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Leishmania infantum: Mixed T-helper-1/T-helper-2 immune response in experimentally infected BALB/c mice

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Abstract

The main goal of the present study was to characterise the course of infection and immunological responses developed by *Leishmania infantum* infected BALB/c mice. Parasite load was determined by Real-time TaqMan® PCR while cytokine and Immunoglobulin G (IgG) production were assessed by ELISA. *Leishmania* DNA was detected in spleen and liver as soon as day 1 post-inoculation (pi) and the parasitism was sustained until the end of the experiment. The cytokine kinetics in spleen and liver was generally associated with the oscillations of parasite load. Overall, it was not observed a distinct Th1 or Th2 pattern of cytokine production during the time of experiment. The infected mice developed a mixed immune response, with concomitant production of IFN-γ, IL-4 and IL-10, both in spleen and liver, and both IgG isotypes. However, our results suggest that, compared to liver, the spleen is more susceptible to *L. infantum* infection.
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Index Descriptors and Abbreviations: *Leishmania infantum*; Mice; Parasite load; Immune response; Spleen; Liver; pi, post-inoculation; Th, T-helper; min, minutes; s, seconds; DNA, deoxyribonucleic acid; IgG, immunoglobulin G; IL, interleukin; IFN, interferon; TGF, transforming growth factor; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay

1. Introduction

The genus *Leishmania* includes around 30 taxa that infect mammals, causing various types of disease. Human leishmaniasis has a wide clinical spectrum, from the naturally healing localised cutaneous lesion to the potentially fatal visceral leishmaniasis, in which parasites disseminate from the site of infection and invade organs and tissues of the mononuclear phagocytic system (Moll, 1993). In canine visceral leishmaniasis, it was proven a strong association between the number of parasites, the severity of the clinical signs, and the intensity of the immune response (Campino et al., 2000). In murine models of cutaneous leishmaniasis, it is widely accepted that resistance to the disease is correlated with expansion of T-helper-1 (Th1) cells and production of IFN-γ, while susceptibility is associated with the development of T-helper-2 (Th2) cells, resulting in IL-4 and IL-10 production (Heinzel et al., 1989; Gumy et al., 2004). However, this Th1/Th2 clear-cut role has not been evident in visceral leishmaniasis (Honore et al., 1998).

The Th1/Th2 balance defines different immune pathways that affect most, if not all, cells of the immune system, controlling the parasite burden observed at the different organs of *Leishmania* infected mice and the pattern of cytokine production can influence the susceptibility or resistance to infection. The main goal of the present study was to characterise the course of infection and immunological responses developed in organs and tissues of the mononuclear phagocytic system of *L. infantum* infected BALB/c mice.

2. Materials and methods

2.1. Mice and infection

Female BALB/c mice were purchased from Harlam Interfauna Ibérica SL (Barcelona, Spain) and housed at the

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Instituto de Higiene e Medicina Tropical (Lisbon), under stable climatic and dietary conditions. In each of the three experiments performed, 45 female BALB/c mice with 4–6 weeks of age were inoculated by i.p. route with 10^7 promastigotes of *L. infantum* MON-1 (MCAN/PT/94/IMT205) in 0.1 ml of saline solution (Group I). Mice from the Control Group (Group C) were inoculated with 0.1 ml of saline solution only. Animal manipulation was conducted according to the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences.

2.2. Sample collection

Five mice from Group I and three mice from Group C were sacrificed at days 1, 3, 7, 14, 28, 42, 56, 70 and 84 post-inoculation (pi). At each time point, biological samples (spleen, liver and peripheral blood) were "pooled", according to each group. The samples were pooled in order to achieve the optimal lymphocyte concentration to use in cytokine assays.

