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Sustained parasite burden in the spleen of Leishmania infantum-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge

Abstract

We analyzed differential responses of spleen and liver, major organ targets for viscerotropic Leishmania species, to experimental infection and examined if

resistance to challenge was organ-specific. In liver, parasites were spontaneously cleared and iNOS trancripts expression paralleled that of amastigote load. In the spleen, amastigote multiplication was only partly controlled, and iNOS transcripts expression was transient. Total numbers of spleen cells, B cells, and T cells were decreased, while F4/80+ and Mac1+ cells were conserved. Expression of splenic MCP-1 transcripts remained constant, indicating its possible contribution to immigration of Leishmania host cells and to sustained parasite load. Spleen cells produced both, Th1- and Th2-type cytokines and Th2-type response was dominant, compatible with the sustained MCP-1 expression. Challenge experiments showed that in contrast to the liver, where initial infection conferred a progressively established immunity, in the spleen there was no induced protection against reinfection. Organ-specific resistance against challenge could be important for designing antileishmanial vaccines.

Full text

INTRODUCTION

Liver, spleen and bone marrow are major organ targets harbouring viscerotropic *Leishmania* species, *L. infantum (chagasi)* and *L. donovani*, in their mammalian hosts. In man, infection by *L. infantum* often remains asymptomatic [1-4], but clinically manifest human visceral leishmaniasis (VL), due to the parasite's uncontrolled multiplication, is a fatal disease if untreated [5].

Most studies of experimental infection of mice with viscerotropic *Leishmania* have been mainly centered on the liver of infected animals [6-19], where the parasite load reaches a maximum approximately 1 month post-infection (pi) and then decreases spontaneously. The liver of mice with the resolved infection shows resistance to reinfection [9]. The infection in the spleen takes quite a dissimilar course and the parasites are not spontaneously cleared [20-30]. No data were available to date on the parasite burden in the spleen after reinfection.

To further analyze the phenomena occurring in the *Leishmania*-infected murine spleen and to identify factor(s) responsible for the chronicity of infection, a role of monocyte chemoattractant protein-1 (MCP-1) is here hypothesized. MCP-1 is a CC chemokine, one of the first to be characterized [31]. Its target cell

spectrum includes mainly monocytes/macrophages, but also memory T lymphocytes, NK cells and basophils. MCP-1 exhibits chemotactic effects on both Th1 and Th2 T cell subpopulations [32, 33]. In human disorders, MCP-1 expression occurs in many pathologies of distinct aetiologies but whose common features include inflammation and infiltration by mononuclear cells [34-36]. Its functional role in inflammatory disorders has been recently documented using disease models in MCP-1-deficient mice [37]. Chemokine expression has been observed in *L. major* infected murine macrophages [38, 39] and in biopsies from patients with American cutaneous leishmaniasis [40]. Chemokine responses in the liver of mice infected by *L. donovani* have been recently examined [19] and shown to occur very early after the infection, to be independent of IL-4 and $NK1.1^+CD4^+$ cells, and to require both $CD4^+$ and $CD8^+$ T cells.

The organ-specific nature of immune responses to *L. donovani* in mice was recently highlighted [25, 28-30, 41]. The aim of this work was to further analyze the differential responses of spleen and liver to experimental infection by *L. infantum* and in particular to examine if acquisition of resistance to a secondary challenge was organ-specific.

MATERIALS AND METHODS

Parasites

L. infantum MON1 (MHOM/FR/94/LPN101), isolated from a patient with visceral leishmaniasis, was maintained by serial passages in Syrian hamsters. The promastigote form was cultured under usual conditions [23, 24] and used for infection of BALB/c mice after 3 *in vitro* passages as stationary phase cells from 7-day-old cultures at 2.5×10^7 promastigotes/ml. Prior to infection, the parasites were washed twice by sedimentation at 2,500 x g, for 15 min, at 4° C, and resuspended in 0.9% NaCl at 2×10^8 cells/ml.

Mice and infections

Seven-week-old female BALB/c mice (Iffa Credo, Arbresle, France) were infected by inoculation into the caudal vein (10^8 promastigotes/mouse), split randomly into groups of 3 to 5 animals, and maintained in a positive pressure chamber (Esi-Flufrance, Wissous, France) for the duration of the experiments. Non-

infected littermates, used as age-matched controls, were bred in the same conditions. Several series of experiments were carried out and mice were examined at various times after the infection, as indicated in the text and in the figure legends. In the three series of challenge experiments, 3 variable periods between the initial and the challenge infections (all carried out with 10^8 promastigotes per mouse) were tested: D (infection 2 - infection 1) = 1 week, or 1 month, or 1 year. Each challenge experiment was performed independently. The period between infection 2 and the time of the parasite burden assessment was constant, 4 weeks. Mice were anesthetized with sodium thiopental (50 mg/g of body weight), sacrificed and livers and spleens were taken under sterile conditions and weighed. The assessment of the amastigote burden was carried out by blinded microscopic enumeration with Giemsa-stained liver and spleen touch prints, by two independent experienced parasitologists and expressed in Leishman Donovan units (LDU = number of amastigotes per 1,000 nucleated cells x organ weight (in grams) x 2 x 10^5), according to Stauber's formula [42].

