



In vivo characterization of two additional *Leishmania donovani* strains using the murine and hamster model

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SUMMARY

Leishmania donovani is a protozoan parasite causing the neglected tropical disease visceral leishmaniasis. One difficulty to study the immunopathology upon *L. donovani* infection is the limited adaptability of the strains to experimental mammalian hosts. Our knowledge about *L. donovani* infections relies on a restricted number of East African strains (LV9, 1S). Isolated from patients in the 1960s, these strains were described extensively in mice and Syrian hamsters and have consequently become 'reference' laboratory strains. *L. donovani* strains from the Indian continent display distinct clinical features compared to East African strains. Some reports describing the *in vivo* immunopathology of strains from the Indian continent exist. This study comprises a comprehensive immunopathological characterization upon infection with two additional strains, the Ethiopian *L. donovani* L82 strain and the Nepalese *L. donovani* BPK282 strain in both Syrian hamsters and C57BL/6 mice. Parameters that include parasitaemia levels, weight loss, hepatosplenomegaly and alterations in cellular composition of the spleen and liver, showed that the L82 strain generated an overall more virulent infection compared to the BPK282 strain. Altogether, both *L. donovani* strains are suitable and interesting for subsequent *in vivo* investigation of visceral leishmaniasis in the Syrian hamster and the C57BL/6 mouse model.

Keywords Flow cytometry, Immunopathology, *Leishmania* spp, Leishmaniasis, Rodent

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Abbreviations: EVL, experimental visceral leishmaniasis; *i.c.*, intracardiac; *i.v.*, intravenous; LDU, Leishman Donovan units; *p.i.*, post infection; PKDL, post-kala-azar dermal leishmaniasis; SSG, sodium stibogluconate; VL, visceral leishmaniasis

INTRODUCTION

Leishmania, a protozoan parasite belonging to the Trypanosomatidae family, is transmitted by phlebotomine sandflies and causes a neglected tropical disease known as leishmaniasis. *L. donovani* is the causative agent of visceral leishmaniasis (VL), also known as Kala-Azar, the most severe form of the disease which can be fatal if left untreated (1). VL is endemic with 90% of the cases found in six countries: India, Bangladesh, Nepal, Sudan, South Sudan and Brazil (2). Every year an estimated 500 000 new people contract VL and about 10 000 people succumb from the disease (2). Medication exists but its effectiveness is hampered by the rise of drug resistant *L. donovani* strains (3) and is associated with high toxicity, difficult mode of administration and high cost (4).

Several *L. donovani* strains have been described in *in vitro* settings to test drug efficacy (5, 6), study intracellular drug resistance pathways (7–9), discover virulence factors (10–12), etc. One major difficulty in *in vivo* studies of *L. donovani* infections is to adapt the strains to the mammalian host. Hence, only a very restricted number of *L. donovani* strains such as the LV9 strain from Ethiopia, first described by R.S. Bray in 1969, and the 1S strain from Sudan, first described by Stauber in 1966 (13), have been adapted to the *in vivo* situation in mice and Syrian hamsters, and have ever since become

reference laboratory strains to study visceral leishmaniasis. *L. donovani* strains from the Indian continent, such as the DD8, AG83 and GE1F8R strains have been described in mice and hamsters (14–18). Recently, a Nepalese clinical isolate *L. donovani* strain (BPK282) was fully sequenced and annotated (19). This strain however has mainly been used as a reference for genotyping studies and its behaviour in mice has only briefly been described, while infection data in hamster is missing (20). Given the many differences which exist between *L. donovani* strains from East Africa and the Indian continent, being on a genomic level (19), their degree of PKDL induction (21) and their differential resistance to sodium stibogluconate (SSG) (3, 22, 23), it is of great importance to have a model of experiment visceral leishmaniasis from both regions.

The clinicopathological features of the hamster model of VL closely mimic the human form of the disease. The infection is characterized by increasing visceral parasite burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinaemia and ultimately death (24). Murine infections represent the subclinical or asymptomatic form of the disease, which happens in 90% of the cases of VL. The C57BL/6 mice are resistant to *L. donovani* and generate an organ-specific immune response. Upon *L. donovani* infection, the parasites rapidly multiply in the liver until 4 weeks postinfection (*p.i.*) followed by clearance after 6–8 weeks. This self-curing mechanism is the result of a Th1-mediated granulomatous response evoked in the liver by the action of interferon γ (IFN- γ) produced by CD4⁺ and CD8⁺ T cells (25–27) and the production of tumour necrosis factor alpha (TNF- α) and nitric oxide (NO) (28, 29). The spleen displays chronic infection characterized by parasite persistence, disruption of the splenic micro-architecture and dysfunctional immune responses (30, 31).

