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Ferroportin-encapsulated nanoparticles reduce infection and improve immunity in mice infected with *Leishmania major*

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ABSTRACT

Inoculation of inbred mice by *Leishmania major* results in two different patterns. C57BL/6 mice display resistance against *L. major* but BALB/c mice show susceptibility to *L. major* with visceral infection, anemia and death. In this study, the effects of treatment of *L. major*-infected BALB/c mice with a ferroportin (Fpn)-encoding construct *via* nanoparticles were evaluated. A fragment encoding Fpn, a major regulator of iron homeostasis, was amplified and sub-cloned to a GFP expression vector to express Fpn-EGFP protein. This construct was incorporated in nanoparticles of alginate/chitosan polymers and orally administered to *L. major*-infected BALB/c mice. Blood hematocrit and iron, footpad size, parasite load and concentration of IFNG, IL4 and IL10 by ELISA were measured in the treated and untreated mice. The results indicated that the treated mice had significantly higher hematocrit and iron levels while exhibited significantly lower footpad size and parasite load measurements. Moreover, lower levels of IL4 and IL10 and higher ratios of IFNG/IL4 or IFNG/IL10 were shown in the treated, compared to the untreated mice. In conclusion, treating BALB/c mice infected with *L. major* with encapsulated Fpn-encoding construct in alginate/chitosan nanoparticles were shown to reduce the infection and improve anemia and immunity in the animal model of leishmaniasis.

1. Introduction

Iron is an essential trace element, required for growth and survival of almost all living organisms. In mammals, iron is required for erythropoiesis, transfer of oxygen, energy metabolism, cellular proliferation and cellular immune responses (Ganz and Nemeth, 2006; Munoz et al., 2009). Iron also plays a strong role in transferring essential components in the human body (Andrews, 1999). A close link between iron and the immune responses has been demonstrated. Most cells of the innate immunity can combat bacterial invasions *via* controlling their own iron contents. This process is mediated by the regulatory factors of two main proteins, known as hepcidin and ferroportin (Fpn) (Maisetta et al., 2010; Singh et al., 2011; Ward et al., 2011). Fpn, introduced by three independent researchers in 2000 (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000), is found in cells such as enterocytes, hepatocytes and macrophages where iron concentration is regulated (Ganz, 2005). Fpn exports iron across the duodenal enterocytes and other cell types into the blood circulation. Fpn interacts with hepcidin, an iron-regulatory hormone which regulates the absorption of iron in enterocytes, the recycling of iron through spleen macrophages and the release of iron from hepatocytes (Ganz, 2007; Oates, 2007). As an exporter of iron, the homeostasis of Fpn has a major influence on growth of microorganisms such as *Salmonella enterica* (Chlosta et al., 2006) or *Mycobacterium tuberculosis* (Van Zandt et al., 2008). Moreover, evidence suggests that strategies exploited by parasites such as *Leishmania* (*L.*) *amazonensis* enable them to acquire iron from their host macrophages for their survival and growth (Huynh and Andrews, 2008). Similar observations emphasize the importance of iron for growth of many pathogens including other *Leishmania* species (Ganz, 2009; Marquis and Gros, 2007). Bulks of reports indicate that the inoculation of *Leishmania major* into inbred mice leads to two different patterns. While resistant C57BL/6 mice develop a small lesion and control the infection within few weeks,

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BALB/c mice display susceptibility to the infection and develop metastatic, visceralized and fatal infections with an anemia (Muller et al., 1992; Reiner and Locksley, 1995).

Nanoparticle-based gene delivery systems have been developed during the last decade. Such non-viral gene delivery systems are considered suitable vehicles since they use polymers to deliver drugs or biomolecules which have advantageous properties such as the ease of production in large quantities, non-toxicity, protection of the drugs, rapid release kinetics and improved intestinal absorption (Koping-Hoggard et al., 2001; Pan et al., 2002). Compared to other polymers, chitosan has been identified as a particularly appealing gene vector in recent years (Mao et al., 2010). The formation of muco-adhesive chitosan coating around DNA has been shown to improve the mucosal absorption by the enterocytes (Illum, 1998). Therefore, this polymer could be considered as a perfect candidate for peptide and gene delivery to the mucosal tissues.

In the present study, the susceptible BALB/c mice were inoculated with *L. major* and the infected mice were simultaneously transfected with chitosan/alginate nanoparticles containing Fpn-encoding fragment. The pathogenicity of the infection was investigated in the treated mice by analyzing the lesion size and the parasite burden in the lymph nodes and the results were compared with data obtained from the untreated mice. Moreover, the profiles of three major cytokines of Th1 and Th2 responses were analyzed using the cultured lymph nodes of the treated and the untreated mice.

2. Materials and methods

2.1. Materials

Polymers for preparation of the nanoparticles included low molecular weight chitosan (Sigma–Aldrich Co., Germany), sodium alginate (Sigma) and calcium chloride (Sigma). L-Gluthamine, penicillin, streptomycin, fetal bovine serum (FBS; Biosera, South Korea) and ^{3}H-thymidine (Amersham, UK) were used in culture media and PolyFect transfection reagent (Qiagen, Germany) was used for transfection.