Spleen cell activation

Preparations of total leishmanial antigens [43] and spleen cell preparation and activation [23, 24] were performed as described previously.

Cell proliferation and lymphokine assays

Spleen cells were seeded at 5 x 10^5 cells in 96-well microtiter plates and stimulated by unfractionated promastigote antigens [43] at 25 mug/ml or left unstimulated. Assays were carried out in triplicate in an incubation volume of 200 mul. After 5 days of culture, cells were pulsed with 1 muCi of [^{3}H]thymidine/well, incubated for an additional 18 hours, and harvested onto a glass fiber filter. Incorporated ^{3}H was measured by beta counting. Data are expressed as (mean cpm ± SD) x 10^{-3}. IL-2, IFN-gamma, IL-4 and TNF-alpha levels in the spleen cell lysates were determined after 48 hours culture of 1.25 x 10^6 cells in 500 mul, by indirect sandwich enzyme-linked immunosorbent assays (ELISA), with a threshold sensitivity of 10 pg per ml for each cytokine, according to the manufacturer's instructions (Duoset [Genzyme, Cambridge, Ma, USA]).

Flow cytometry

Phenotypic analysis of spleen cell subpopulations was performed after triple staining with FITC-conjugated anti-MHCII (clone FD11-54.3, Pharmingen), phycoerythrin-conjugated anti-CD8 (clone 53-6.72, Pharmingen) and anti-CD4 (clone CT-CD4, Caltag), and biotin-conjugated anti-CD11b (clone Mac1, Caltag) or F4/80 (clone C1-A31, Caltag) or anti-B220 (clone RA3-6B2, Caltag), followed by streptavidin-tricolor (TC, Caltag). All the staining steps were carried out at 4° C in PBS containing 5 mM EDTA and 3% (v/v) FCS. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) using Cellquest software (Becton Dickinson).

Semi-quantitative RT-PCR

Spleens and livers were isolated on days 2, 7, 15, 29, 70 and 400 post-infection, tissue fragments (10-15 mg/spleen, 20-25 mg/liver) were separated from the rest of the organ, immediately immersed in liquid nitrogen, pooled within each experimental group and stored in liquid nitrogen until use. RNA preparations were carried out from ground tissues using SV total RNA system (Promega), following the manufacturer's instructions. cDNA were prepared as described previously [23]. The following primers were used for cDNA amplifications: sense (S)-hypoxantine phosphoribosyl transferase (HPRT) (5'-GTTGGATACAGGCCAGACTTTGTTG-3'), antisense (AS)-HPRT (5'-GATTCAACTTGCGCTCATCTTAGGC-3'), S-MCP-1 (5'-CATTCACCAGCAAGATGATCC-3'), AS-MCP-1 (5'-CTCATTTGGTTCCGATC-CAG-3'), S-iNOS (5'-TCACGCTTGGGTCTTGTTCACT-3'), AS-iNOS (5'-TTGTCTCTGGGTCCTCTGGTCA-3'). Reactions were carried out using 3 mul of a cDNA template in 25 mul of reaction mixture [1X Taq polymerase buffer (Appligene), 0.2 mM deoxynucleotides (Promega), 1 muM primers (Eurogentec), 0.014 U/mul Taq DNA polymerase (Appligene)], under the following conditions: for MCP-1 and HPRT, the initial denaturation was 2 min at 94° C, followed by cycles of denaturation 1 min at 94° C, annealing 1 min at 55° C, polymerization 1.5 min at 70° C, and terminated by elongation at 70° C for 7 min. For iNOS, annealing was carried out at 60° C. The specificity of amplicons was confirmed by sequencing. For the semi-quantitative analysis for each cDNA under study (MCP-1, iNOS, HPRT) the minimal number of cycles leading to a signal detectable by dot blot analysis (see below) was first determined: 25 cycles for MCP-1, iNOS, HPRT in the liver samples, 27 cycles for MCP-1, HPRT and 35 cycles for iNOS in the spleen samples. The sequential dilutions (in 20X SSC buffer) of amplicons obtained under the conditions defined above, were transfered onto Hybond N^+

