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Vaccination with Nucleoside hydrolase (NH36) *of L.(L.) donovani* or its C-terminal portion (F3) in formulation with saponin prevents the increase of the proportions of spleen dendritic cells in murine experimental visceral leishmaniasis

Dirlei Nico, Alexandre Morrot, Clarisa Beatriz Palatnik-de-Sousa*

Insituto de Microbiologia Paulo de Góes, Universidade Federal de Rio de Janeiro, Avda Carlos Chagas 373. Cidade Universitaria, Ilha do Fundão,Rio de Janeiro, CEP 21941-902, Brazil.

Abstract

Visceral leishmaniasis is a chronicand lethal parasite disease against which no human vaccine is available.Hepato-splenomegaly and a progressive suppression of the cellular immune response are among its most important clinical signs. The characteristic cellular immunosupression was described as being mediated in part, through the spatial segregation of dendritic cells (DCs) and T cell lymphocytes due to altered frequencies and migration capabilities of DCs. In this investigation, we measured the spleen/body relative weight, the spleen parasite load and the total counts of spleen DCs of C57BL6 mice infected with *Leishmania chagasi*. All the variables achieved their maximum at 30 days after infection. We detected in infected animals a 5.08 fold increase of spleen relative weight, a 19.6 fold increase of parasite load and a 4.55 increase of total DCs counts, when compared to naïve controls. We further analysed the efficacy of the NH36 and F3 vaccines formulated in saponin in prevention of visceral leishmaniasis. When compared to the infected controls, both vaccines determined strong protection. The F3 vaccine induced the highest efficacy showing 95% and 49% reduction the parasite load and splenomegaly, respectively. The NH36 vaccine, on the other hand, developed a slightly lower but still significant protection reducing by 87% the parasite load and by 39% the spleen relative weight. Both vaccines also prevented the increase in total counts of DCs with no significant difference between them (36% by the NH36 and 26% by the F3 vaccine). Our results suggest that vaccination against murine visceral leishmaniasis with the NH36 vaccine can prevent the development of the disease by preventing the DCs dysfunction-related immunosupression. Additionally, they disclose the potential use of the NH36 C-terminal moiety, the F3 peptide for optimization of the vaccine efficacy.

Keywords: Visceral leishmaniasis; *Leishmania donovani*; *Leishmania chagasi*; C57BL6 mice; spleen/body relative weight; splenomegaly; dendritic cells

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*Corresponding author. Tel.: +55-21-25626742; fax:+55-21-25608344

E-mail address: immgcpa@micro.ufrj.br

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Visceral leishmaniasis is a chronic parasite disease which occurs as a canine zoonosis caused by *Leishmania infantum/ L. chagasi* in the Americas, the Middle East, Central Asia, China and the Mediterranean and as an anthroponosisprovoked by *L. donovani* in India and Central Africa. No human vaccine against VL is available [1]. Annually, 0.2 to 0.4 million new human cases of VL are registered [2]. More than 90% of them occur in India, Bangladesh, Sudan, South-Sudan, Ethiopia andBrazil [2].The human disease is lethal if not treated early after the onset of clinicopathological abnormalities that include: malaise, anaemia, cachexia, hypergammaglobulinaemia, hepato-splenomegaly and progressive suppression of the cellular immune response [1].

The geographical encounter between DCs and T cells in the T cell areas of secondary lymphoid organs determines the success of cellular immunity after infection. In spleen, DCs should migrate from the marginal zone (MZ) to the periarteriolar sheath (PALS), to meet T cells in response to constitutively express chemokine gradients of CCL21 and CCL19. In contrast to that, a defective DC migration plays a major role in the pathogenesis of VL. Indeed, the characteristic cellular immunosupression of VL is mediated in part, through the spatial segregation of DCs and T cells [3].

In the BALB/c and C57BL/6 mice models of VL, an increase in the absolute numbers of spleen CD11c+ DCs during the progression of *L. donovani*infection was described [3]. During this period, the parasite number increased and severe splenomegaly became evident [3]. Furthermore, DCs of *L. donovani* infected mice showed a defective migration to the periarteriolar lymphoid sheath (PALS) which prevented the antigen presentation to T cells and the subsequent T cell activation and optimal induction of immune response against the parasite [3].

We developed the first vaccine licensed against canine VL (Leishmune®) which is composed of the FML (Fucose mannose ligand) antigen of *L. donovani* and QS21 and deacylated saponins of *Quillaja saponaria*. The vaccine induces 98% protection to canine VL in healthy dogs [4], reduces the human and canine incidence of disease [5], the number of exposed parasites to insect vectors [6] and is a transmission blocking vaccine [7, 8]. The main antigen of FML is the Nucleoside hydrolase of *Leishmania donovani* (NH36) which protects mice and dogs from VL [revised in 8]. Vaccination with the NH36 C-terminal moiety, the F3 peptide, formulated with saponin, induced the strongest protection (88-97%) against VLthat was mediated by a CD4+-T cell driven immune response [9].