2.2. Mice and parasite

Female BALB/c mice, 5–6 weeks old and bred at the animal facility of Production Complex of Pasteur Institute of Iran at Karaj, were used. Mice were inoculated in their footpad with stationary phase promastigotes of *L. major* reference strain of Iran (MRHO/IR/75/ER) for vaccine studies. Treatments of the mice were in accordance with the national guidelines and the approval for the study was given by Ethics Committee of Pasteur Institute of Iran.

2.3. Methods

2.3.1. RNA extraction, cDNA synthesis, cloning and expression

RNA extraction, cDNA synthesis and cloning were carried out as described previously (Rafiee et al., 2012). Briefly, total RNA was extracted from intestinal tissue of Indian zebrafish by RNX™ reagent (Cinnagen, Iran). The extracted RNA was used for cDNA synthesis by High Fidelity Prime Script™ RT-PCR kit (Takara, Japan) according to the manufacturer's protocol. The amplicon (1714 bp) was initially treated with *Taq* DNA polymerase at 72 °C for 10 min and then cloned into TOPO TA Cloning plasmid (Invitrogen, CA, USA). Following the extraction of recombinant plasmid from the transformed TOP10 *Escherichia coli* cells by AccuPrep® Plasmid Mini Extraction Kit (Bioneer, South Korea), the plasmids encoding Fpn protein were sub-cloned in pEGFP-N1 expression vector (Clontech, CA, USA) with SV40 promoter to construct the final recombinant plasmid pEGFP-ZFpn. The constructs were sequence-confirmed by SeqLab laboratories (Germany). To confirm the expression of Fpn protein, pEGFP-ZFpn construct was transfected into HEK 293T cell line by PolyFect transfection reagent (Qiagen). The cells emitted green fluorescence *in vitro* under the immunofluorescent microscope, 48 h post-infection.

2.3.2. Preparation and characterization of nanoparticles for treatment of mice

Nanoparticles for each treatment of mice were prepared from alginate/chitosan (alg/chi) polymers by pre-gel preparation method with a few modifications (Gazori et al., 2009). In practice, optimum conditions for preparation of the nanoparticles with appropriate size (100–400 nm) for absorption by the digestive tract were determined based on to the above reference and our experiments in which the N/P ratio (*i.e.* the stoichiometric ratio of the positive charge of the chitosan amine groups (N), to the negative charge of the plasmid phosphate groups (P)) was considered as 5, alg/chi ratio as 1, $CaCl_2$/alg ratio as 0.2 and pH at 5.3. Initially, 0.1% (w/v) stock solutions of sodium alginate, calcium chloride and chitosan were prepared in 50 ml total volumes using deionized water as solvents and filtered (0.22 μm). In case of chitosan stock solution, the solvent contained 1% (v/v) acetic acid. An alginate/calcium chloride solution was then prepared in 0.2 (v/v) ratio while 702 μl calcium chloride stock solution was added drop-wisely to 3510 μl sodium alginate stock solution. Afterward, a mixture of 270 μg of each constructed plasmid with 3.51 ml of the chitosan stock solution was prepared and after a brief vortex, the mixture was incubated at room temperature for 10 min. Following bringing the volume of this mixture to 10 ml by deionized water, the diluted mixture was added slowly to the above alginate/calcium chloride solution. At the same time, in order to prevent the particles from getting bigger than the required dimensions, the total volume of the mixture was slowly brought to 1.5 l while stirring for 30 min at room temperature. This mixture was then concentrated by centrifugation ($3220 \times g$ for 30 min at 20 °C) in an Amicon 8050 device with 100 kDa filter (Millipore, MA, USA) to separate free polymers from the nanoparticles. Morphological characteristics of the nanoparticle were examined by scanning electron microscopy (LEO1455 VP, 10 KV Cambridge, UK). The particle size and zeta potential were detected using Scattering Particle Analyzer and Malvern zeta sizer ZS series (Malvern, Co., UK), respectively.

2.3.3. Treatment of the infected mice with pEGFP-ZFpn

Three groups of BALB/c mice ($n = 18$ per group), infected with 1×10^5 stationary phase promastigotes in 50 μl PBS into the hind right footpad were used for treatment as follows: (i) the first group received alg/chi nanoparticles containing pEGFP-ZFpn construct, simultaneously with infection; (ii) the second group received alg/chi nanoparticles containing pEGFP plasmid alone (henceforth named the plasmid control group) simultaneously with infection; (iii) the third group received nothing and was used as *L. major* infection control. The treatments for the first and second groups were administered in 5 time-points by complete delivery of 1 ml of the nanoparticle solutions containing 30 ng of the appropriate construct to each mouse stomach by oral gavage at day 0, followed by weeks 1, 2, 3 and 4, post-infection for a total dose of 150 ng per mouse at the end of the treatments.

2.3.4. Determination of hematocrit and serum iron

Hematocrit was determined by centrifugation of whole blood from mice ($n = 6$ per group) infected and treated as described in Section 2.3.3, at weeks 3, 4, 5 and 6 post-infection in 75 mm NRRI/E