(Amersham) membrane, which was incubated for 5 min in the denaturation buffer (NaCl 1.5 M, NaOH 0.5 M), then 5 min in the neutralisation buffer (NaCl 1.5 M, Tris 0.1 M, pH 7.0), then fixed by heating (15 min at 80° C) and UV irradiation (few seconds). Samples were revealed after incubation of the membrane with alkaline phosphatase-labelled probe (see below) at 10 ng/ml in hybridation buffer (AlkPhos, Amersham) for 18 hours at 55° C, 4 washings [twice in 500 ml of buffer 1 (urea 2M, SDS 0.1%, $MgCl_2$ 10 mM, NaCl 150 mM, NaH_2PO_4 50 mM pH 7.0) at 55° C for 10 min, and twice in 500 ml of buffer 2 (Tris 50 mM, $MgCl_2$ 2 mM, NaCl 100 mM, pH 10.0) for 5 min at room temperature], followed by incubation with chemiluminescent agent containing revelation buffer (AlkPhos, Amersham) for 3 min. After 2 to 10 min exposure to the hybridized membrane, Hyperfilm-MP (Amersham) was scanned by UMAX laser densitometer and signal intensities (SI) were analysed using VisioLab software. The MCP-1 and iNOS transcript levels were determined after normalisation to the HPRT signal and the results are expressed in arbitrary units.

Probe preparation and labelling

Amplified cDNAs were purified using a High Pure PCR Product Purification kit (Boehringer Mannheim) and labelled using AlkPhos kit (Amersham), following the manufacturers' instructions.

Statistical analysis

The differences between experimental groups were analyzed using Student's t test for unpaired data (StatWorks). $P < 0.05$ was taken as statistically significant.

RESULTS

Monitoring of the parasite load and of iNOS and MCP-1 transcripts in spleen and liver over 400 days after L. infantum *infection*

In the first series of experiments, the parasite burden resulting from an iv inoculation of 10^8 stationary phase promastigotes and the concomitant expression of iNOS and MCP-1 mRNA were recorded in liver and spleen at various intervals during acute and chronic stages of the infection. Figure 1a shows that in the liver, the parasite proliferation leads to a heavy amastigote load, with a

maximum reached at 2 to 4 weeks post-infection (pi), then the parasite burden decreases spontaneously. The associated increase of the liver weight was moderate (at most 20%) and transient (not shown). The expression of both iNOS and MCP-1 transcripts (Figure 1c and e) peaked around the time of the maximal parasite load and declined to low values together with the number of amastigotes (Figure 1a). In contrast, in the spleen, a plateau of the amastigote burden was attained in 8 to 10 weeks pi, and did not decline over 400 days (Figure 1b). A severe splenomegaly was systematically associated with the infection (Figure 2c). The transcripts of iNOS (Figure 1d) were present only transiently, in spite of the sustained amastigote load. The increase of MCP-1 mRNA was less important than that in liver, but, unlike in liver, it did not decline (Figure 1f). Similarly to the spleen parasite burden, MCP-1 transcripts maintained their level from one month to over one year after the infection. These data bear out and extend previous observations on the distinct pattern of processes elicited by viscerotropic *Leishmania* species in the organs under study.

Cell subpopulations are altered differentially in the spleen of long-term infected mice

To further examine the *L. infantum*-infected murine spleen, we next compared the distribution of different spleen cell subpopulations in long-term-infected mice to that in the non-infected age-matched controls. Flow cytometry experiment illustrated in Table 1 showed that in leishmanial mice the total number of cells was reduced by 50% and that the distribution of spleen cell subpopulations was profoundly altered. The fraction of $CD4^+$ T cells was unchanged (around 40%), but $CD8^+$ T cells dropped from 20 to 14% and B cells from 26 to 15%. As a result, the total number of B and T lymphocytes was notably decreased in infected mice (B cells were reduced by over 70%, T $CD4^+$ cells by 40% and T $CD8^+$ cells by 65%). At the same time, the fraction of cells, comprising monocytes and macrophages, which were stained by F4/80 or Mac1 antibodies increased from 11 to 26% and from 6 to 15% respectively, and the total number of these cells was approximately the same as that in uninfected controls.