The present study describes the immunopathological behaviour of two additional *L. donovani* strains in both the C57BL/6 mouse model and the Syrian hamster model. Both strains have different historical and geographical backgrounds: the *L. donovani* L82 strain was first isolated from an Ethiopian patient in 1967, whereas the *L. donovani* BPK282 strain originated from a Nepalese patient in 2003 and encountered a low number of passages since isolation from the patient. These two strains differed phenotypically and genotypically from each other as well as from the reference laboratory *L. donovani* strain LV9 and are both suitable for *in vivo* studies of chronic visceral leishmaniasis providing new models with well-characterized differences in pathology.

MATERIALS AND METHODS

Animals, parasites, and infections

The *L. donovani* BPK282 strain (MHOM/NP/03/BPK282A1 also known as BPK282/0 cl4) and L82 strain (MHOM/ET/62/L82), from Dr. J.C. Dujardin, Institute of Tropical Medicine, Antwerpen and Dr. Louis Maes, University of Antwerp, Antwerpen, were maintained by passage in Syrian hamsters. Hamsters were infected with 2×10^7 *L. donovani* amastigotes by intracardiac (*i.c.*) injection. Amastigotes for murine infection were obtained from the spleen of infected donor hamsters. The hamsters developed progressive illness resulting in death by 10 weeks. Infected and control noninfected hamsters were euthanized at 8 weeks post-infection.

Female 8–10 week old C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France) and housed in cages containing a maximum of 8 mice, with enriched environment and unrestricted access to food and water. Mice were infected by intravenous (*i.v.*) injection of 2×10^7 amastigotes, collected from infected hamster spleens, with either the *L. donovani* BPK282 strain or the *L. donovani* L82 strain. Mice and hamsters were housed at the animal facility of the Vrije Universiteit Brussel, Brussels.

Ethic statements

All the experiments were performed according to directive 2010/63/EU of the European Parliament for the protection of animals used for scientific purposes and approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (clearance number 14-220-17).

Determination of parasite burden and hepatosplenomegaly

At different times after infection, mice were sacrificed and parasite burden in the spleen and the liver was determined by limiting dilution as described by Titus *et al.* (32). Briefly, the spleen and the liver were collected and homogenized in 4 mL M199 medium (GIBCO) supplemented with 20% foetal calf serum (FCS), 100 U/mL penicillin (GIBCO), 100 μ g/mL streptomycin (GIBCO), 4 mM sodium NaHCO₃ (Merck), 0.0005% hemin (Sigma-Aldrich, Saint-Louis, Missouri, USA) and 0.1 mM adenosine (Sigma-Aldrich, Saint-Louis, Missouri, USA). A part of the suspension (200 μ L) was plated in 96-well plates in triplicates at a twofold serial dilution, incubated for 7 days at 26°C and assessed for parasite growth under an inverted light microscope. Parasite burden in the spleen of hamsters was determined from Giemsa-stained impression

smears and expressed as Leishman-Donovan units (LDU) (number of parasites per 1000 host cell nuclei \times organ weight in mg).

Hepatosplenomegaly was assessed by weighing the spleen and the liver of the infected animals at different time points after infection. For each time point, spleen and liver indices were calculated as follows: (weight of organ/weight of animal) \times 100.

Flow cytometry

Spleen and liver were harvested from CO₂ euthanized non-infected control, *L. donovani* BPK282 and *L. donovani* L82-infected mice. Spleen cells were obtained by homogenizing the organs in 10 mL RPMI medium containing 5% FCS and filtered (70 μ m pore filter).

Hepatic nonparenchymal cells were prepared from the liver of naive or infected mice digested with 100 U/mL collagenase III (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 50 U/mL DNase I (Worthington Biochemical Corporation) in RPMI medium. The digestion was blocked with 20 mL of 2.5 mM EDTA in RPMI medium and the suspension filtered (70 μ m pore filter). The nonparenchymal cells were then separated from hepatocytes with a 33% percoll in PBS solution by centrifugation. Next, cell suspensions were centrifuged (1400 rpm, 7 min, 4°C) and the pellet was treated with RBC lysis buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂-EDTA). Cells were washed with FACS medium (5% FCS in RPMI) and non-specific binding sites were blocked by incubating 20 min at 4°C with a Fc-blocking antibody (anti-CD16/32, clone 2.4G2, BD Bioscience, San Jose, CA, USA).