As a first step in the evaluation of the impact of vaccination with NH36 or F3, in prevention of the defective distribution and function of spleen´s DCs, we here assessed the variation of splenomegaly, parasite load and absolute numbers of CD11c+ DCsin spleens of C57BL6 vaccinated mice that were further infected with *L. chagasi*.

Eight week old C57BL6 females were vaccinated with three doses of 100 µg of NH36 or the F3 peptide obtained as described before [9] and formulated in saponin, through the *sc* route, with weekly interval. On week four,the intradermal response against *L. donovani* promastigotes was evaluated and a challenge with 3 x 10^7 amastigotes of *L. chagasi* obtained from infected hamsters was performed by intravenous injection via the tail vein. Mice were sacrificed at day 15, 30 and 45 after infection, for evaluation of splenomegaly (% of spleen weight/ body weight), parasite load determination in Giemsa stained spleen smears (Leishman Donovan units-LDU= number of amastigotes/ 500 spleen cells x spleen weight in mg) and total counts of spleen DCs. To isolate the DCs spleen were harvested aseptically and disrupted with DNAse and collagenase. The cell suspensions were counted in haemocytometer and labelled with anti-CD11c (DCs surface marker)-magnetic beads. DCs were further separated by chromatography in magnetic columns, recovered by mechanical force, centrifuged, incubated with CD11-c-FITC antibody and counted.All mouse studies followed the guidelines set by the National Institute of Health, USA, the EU Directive 2010/63/EU and the Institutional Animal Care and Use Committee approved the animal protocols (Biophysics Institute-UFRJ, Brazil, protocol IMPPG-007).We use the Kruskall Wallis and Mann Whitney non-parametrical tests for comparison of means.

The respective evolution of the spleen/body relative weight, spleen parasite load and total counts of spleen DCs along the time are summarized, respectively in Figure 1 A, B and C. When compared to normal uninfected mice (Figure 1A-C), the splenomegaly, the number of parasites (LDU units) and the number of DCs of infected mice were significantly increased and developed exponential slopes from day 15 to day 30 followed by a plateau detected from day 30 to day 45 after infection. Our data slightly differ from the results obtained by Ato *et al*. [3]who used the same mice model but *L. donovani* infection. Ato *et al*. [3] expressed the relative splenomegaly, as the

ratio of the spleen weight to body weight of infected mice divided by that of naïve mice. Despite they described ratios close to 1on day 14, the peak achieved on day 28 was lower (1.7) than the one we detected in *L. chagasi* infected mice (5.08 = 3.41 in infected/0.67in naïve mice) suggesting a more advanced pathology caused by infection with *L. chagasi*. Ato *et al*. (2002) did not follow up the infection up to 45 days [3].In agreement with the higher levels of spleen relative weight, the numbers of spleen LDU achieved after *L. chagasi* infection (39.2 at day 15 and 86.2 at day 30) (Figure 1 B) were much higher than those reported after *L. donovani* infection (2 at day 15 and 10 at day 30) [3]. Indeed, the *L. chagasi* parasite loadshowed a 19.6 and 8.62 respective fold increases, for day 15 and day 30, respectively,above those achieved in the *L. donovani* model [3].Additionally, the total number of DCs was higher in our *L. chagasi* model (45.55 x 10^6 DCs at day 30)(Figure 1 C) than in the *L. donovani* model (10 x 10^6) [3].Ato *et al*., used an infective challenge only 1.5 fold lower (2 x 10^7 amastigotes of *L. donovani*) [3] than the one we used in this *L. chagasi* model of infection (2 x 10^7 amastigotes). Therefore, the 5.08 fold increase in spleen relative weight, 19.6 fold increase in parasite load and 4.55 increase in total DCs counts could not attributed to the higher inoculum but instead, to moreaggressive aspects of the infection by *L. chagasi.*