Th1- and Th2-type cytokines are produced in vitro *in response to leishmanial antigens in the spleen of long-term-infected mice*

To assess the specific cellular responses in the chronic *L. infantum* infection, the *in vitro* production of type 1 and type 2 cytokines by spleen cells, and spleen cell proliferation were examined. The results are summarised in Table 2. The cells isolated from mice spleens 10 weeks and 12 months after *Leishmania* infection were challenged by total leishmanial antigens, or left unstimulated. The cells from infected mice produced IL-2, IFN-gamma and IL-4, while the cytokines produced by cells from uninfected controls were at thresholds of detection. The capacity of cells isolated 12 months post-infection (pi) to become activated by addition of exogenous antigens, was higher than that of cells isolated 10 weeks pi. The ratio of IFN-gamma production/IL-4 production was approximately 3 (6) in the cells isolated 10 weeks (12 months) pi. The proliferation of spleen cells from the long-term-infected mice measured in the absence ($8.7 \pm 5.6 \times 10^3$ cpm) and in the presence ($15.2 \pm 3.6 \times 10^3$ cpm) of exogenous antigens, as compared to proliferation of cells from uninfected controls ($3.7 \pm 0.3 \times 10^3$ cpm), further indicated that in the infected spleen the T cells remained functional and responsive to the parasites presence *in vivo* and to an additional *in vitro* stimulation. Finally, the spleen cells from the chronically-infected mice produced TNF-alpha (192 ± 43 pg/ml), in contrast to uninfected controls (34 ± 15 pg/ml), and this production was not enhanced by the cell stimulation *in vitro*.

Liver but not spleen is protected against reinfection by L. infantum

To analyse the development of protective immunity in *L. infantum*-infected BALB/c mice, we performed a series of challenge experiments in which the periods between the initial and the challenge infections were variable [D (infection 2 - infection 1) = 1 week, or 1 month, or 1 year], and the period between infection 2 and the time of the parasite burden assessment was constant, 4 weeks. The experiments shown in Figure 2a, confirm the long-term hepatic protection against *L. infantum*, and provide further information on the chronology of its progression. First, when the challenge infection was administered one week after the initial infection, the hepatic parasite burden resulting from double infection was higher than that resulting from both, infection 1 ($p = 0.024$) and infection 2 ($p = 0.015$). Next, a partial hepatic protection was elicited by an initial infection when it was delivered one month before challenge, since the hepatic amastigotes resulting from the double infection was decreased roughly 2-fold, as compared to a single infection ($p = 0.005$). Finally, in the liver of mice infected one year before, the challenge parasites were barely detectable before and after reinfection. In all experiments

performed, hepatomegaly was moderate and transient, without significant correlation between the density of the hepatic amastigote burden and liver weight (not shown). The whole picture proved to be quite different in the spleen. Figure 2b shows that the load of amastigotes resulting from double infection was higher than that resulting from the challenge infection, whatever was the period which elapsed between the initial infection and the challenge ($p = 0.009$, $p = 0.003$, $p = 0.011$ for challenge after 1 week, 1 month, 1 year, respectively). Furthermore, an important increase of spleen weight (Figure 2c) was associated with all infections (except the challenge infection of 1-year-infected mice, which happened to be rather modestly successful). These experiments showed that in contrast to the liver, where the initial infection self-cured and conferred immunity against the subsequent infections, in the spleen the initial infection was not cleared and not only did not induce protection against the challenge, but brought about a higher parasite load.

DISCUSSION

In this work we investigated differential responses in murine liver and spleen towards short- and long-term infection by *L. infantum.* The liver is a tissue where parasites are spontaneously cleared. In contrast, the spleen is a compartment which supports the sustained parasite load over very long periods. The development of the parasite burden is associated with severe splenomegaly indicating the existence of inflammatory phenomena, persisting in the long-term infected mice.

Although the amastigote multiplication was controlled in part, since the parasite load reached a plateau, the splenic expression of iNOS transcripts was found to be only transient, and late in infection there was no detectable iNOS mRNA. In the liver, the expression of iNOS trancripts paralleled that of the amastigote load. A lack of correlation between the parasite burden and the ability of cells to synthesize NO has been already reported [44-46]. The loss of cytotoxic antileishmanial activity by macrophages might be due to a secondary maturation and/or differentiation of cells exposed to the inflammatory environment in the spleen [47]. For instance, the high basal production of TNF-alpha, unresponsive to *in vitro* activation, might originate from such activated macrophages. The mechanisms involved in the initiation of these splenic inflammatory phenomena are not known, but the early parasite destruction by

polymorphonuclear cells (D. Rousseau, submitted) might represent one of responsible factors.

The total cell number and the number of B and T lymphocytes were notably decreased. The loss of B cells, associated with the loss of follicular dendritic cells, has been previously reported in the spleen of chronically *L. donovani*-infected BALB/c mice [25], although the anti-*Leishmania* antibody production has been shown to be conserved [24]. The exact causes of lymphocyte depletion in the long-term *L. infantum*-infected murine spleen are not known. A differential migration of naive *versus* memory cells, and differential rates of naive *versus* memory cell proliferation are, as yet, subjects of controversy [48]. But the responsiveness of T cells to MCP-1 (see below) was reported to be confined to memory or activated subsets [31, 49-51], and the cell deletion due to an ongoing, antigen-driven cell activation [52] might be among responsible factors. The disintegration of the white pulpe and the depletion of lymphocytes have been also reported in human VL [53].