Isolated cells were labelled with fluorochrome-conjugated antibodies to CD45R, CD93, CD4, CD8a, Ly6C, CD11b for 30 min at 4°C. Fluorescent antibodies: anti-CD93-APC (clone AA4.1, eBioscience, San Diego, CA, USA), anti-CD45R (B220)-APC-Cy7 (clone RA3-6B2, BD Bioscience), anti-CD4-PECy7 (clone GK1.5, eBioscience), anti-CD8a-APC (clone 53-6.7, eBioscience), anti-CD11b-PECy7 (clone M1/70, BD Biosciences), anti-Ly6C-APC (clone AL-21, BD Biosciences). Cross-reactive antibodies for analysing isolated cells from hamster spleen and liver (33, 34): rat anti-mouse CD4 (clone GK1.5, eBioscience), mouse anti-mouse/rat major histocompatibility complex II (MHCII I-E^k clone 14-4-4S, eBioscience). Analyses were performed using a FACS Canto II flow cytometer (BD Biosciences) and data were processed using FLOWJO software (Tree Star Inc., Ashland, OR, USA). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of negative or positive gates by the total cell number determined from the cell suspension of each sample under the microscope.

Enzyme-linked immunosorbent assay (ELISA)

Concentration of IFN- γ in serum was determined by ELISA according manufacturers' protocol (BD Pharmingen cat no 551216).

Restriction fragment length polymorphism (RFLP)

Parasites were harvested from culture in M199 + 20% FCS and DNA was extracted using QIAamp DNA mini kit according to manufacturer instructions (Qiagen, Valencia, CA, USA). Genomic DNA (5 ng) was used to amplify a part of Leishmania mini-circle kDNA (35), PCR product was cleaned up using Wizard SV Gel and PCR Clean-Up kit (Promega, Fitchburg, WI, USA) before digesting 500 ng of the amplification product by restriction enzyme *HaeIII* (Thermo Fisher Scientific, Waltham, MA, USA) for 4 h at 37°C. Digestion product was migrated on a 3% small fragment agarose gel (Eurogentec, Seraing, Belgium) for 5 h at 50V prior revelation using ethidium bromide.

Statistical analysis

Statistical analysis was performed using Student *t*-test with GRAPHPAD PRISM software (GraphPad 6, San Diego, CA, USA). Values are expressed as mean \pm standard deviation (SD) values of $P \leq 0.05$ are considered to be statistically significant.

RESULTS

The *L. donovani* strains LV9, L82 and BPK282 are genotypically distinct strains based on RFLP of minicircle kinetoplast DNA

The *L. donovani* LV9 and 1S strains have previously been used as reference laboratory strains in several *in vivo* studies on the immunopathology of visceral leishmaniasis. Little information is available about the *L. donovani* L82 strain. It is also a strain that was isolated in Ethiopia in 1967 but which has mostly been described *in vitro* (6, 36–38) and poorly studied in *in vivo* mouse models (39, 40) or in the Syrian hamster model (5, 41). Very few studies on *L. donovani* clinical isolate strains exist due to the difficulty to adapt those strains to the animal host (20, 42–44) and the Nepalese BPK282 strain is such an example and has been kept *in vitro* at the Institute of Tropical Medicine (ITM), Antwerp. In a first step a RFLP assay, as described by Bhattarai *et al.* (35), was performed to investigate how different the *L. donovani* L82 and the BPK282 strains were compared to the LV9 strain and to each other. The PCR products were amplified successfully

