Studies in a co-infection murine model of *Plasmodium chabaudi chabaudi* and *Leishmania infantum*: interferon-γ and interleukin-4 mRNA expression

Cláudia S Marques, Nuno Rolão, Sónia Centeno-Lima*, Hélder Lousada, Carla Maia, Lenea Campino, Virgílio E do Rosário*, Henrique Silveira*/+

Unidade de Leishmanioses *Unidade de Malária, Centro de Malária e Doenças Tropicais, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 96, 1349-008 Lisboa, Portugal

This work aimed to study the T helper type 1/2 (Th_1/Th_2) cytokine profile in a co-infection murine model of Plasmodium chabaudi chabaudi *and* Leishmania infantum. *Expression of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) was analyzed, in spleen and liver of C57BL/6 mice, by reverse transcriptase-polymerase chain reaction. High levels of IFN-γ expression did not prevent the progression of* Leishmania *in co-infected mice and* Leishmania *infection did not interfere with the Th_1/Th_2 switch necessary for* Plasmodium *control. The presence of IL-4 at day 28 in co-infected mice, essential for* Plasmodium *elimination, was probably a key factor on the exacerbation of the* Leishmania *infection.*

Key words: co-infection - *Plasmodium* - *Leishmania*

Malaria and leishmaniasis are currently two of the major causes of mortality and morbility among human parasitic infections, with an enormous social and economic impact. Human cases of concomitant malaria, *Plasmodium falciparum* and leishmaniasis, *Leishmania donovani* have been reported (Nandy et al. 1995, Sah et al. 2002).

In 1980s, Coleman and his colleagues developed several studies of interactions betweeen *L. mexicana* and *P. yoellii* in the murine model, that demonstrated an elevation of severity and susceptibility to the diseases during co-infection. However, they did not investigate the immunological mechanisms involved (Coleman et al. 1988a, b, c). The *P. chabaudi chabaudi/L. infantum* murine model could reveal parasite-specific immune responses and mechanisms of regulation of T-cell subsets during a concomitant infection. Regulation of immune response by $CD4^+$ T helper (Th) cells and cytokine products have in fact been shown to be essential during both *Plasmodium* and *Leishmania* infections.

P. c. chabaudi strain AS infection, in C57BL/6 mice strain, induces a sequential immune response by the two major subsets of $CD4^+$ Th cells (Langhorne 1989). The acute primary parasitaemia, with a peak level around day 7, is resolved by the Th_1 type response and related cytokines, mainly interferon-gamma (IFN-γ) (Langhorne 1989, Zhong & Stevenson 2000). The control and clearance of chronic subpatent blood infection during the second phase of infection are related with the Th_2 pattern of cytokine production, namely interleukin-4 (IL-4), that is crucial for B cells maturation and subsequent antibody release (Langhorne 1989, Taylor-Robinson & Phillips 1994).

+Corresponding author. E-mail: hsilveira@ihmt.unl.pt
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The murine malaria *P. c. chabaudi* is a recognized model for analyzing the acquired immunity to the asexual erythrocytic stages of malaria parasites especially for *P. falciparum* infections of humans (reviewed by Taylor-Robinson 1998).

In murine models of leishmaniasis, it is widely assumed that resistance to the disease is correlated with the production of IFN-γ by Th_1 cells, while susceptibility is associated with Th_2 response, resulting in IL-4 production (Heinzel et al. 1989, Gumy et al. 2004), although in visceral leishmaniasis this clear-cut role of Th_1/Th_2 is less evident (Honoré et al. 1998).

The main goals of this study were to evaluate whether the initial immune responses to *P. c. chabaudi* could be blocked, when mice were co-infected by *L. infantum*, thus preventing the Th_1/Th_2 switch, and to investigate if resistance or susceptibility to *L. infantum* could be modulated by a previous activation of Th_1 cells. In order to clarify these questions, we analysed mRNA expression of IFN-γ and IL-4, both in spleen and liver of co-infected C57BL/6 mice, by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

C57BL/6 female mice, 5 to 8 weeks old were purchased from Harlam Interfauna Ibérica SL (Barcelona, Spain) and kept in the IHMT animal facilities, according to the European Union requirements (86/609/CEE) recognized in Portuguese law (DR DL129/92 and Portaria 1005/92). *P. c. chabaudi* AS was obtained from the Institute of Cell, Animal and Population Biology, University of Edinburgh and maintained by continuous blood passage. *L. infantum* MON-1 amastigotes were isolated from spleen and liver of infected golden hamsters (*Mesocricetus auratus*), according to Cenini et al. (1989).