In this context of an overall cell number decrease, the only cell subpopulations the percentage of which doubled and total number remained constant, were monocytes/macrophages. These cells are the major *Leishmania* target cells, and a dense infiltration of the white pulpe by *L. donovani*-infected macrophages has been reported [25, 54]. We also showed that in the spleen, the expression of the MCP-1 transcripts remained constant throughout the infection, suggesting a sustained production of MCP-1 which might be responsible, at least in part, for the maintenance of the total number of monocytes/macrophages. Both, Th1- and Th2-type, cytokines were produced *in vitro* by *Leishmania*-infected spleen cells, and this production was enhanced by leishmanial antigens. The ratio of IFN-gamma to IL-4 produced, of the order of 3 to 6, indicates the predominance of the Th2-type response. Indeed, given their respective molecular weights and dissociation constants, IFN-gamma and IL-4 generate comparable biological effects at a IFN-gamma to IL-4 ratio of the order of 10. In *L. major*-infected mice, it has been shown in fact that the Th1-type response prevails when this ratio attains 100 to 1,000 [55]. This Th2 dominance in the spleen is compatible with the sustained expression of MCP-1 transcripts during the infection, since it has been shown that MCP-1 induces polarization toward Th2-type response [56]. The cellular source of the chemokine was not identified, but it may be argued that it could be produced by macrophages, infected or bystander, as was shown for IL-12 [29], since all other cells were depleted. In contrast to the spleen, in the

liver, the increase of MCP-1 transcipts was transient, peaking around the maximal hepatic parasite burden. Therefore, MCP-1 could be a factor of aggravation of the splenic disease in two ways (Figure 3). Firstly, by contributing to the influx of macrophages, usually effector cells, but the target cells for *Leishmania*, and thus providing the new host cells and allowing parasite multiplication and persistence. Secondly, MCP-1, a chemokine associated with several inflammatory diseases [32-36], could participate in the local inflammation and perpetuate it, thus contributing to the destruction of the spleen architecture [25, 28]. The involvement of the inflammatory response in the development of metastatic cutaneous lesions in hamsters infected with *L. panamensis* has been reported [57].

Finally, the challenge experiments showed the differential reaction in murine liver and spleen towards reinfection by *L. infantum.* In the liver, the initial infection conferred immunity against subsequent infections. This acquired resistance was established progressively and became partially effective already one month post-infection. In contrast, in the spleen, the initial infection was not cleared, although a partial control of the parasite proliferation is likely to exist, since the amastigote load remains quite stable over long periods of time. In spite of this partial control, due quite probably to the presence of effective, *Leishmania*-specific, T cells, there was no protective immunity induced in the spleen. Indeed, all challenge infections resulted in a significant increase of the total parasite load.

Taken together, our data indicate that the chemokine MCP-1, by its inflammatory and monocyte attractant properties, could contribute to the self-amplified chronic infection of the spleen. The possibility of acquired resistance against reinfection in one target organ (the liver), together with lack of protection in another parasite-invaded organ (the spleen) should highlight the need for caution in the future design of antileishmanial vaccines.