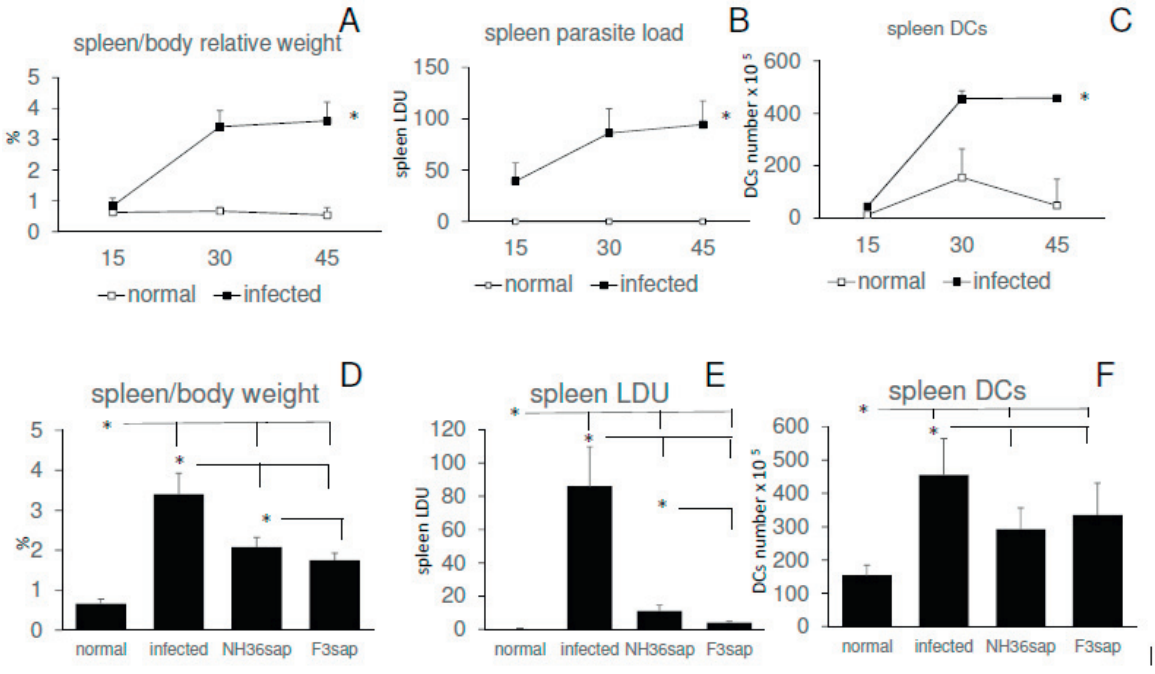

Fig. 1. Variation of the (A) spleen/body relative weight, (B) spleen parasite load and (C) total counts of DCs in spleen, along the time (days). Effect of the preventive vaccination with NH36sap and F3sap on the (D) spleen/body relative weight, (E) spleen parasite load and (F). Results of two identical experiments with n=7 mice per group in each experiment.

We further analysed the efficacy of the NH36 and F3 vaccines formulated in saponin (NH36sap and F3sap) in prevention of the increase in spleen relative weight, spleen parasite load and increase of DCs total provoked by the challenge with *L. chagasi*(Figure 1 D-F). The comparison was performed at day 30 after infection, when these deleterious effects showed their highest incidence (Figure 1 A-C). As expected, the infection enhanced the levels of spleen/body relative weight (Fig. 1D), and DCs counts (Fig. 1 F) above those exhibited by naïve controls. Additionally, when compared to the infected controls, both vaccines determined strong protection.A significantly higher efficacy was developed by the F3 vaccinewhich reduced by 95% the parasite load (Fig. 1 E) and by 49% the splenomegaly (Fig. 1 D). The NH36 vaccine, on the other hand, developed a slightly lower but still significant protection reducing by 87% the parasite load and by 39% the spleen relative weight. Both vaccines also prevented

the increase in total counts of DCs with no significant difference between them (36% by the NH36 and 26% by the F3 vaccine) (Figure 1 D-F).

Our results of the *L. chagasi* model support the previous report of Ato *et al*., [3] who described the increase of the frequency of total DCs in spleens of *L. donovani* infected mice during the period of increase of parasite load and pathology. Furthermore, we showed that the severity of parasitic and clinical signs is higher during the infection caused by *L. chagasi*. The NH36 and its C-terminal moiety, the F3 peptide, showed high efficacy in vaccination against this very severe infection. The F3 peptide was identified before as the domain of the NH36 of *L. donovani* responsible for its immunogenicity and protective efficacy against murine VL [9]. Immunization with F3 exceeds in 36.73 ± 12. 33% the protective response induced by the cognate NH36 protein. Increases in IgM, IgG2a, IgG1 and IgG2b antibodies, CD4+ T cell proportions, IFN-γ secretion, ratios of IFN-γ/IL-10 producing CD4+ and CD8+ T cells and percents of antibody binding inhibition by synthetic predicted epitopes were detected in F3 vaccinated mice [9]. The increases in DTH and in ratios of TNFα/IL-10 CD4+ producing cells were however the strong correlates of protection which was confirmed by *in vivo* depletion with monoclonal antibodies, algorithm predicted CD4 and CD8 epitopes and a pronounced decrease in parasite load (90.5-88.23%; p=0.011) that was long-lasting [9]. In this investigation we further demonstrate that, in the more susceptible C57BL6 mice model, protection induced by the NH36 vaccine is also directed to its F3 peptide. In addition, we showed that the modulation of the immunoprotective response against VL induced by F3 is related also to the prevention of the increase of the total numbers of DCs in spleens. The analysis of the migration capabilities of the spleen DCs is under progress.

Acknowledgements

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