Mice were divided into the following groups: (i) intraperatonial inoculation with isotonic saline solution, (ii) intraperatonial inoculation with 10^6 *P. c. chabaudi* AS

parasitised red blood cells ($pRBC_s$), (iii) intraperatonial inoculation with 10^6 *L. infantum* (MON-1) amastigotes, and (iv) intraperitonial inoculation of 10^6 *P. c. chabaudi* AS $pRBC_s$ and seven days later with 10^6 *L. infantum* (MON-1) amastigotes.

Three mice of each group were sacrificed by cervical dislocation and fragments of spleen and liver were collected at days 0 (*P. c. chabaudi* inoculation day), 7 (*L. infantum* inoculation day), 14, 28, 35, and 56.

DNA extraction from sample tissue (spleen and liver) was carried out according to Boom et al. (1990) and PCR amplification for the diagnosis of infection caused by *L. infantum* was performed using primers based on a complete DNA sequence of *L. infantum* kinetoplast minicircle (Cortes et al. 2004).

L. infantum load was monitored by determination of parasite density in spleen and liver of groups 3 and 4, using the limiting dilution method (Buffet et al. 1995).

P. c. chabaudi parasitaemia was monitored and determined during the course of infection of groups 2 and 4 by optical microscopic observation of thin blood smears made from mice tail snips and stained with Giemsa.

Fragments of spleen and liver collected for RNA extraction were stored in liquid nitrogen until use. Extraction of total RNA was performed with TRIzol® reagent (Invitrogen-Life Technologies, New York, US), according to the manufacturer's protocol. Synthesis and amplification of cDNA were performed as described by Centeno-Lima et al. (2002). Specific primers for murine IFN-γ, IL-4 and hypoxanthine-guanine phosphoribosyl transferase (HPRT) as an internal standard were used (Centeno-Lima et al. 2002).

After electrophoresis on 1% agarose gel, DNA was transferred to a positively charged nylon membrane by common blotting procedures. Blots were hybridised with a specific digoxigenin (DIG) labelled oligoprobe for each cytokine and HPRT as previously described by Centeno-Lima et al. (2002). The oligonucleotide probe-labelling protocol was performed according to the manufacturer's protocol (Roche Applied Science). Hybridization chemiluminescent signals were quantified and converted to optical density units (OD) using Phoretix 1D plus software (Phoretix International). Results were normalized for the relative quantity of cytokine mRNA (arbitrary units) by dividing the OD of the cytokine by the corresponding OD of HPRT, for each sample. Data analysis was carried out using Statistical Package for Social Sciences 11.0 Program (SPSS®). The non-parametric Mann-Whitney rank sum test was used to assess statistical significance for values of $P < 0.05$. The results of the following groups were statistically compared at days 14, 28, 35, and 56 after *P. c. chabaudi* infection: group 2 vs group 4 and group 3 vs group 4.

RESULTS

Mice infected with *P. c. chabaudi* alone (group 2) showed an expected peak of parasitaemia at day 7 (37%) that was followed by a drastic reduction of parasitaemia. Two recrudescences were noted at days 16 and 30 before total eradication of blood parasites, verified by optical microscopy observation (Fig. 1).

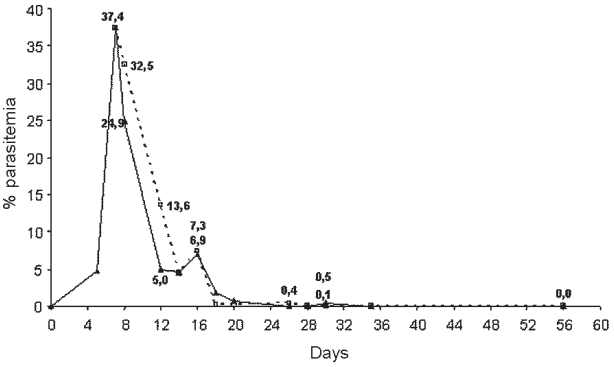

Fig. 1: course of blood stage *Plasmodium chabaudi chabaudi* infection in C57BL/6 mice infected with 10^6 *P. c. chabaudi* parasitized red blood cells (pRBC) (full line) and in C57BL/6 mice co-infected with 10^6 *P. c. chabaudi* pRBC's and 7 days later with 10^6 *Leishmania infantum* amastigotes (dotted line). Parasitaemia was determined after observation of thin blood smears stained with Giemsa and by counting total and infected red blood cells in five microscopic fields. The total number of red blood cells was equal or superior to 2000. Data represent the geometric mean of 3 mice per group, from the most representative experiment.

All cultures from the spleen and liver samples, collected in mice inoculated with *L. infantum* only (group 3) were negative throughout the experiment, however the presence of *Leishmania* DNA was confirmed in both organs by PCR.