CONCLUSION

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Figures

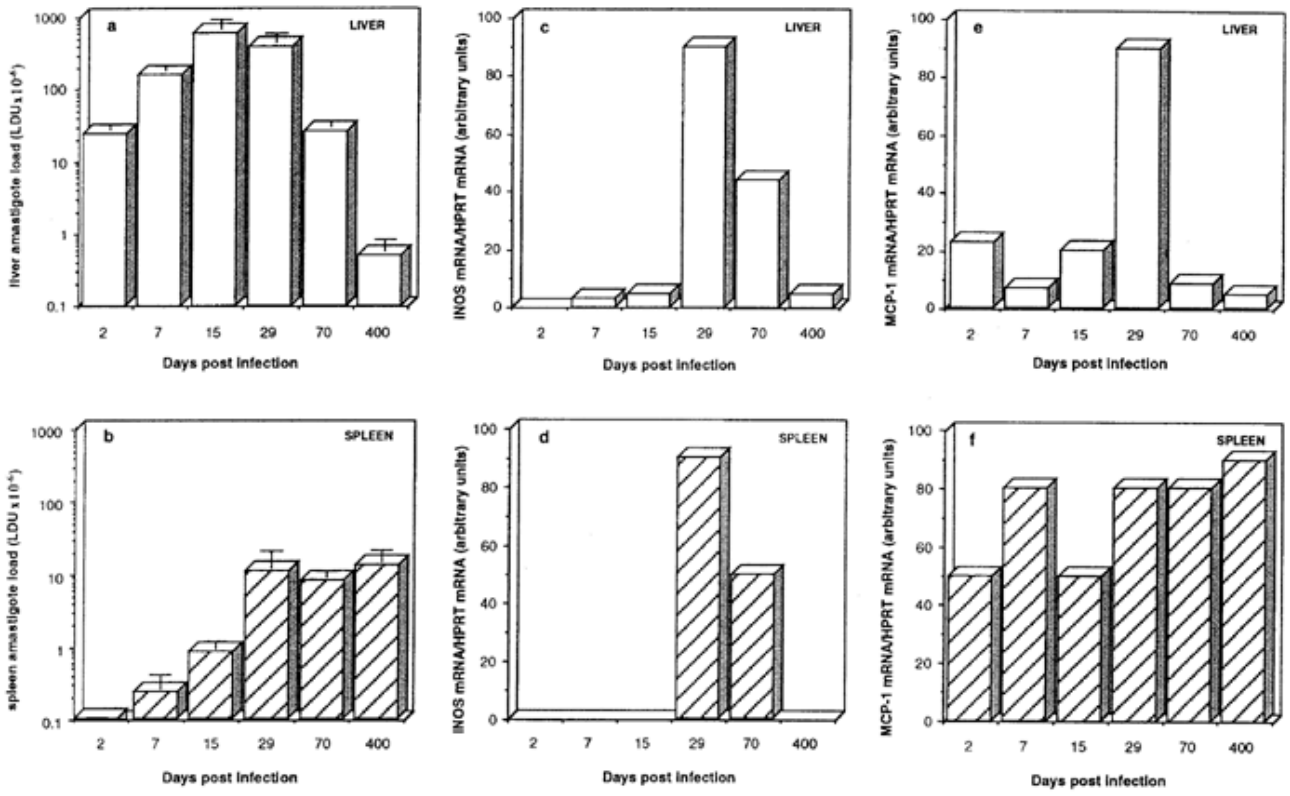
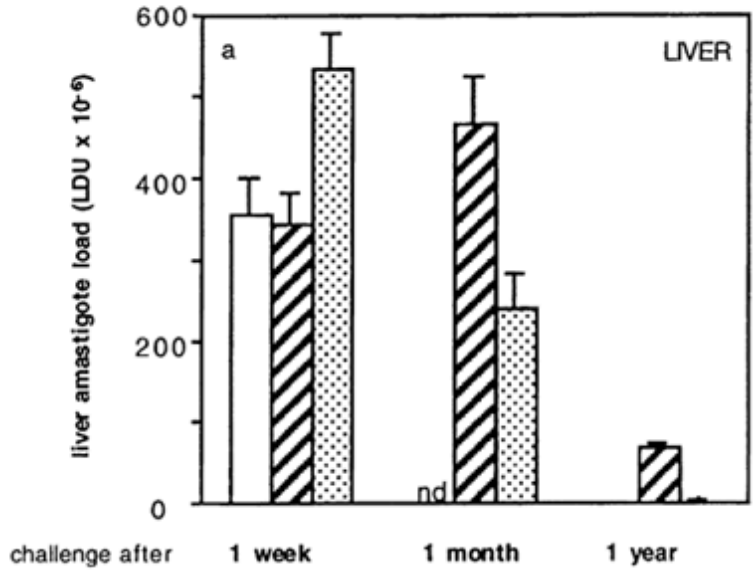


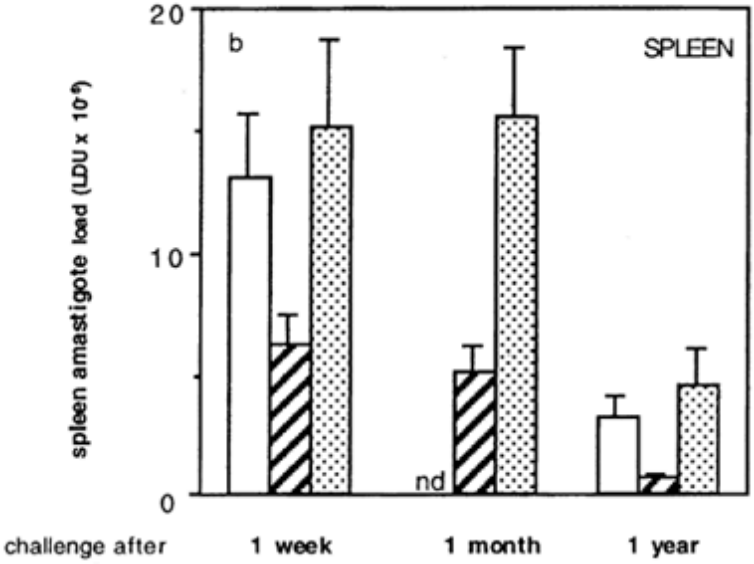
Figure 1

Differential evolution of amastigote burden, and iNOS and MCP-1 mRNA expression in liver and spleen of *L. infantum*-infected BALB/c mice.

