joshi et al. in PBMCs and lymphocytes of treated *Leishmania* patients and hamsters, respectively, indicating that the partial 50 kDa domain of EF-2 as one of the most promising vaccine candidates against VL (Joshi et al., 2016).

Extending the above findings, in the present study, we identified through in silico analysis the N-terminal domain of *L. infantum* EF-2 ranging from 1 to 357 amino acids as the domain having more potentials to induce protective cellular immune responses. This domain was used in order to design a DC-based vaccine with enhanced immunogenicity and prophylactic potential against VL using the experimental model of disease in BALB/c mice. Given the fact that only mature DCs are effective antigen-presenting cells and this is accomplished by recognition of pathogen-derived antigens though TLRs, we triggered DCs with CpG ODNs, potent TLR9 ligands, in order to potentiate antigen-specific T_H1 and $CD8^+$ T cell responses. Subsequently, we aimed to evaluate the efficacy of CpG-triggered and N-terminal *Li*EF-2-pulsed DCs to induce protective immune responses after challenge with *L. infantum* in mice.

2. Materials and methods

2.1. Ethics statement

This study was conducted in strict accordance with the National Law 2013/56, which adheres to the European Directive 2010/63/EU for animal experiments and complied with the ARRIVE guidelines. All the protocols received prior approval by the Institutional Animal Bioethics Committee. All efforts were made to minimize animal suffering.

2.2. Mice, parasite culture, preparation of soluble Leishmania antigen and CpG ODNs

Studies were performed with female 6–8 weeks old BALB/c mice reared in the pathogen-free animal care facility at Hellenic Pasteur Institute. They were housed in a climatically controlled room receiving a diet of commercial food pellets and water ad libitum.

L. infantum (MHOM/GR/2001/GH8) strain was cultured in vitro in RPMI-1640 (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$, 100 U/ml penicillin, 10 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Paisley, UK) which will be referred as complete RPMI. For preparation of soluble *Leishmania* antigen (SLA) previously published protocol was followed (Papadopoulou et al., 1998).

The CpG ODNs 1826 (5′-tccatgacgttcctgacgtt-3′) that contain two unmethylated CpG motifs were purchased from Invivogen (San Diego, CA), resuspended according to manufacturer's instructions and stored at −20 °C until use.

2.3. In silico prediction of MHC class I and II binding epitopes

The protein sequence of *L. infantum* EF-2 (*Li*EF-2) was retrieved from the GeneDB protein database (LinJ.36.0190) and is composed by 845 amino acids (aa). The protein sequence was screened individually using SYFPEITHI (www.syfpeithi.de), NetMHC (http://www.cbs.dtu.dk/services/NetMHC/), NetMHCII (http://www.cbs.dtu.dk/services/NetMHCII/) and IEDB (http://tools.iedb.org) for the identification of potential epitopes able to bind to BALB/c MHC class I ($H2\text{-}K^d$, $H2\text{-}L^d$ and $H2\text{-}D^d$) and class II ($H2\text{-}IA^d/IE^d$) molecules. The cut-off score was adjusted ≥20.0 for SYFPEITHI (Rammensee et al., 1999) and a default prediction threshold (binding affinity < 500 nM) depicting accuracy > 85% was used for NetMHC (Andreatta and Nielsen, 2016; Nielsen et al., 2003) and NetMHCII (Nielsen and Lund, 2009; Nielsen et al., 2007). The MHCI and MHCII binding predictions were also made using the IEDB analysis resource Consensus tool (Kim et al., 2012) and the peptides predicted with percentile ranks below 1.0 or less than or equal to 10.0 for MHC class I and II, respectively, were chosen (Fleri