2.3. Parasite load

Parasite load determination was accessed by Real-time TaqMan® PCR, as previously described (Rolão et al., 2004). Briefly, 10 mg of spleen and liver from each sacrificed mouse were pooled according to each group (50 mg of each organ per group). Biological samples were then processed for DNA extraction (PCR-template Preparation kit, Roche Diagnostics GmbH, Mannheim, Germany) and quantification (GeneQuant, Amersham Biosciences, Buckinghamshire, United Kingdom). All DNA samples were amplified for parasite load determination.

Mass cultures of *L. infantum* promastigotes spiked with mouse DNA were used to construct the standard curve. Parasites were counted using a Neubauer hemacytometer (mean value of 10 counts) and cultures were diluted in series of 10-fold dilutions ranging from 10^5 to 1 parasite. The diluted parasite cultures were then processed for DNA extraction, as above (PCR-template Preparation kit, Roche) and mixed with DNA from healthy mice.

Primers and probe were designed from a kinetoplastid DNA (kDNA) minicircle sequence of *L. infantum* (Genebank A/N AF169140). DNA samples were analysed using the following oligos (Applied Biosystems, Foster City, California): primers LshNRf (forward, 5′-GGTTAGCC GATGGTGGTCTT-3′), LshNRr (reverse, 5′-GCTATA TCATATGTCCAAGCACTTACCT-3′) and TaqMan® internal probe LshNRp (5′-ACCACCTAAGGTCAACC C-3′). PCRs were performed in the ABI PRISM® 5700 System (Perkin-Elmer, Applied Biosystems). Two microlitres of each DNA sample were added to a reaction mix consisting of 10 μl of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) and 1 μl of unlabeled primers and TaqMan® MGB probe (FAM™ dye-labelled) mix (Applied Biosystems), in a final volume of 20 μl. All samples were performed in duplicate and optimal conditions for PCR amplification were: 95 °C for 10 minutes (min) and 40 cycles of 95 °C for 15 seconds (s), and 60 °C for 60 s. The threshold of detection was automatically set at 10 times the standard deviation above the mean of baseline emission, representing the background level calculated from cycles 6 to 15. To overcome possible quantification errors due to variations in tissue weighing, results were normalized to the DNA concentration of the samples, as described elsewhere (Rolão et al., 2004).

2.4. Immune response

Spleen and liver samples were homogenized and processed for mononuclear cell separation by means of Ficoll (Maluish and Strong, 1986) and Percoll (Goossens et al., 1990) gradients, respectively. One hundred and fifty microlitres of cell suspension (2×10^6/ml) was added to each well of micro-ELISA U-bottom plates and left alone or incubated with crude *Leishmania* antigen (10 μg/ml per well), in a final volume of 200 μl/well. After incubation in a humidified chamber for 96 hours (h) at 37 °C/5% CO_2, supernatants were collected and processed for cytokine quantification by "sandwich" ELISA (BD Pharmingen, San Diego, USA), namely IFN-γ, IL-12, IL-4, IL-10 and TGF-β.

Peripheral blood samples were centrifuged at 425*g* for 15 min at room temperature. Sera were removed and processed for IgG1 and IgG2a detection by ELISA (Caltag Laboratories, Burlingame, USA).

2.5. Statistical analysis

The correlation between the kinetics of parasite load and cytokine production was determined by Spearman's rank correlation analysis.

3. Results

Presented results are representative of the three experiments performed.

3.1. Parasite load

Leishmania DNA was detected in all time-points after inoculation in infected group (Fig. 1). Parasite load reached higher values in spleen than in liver. It peaked at days 7 and 56 pi (maximum) in the spleen, and reached a maximum at day 84 pi in the liver. Parasite DNA was not detected on samples from Group C.

PCR efficiency of amplification was 95.82% and the inter-assay coefficient of variance, calculated from four replicates of the same 10-fold DNA dilutions performed on separated runs, was de 1.13, 2.37, 4.09, 0.27, 0.91, and 1.89% for 10^5, 10^4, 10^3, 10^2, 10, and 1 parasite, respectively. Intra-assay coefficient of variation, calculated from a single run of four duplicated 10-fold dilution samples on the same

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