Livers **(a, c, e)** and spleens **(b, d, f)** were isolated on days 2, 7, 15, 29, 70 and 400 post-infection from mice infected by the iv route (10^8 promastigotes/mouse). Amastigote burden **(a, b)**, determined by blinded microscopic enumeration of Giemsa-stained liver and spleen touch prints, is expressed in Leishman Donovan units (LDU = number of amastigotes per 1,000 nucleated cells x organ weight (in grams) x 2 x 10^5). iNOS **(c, d)** and MCP-1 **(e, f)** transcript levels were determined using semi-

quantitative RT-PCR analysis, quantification by densitometry and normalization to HPRT expression, as described in Materials and Methods. The results are expressed in arbitrary units: the figures allow estimation of the variation in time of a given mRNA, but do not reflect the relative amounts of MCP-1 and iNOS transcripts in the same organ, or of the same mRNA in both organs. There was no detectable expression of iNOS transcripts, as measured by RT-PCR either in liver or in spleen of uninfected control mice. MCP-1 expression was determined (after normalisation to HPRT expression) to be 1 (arbitrary unit) in liver and 40 (arbitrary units) in spleen.


a
LIVER
liver amastigote load (LDU x 10⁻⁶)
600
400
200
0
nd
challenge after
1 week
1 month
1 year


b
SPLEEN
spleen amastigote load (LDU x 10⁻⁶)
20
10
0
nd
challenge after
1 week
1 month
1 year

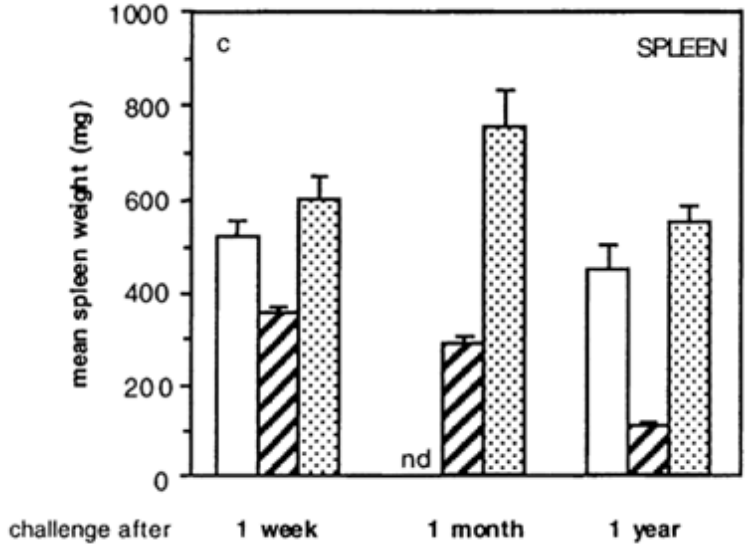

c
SPLEEN
mean spleen weight (mg)
1000
800
600
400
200
0
nd
challenge after
1 week
1 month
1 year

Figure 2

Liver but not spleen is protected against reinfection by *L. infantum* in BALB/c mice.

Mice were infected by the iv route with 10^8 promastigotes/mouse (infection 1, open bars). After a period of 1 week (experiment 1), or 1 month (experiment 2), or 1 year (experiment 3) as indicated, a second inoculation (10^8 promastigotes/mouse) was carried out in half of the mice already infected (infection 1 + infection 2, grey bars) and to uninfected age-matched littermates (infection 2, hatched bars). Four weeks after the second infection, livers and spleens were examined. The assessment of the parasite burden was carried out on liver and spleen touch prints and is expressed in LDU (mean ± SEM) as in figure 1. Each challenge experiment was performed independently, with 5, 10, 6 mice per group in experiment 1, 2, 3, respectively. Mean weight of uninfected spleen was (105 ± 8) mg, and quite independent of mouse age. nd = not done.