The course of blood stage *P. c. chabaudi* infection in co-infected mice (group 4) was similar to what was described for group 2, though mice from group 4 reached higher levels of parasitaemia until day 14, exhibiting recrudescence at days 16, 26, and 30 (Fig. 1).

Spleen from co-infected mice showed positive promastigote cultures at day 56, with a parasite density mean value of 8×10^4 parasites per g of organ, as determined by the limiting dilution method. The high *Leishmania* parasite density observed at day 56 was accompanied by an evident splenomegaly on all three mice.

The IFN-γ expression in the spleen and liver of mice infected with *P. c. chabaudi* only, reached a peak at day 7 that coincided with peak of parasitaemia. The expression level of IFN-γ decreased afterwards (Fig. 2a, b).

Co-infected mice showed elevated IFN-γ expression at day 56, both in the spleen and liver, and significant differences were noted in the liver between this group and mice infected *P. c. chabaudi* only (Fig. 2a, b).

In mice infected with *L. infantum* only the IFN-γ expression levels were generally lower than in co-infected mice and in spleen, IFN-γ expression was only detectable at day 28. Significant differences between group 3 and group 4 were observed in the liver at days 14 and 56 (Fig. 2a, b).

The IL-4 gene expression was detected in the spleen of groups 2 and 4, with a peak at day 28. At this day, the IL-4 expression levels were significantly higher in co-infected mice than in *Leishmania* infected mice (Fig. 2c). This cytokine was not detected in the liver of mice from any group (data not showed).

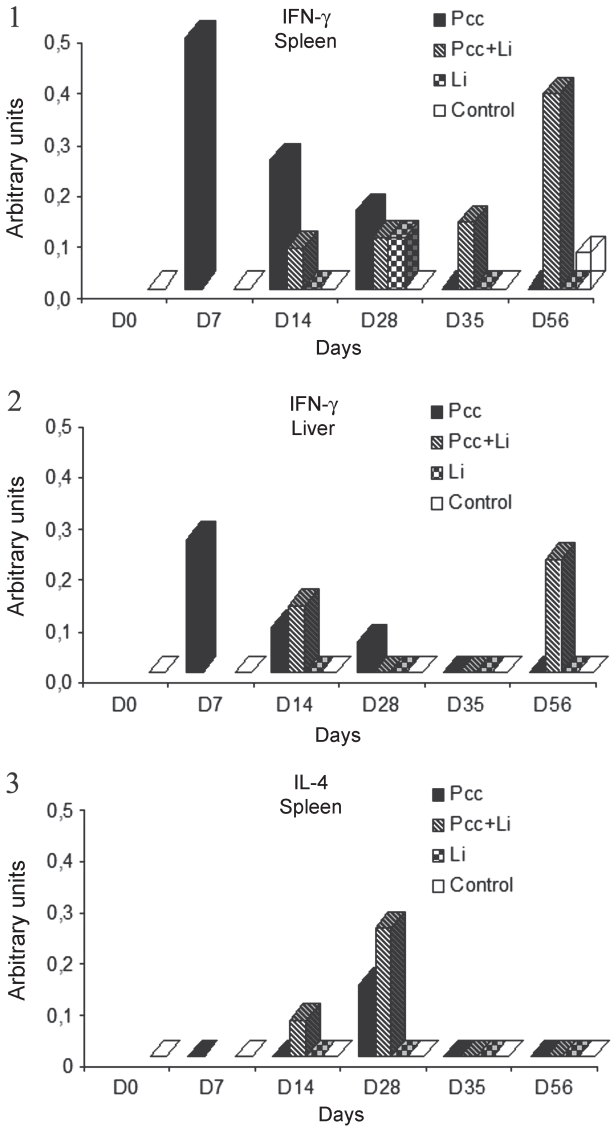

Fig. 2: interferon-gamma (IFN-γ) and interleukin-4 (IL-4) relative gene expression in liver and spleen of C57BL6 mice during *Plasmodium chabaudi chabaudi* and/or *Leishmania infantum* infections. Co-infected mice were inoculated at day 0 with *P. c. chabaudi* and at day 7 with *L. infantum*. Cytokine reverse transcriptase-polymerase chain reaction data was normalized by dividing the OD of the cytokine by the corresponding OD of the constitute gene HPRT (arbitrary units). Data represent median of 3 mice per group, from the most representative experiment. 1 and 3 - spleen; 2 - liver, Pcc - *P. c. chabaudi* infected mice (group 2); Pcc+Li - *P. c. chabaudi* and *L. infantum* infected mice (group 4); Li - *L. infantum* infected mice (group 3); Control - mice inoculated with isotonic saline solution (group 1).