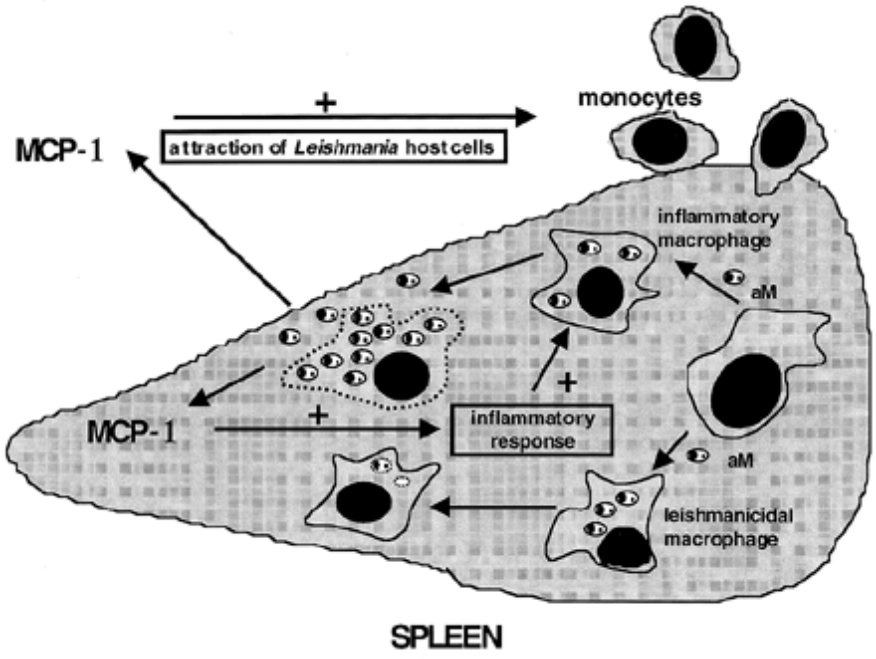


Figure 3

Involvement of monocytes chemoattractant protein-1 in sustained parasite burden in the spleen of *L. infantum* infected mice.

Spleen-derived MCP-1 leads to immigration of monocytes into the organ, thus providing new host cells for *Leishmania*. Locally, MCP-1 participates in inflammation, thus contributing to an environment in which infected macrophages do not respond to Th1-generated signals and do not acquire leishmanicidal activity. Ongoing monocytes influx and inflammatory phenomena allow the parasite multiplication and the destruction of the spleen architecture, and perpetuate self-amplified chronic splenic leishmaniasis. aM = amastigote.

Table 1
Cell subpopulations in the spleen of long-term-infected mice

	All cells	CD4⁺ cells	CD8⁺ cells	B cells	F4/80⁺ cells	Mac1⁺ cells	MHCII⁺ cells
Infected mice	40.9	16.2 (39)	5.9 (14)	6.2 (15)	10.4 (26)	6.2 (15)	7.9 (19)
Control mice	88.7	31.4 (36)	17.3 (20)	23.5 (26)	10.2 (11)	5.6 (6)	22.5 (25)

Data were obtained by flow cytometric analysis of homogenized spleen cells isolated from mice, 14 months post-*L. infantum* infection or from age-matched littermates. Cell counts are expressed in millions per organ; numbers in parentheses represent percentage of total counts. MHCII⁺ cells include B cells and some macrophages; Mac1⁺ cells include monocytes, macrophages and granulocytes.

Table 2
***In vitro* cytokine production by spleen cells[a]**

	IFN-gamma (pg/ml)	IL-4 (pg/ml)	IL-2 (pg/ml)
10 weeks pi			
Unstimulated cells	121 ± 18	39 ± 8	54 ± 5
Stimulated cells	262 ± 23	84 ± 16	84 ± 15
Controls	16 ± 5	28 ± 4	14 ± 2
12 months pi			
Unstimulated cells	480 ± 125	83 ± 38	78 ± 8
Stimulated cells	1,547 ± 411	258 ± 39	282 ± 38
Controls	51 ± 11	24 ± 3	18 ± 5

a) Mice inoculated with *L. infantum* (10^8 promastigotes/mouse) were examined 10 weeks or 12 months post-infection (pi). Uninfected littermates were used as age-matched controls. One representative experiment out of three performed is shown. Spleen cells were seeded in 500 mul at 2.5 x 10^6 cells/ml on 48-well plates and incubated for 48 hours with unfractionated promastigote antigens at 25 mug/ml or left unstimulated. Cytokines were measured by indirect sandwich ELISA (in duplicate) for each individual mouse. Data represent mean ± SEM for five (10 weeks pi) or three (12 months pi) animals per group. Spleen cells from uninfected controls were incubated with or without antigen, similarly to cells from infected mice, and there was no difference between the two measures. Differences between different cell stimulation (infected stimulated *versus* infected unstimulated *versus* controls) are significant (t test, $p < 0.05$) except for IL-4 production by infected unstimulated *versus* control cells at both times tested.

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THP-1 cells, macrophages, estrogen receptors, estrogens, monocytes.

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