DISCUSSION

Despite the existence of overlapping endemic areas, scarce information is known on concomitant malaria and leishmaniasis and no references are available regarding cytokine analysis in mixed infections involving *Plasmodium* and *Leishmania*. In the present study, the analysis of cytokine mRNA expression in spleen and liver cells during the early phase of erythrocytic *P. c. chabaudi* infection, confirmed that in C57BL/6, there is a Th_1 inflammatory response, associated with high expression of IFN-γ by day 7 post-infection (peak of parasitaemia) in order to control parasitaemia (Langhorne 1989, Zhong & Stevenson 2000). In parallel, our results confirmed a progressive elevation of IL-4 expression, a Th_2 cytokine characteristic of the chronic stage of *P. c. chabaudi* infection, responsible for B cells maturation and antibody release, which are necessary for recrudescence control and total elimination of blood parasites (Langhorne 1989, Taylor-Robinson & Phillips 1994). When all *Plasmodium* parasites are eradicated from the blood (around day 35), the IL-4 expression becomes undetectable, both in plasmodium-only and co-infected mice.

Although the course of parasitaemia was similar in mice infected only with *P. c. chabaudi* and in co-infected mice, the IFN-γ expression level seemed to persist from day 14 onwards in the co-infected group, mainly in the spleen, and then markedly increased at day 56. This sustained IFN-γ expression may be related to the fact that on the day of *Leishmania* inoculation, a Th_1 immune response had already been established as a result of the *Plasmodium* infection. Although some authors point out to the importance of IFN-γ in the resolution of leishmaniasis infection (Kaye et al. 1991, Carvalho et al. 1994), the IFN-γ expression levels detected at day 7, when *Leishmania* parasites were inoculated, apparently did not restrain the progression of *L. infantum* infection in co-infected mice. According to Melby et al. (2001), syrian hamsters infected with *L. donovani* are not able to control parasite replication despite the high levels of Th_1 cytokine expression, namely IFN-γ, as observed in this study. In fact, co-infected mice presented an evident splenomegaly on day 56, which is a typical clinical symptom of visceral leishmaniasis, and positive cultures with high values of parasite density were observed. In addition, there was no detectable erythrocytic *Plasmodium* parasites. Concomitant *Plasmodium* infection seems to increase the susceptibility to *Leishmania*, as there were no clinical signs or positive cultures from mice infected with *Leishmania* only.

L. infantum infection had little effect on the Th_1/Th_2 switch necessary for *P. c. chabaudi* control. The differences on IFN-γ expression observed at day 14 in co-infected group were not sufficient to interfere with *P. c. chabaudi* parasitaemia as it was probably already under control. On the other hand, the presence of IFN-γ at the time of *Leishmania* infection might have determined the high levels of IFN-γ observed at day 56. Himmelrich et al. (2000) mention that exogenous IL-4 administration to C57BL/6 mice does not modify their resistant phenotype nor redirect effector cells to a Th_2 phenotype. However, the presence of IL-4 at day 28, essential for *P. c. chabaudi* elimination, was probably a key factor on the outcome of *Leishmania* infection (IL-4 was not detected in *Leishmania* infected only) leading to an increase in the *Leishmania* parasite load at day 56 associated with a high IFN-γ production. The higher expression levels of IL-4 on the day 28 might have been determinant, as it probably disturbed the fine tune of the mixed Th_1/Th_2 response observed in *L. infantum* infected mice (Honoré et al. 1998).

On the other hand, we can not exclude the importance

of other cytokines during this concomitant parasitic infection. For example, a high expression and production of IL-10 as well as IFN-γ, were detected in dogs experimentally infected with *L. infantum* (Santos-Gomes et al. 2002) and by day 28 and 56 after infection with *L. donovani*, there was a high splenic and hepatic mRNA expression of IL-10 and TGF-β in hamsters (Melby et al. 2001). Belkaid et al. (2002) also showed that the persistence of parasites of *L. major* in C57BL/6 is related with the production of IL-10 by $CD4^+CD25^+$ regulatory T cells.

In conclusion, a fine tuning between Th_1 and Th_2 type responses is essential for the resolution of these parasitic infections. However the co-infection, that often occurs in nature, can disrupt this balance, leading to disease exacerbation.

Although this study comes across as a preliminary investigation, to our knowledge, is the first work in the murine model, that approaches immunological aspects in concomitant infection of *P. c. chabaudi* and *L. infantum*. Nevertheless, new research is currently undergoing in our laboratory, in order to explore and firmly define the impact of co-infection on Th_1/Th_2 immune responses.

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