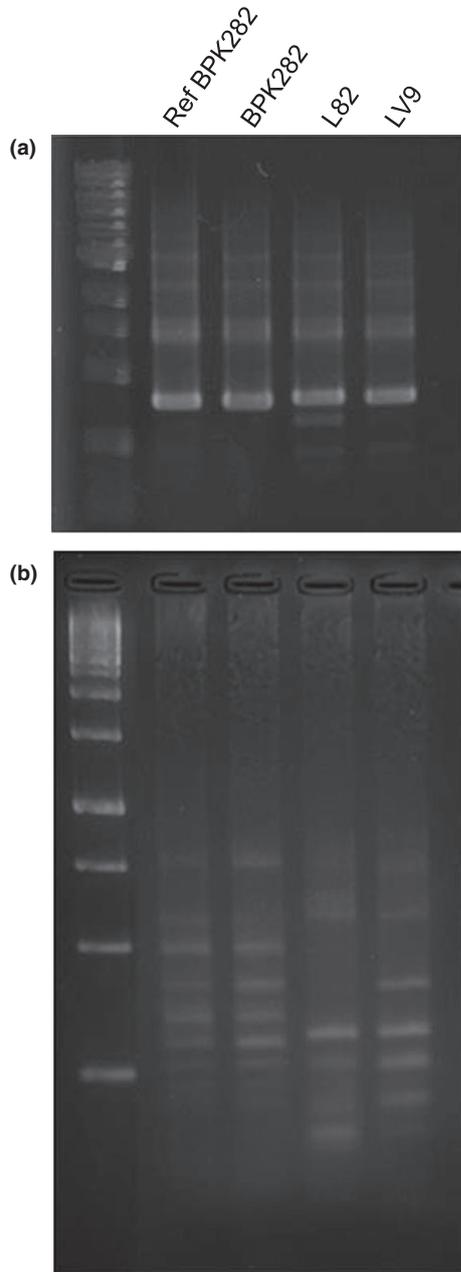


Figure 1 BPK282 strain shows significant genotypic differences compared to L82 in RFLP pattern. DNA from *in vitro* cultured *L. donovani* promastigotes was extracted. Ref BPK282 refers to the *L. donovani* BPK282 strain, which was kept *in vitro* at the Institute of Tropical Medicine, Antwerpen (19). (a) A part of *Leishmania* mini-circle kDNA was amplified and run on a 3% agarose gel. (b) Subsequently, the amplification product was digested by the restriction enzyme *HaeIII* and run on a 3% agarose gel.

(Fig. 1a) and digestion with *HaeIII* restriction enzyme indicated the presence of three distinct *L. donovani* kinetoplast minicircle genotypes (Fig. 1b). Firstly, it was

observed that the BPK282 strain that was passaged in hamsters for more than 2 years remained identical to the BPK282 which was continuously passaged *in vitro*. This observation gave a first indication that changes were minimal between *in vivo* and *in vitro* conditions within this particular sequence of 800 nucleotides in the kinetoplast minicircle DNA. Secondly, the RFLP results obtained indicated that the L82 strain and the BPK282 strain were clearly distinct from each other. Moreover, both the L82 strain and the BPK282 strain were different from the LV9 strain.

The *L. donovani* BPK282 strain is less virulent than the L82 strain in Syrian hamsters

A parameter that is commonly used to assess the virulence of *L. donovani* infections in Syrian hamsters is weighing the animals over the course of infection. L82-infected hamsters reached a maximum weight approximately after 4 weeks of infection and rapidly started losing weight after that until approximately 8 weeks *p.i.* when they were sacrificed due to their declining health status (Fig. 2a). In contrast, BPK282-infected hamsters continuously gained weight until 12 weeks *p.i.* (Fig. 2a). At rare occasions, however it appears that one hamster (out of three) would significantly lose weight as of week 24 *p.i.* (data not shown). In line with these results, the increase in relative spleen and liver weights, expressed as spleen and liver index, were significantly higher for L82-infected hamsters compared to BPK282-infected hamsters after 8 weeks of infection (Fig. 2b). Moreover, the spleen of L82-infected hamsters contained significantly higher parasite loads than the one from BPK282-infected hamsters (Fig. 2c). CD4⁺ T cells are important for the establishment and maintenance of anti-parasitic immunity in the liver as well as immune surveillance and suppression of parasite outgrowth in the spleen in the murine model of VL (45). B cells have been shown to promote parasite persistence and to be involved in dampening neutrophil-mediated inflammatory responses in the C57BL/6 model (46). Little is known on the impact of *L. donovani* infection on T cells and B cells in hamsters. Although few reagents are available for hamster immunologic analyses, cross-reactive anti-mouse antibodies have been described previously to detect CD4⁺ T cells (33) and MHCII⁺ cells, like B cells, (34) by flow cytometry. The total amount of hematopoietic cells present in the spleens of L82- and BPK282-infected hamsters at 8 weeks *p.i.* was significantly higher than in naive mice (Fig. 2d). The spleen of L82-infected hamsters had similar numbers of CD4⁺ T cells and higher numbers of MHCII⁺ B cells after 8 weeks of infection

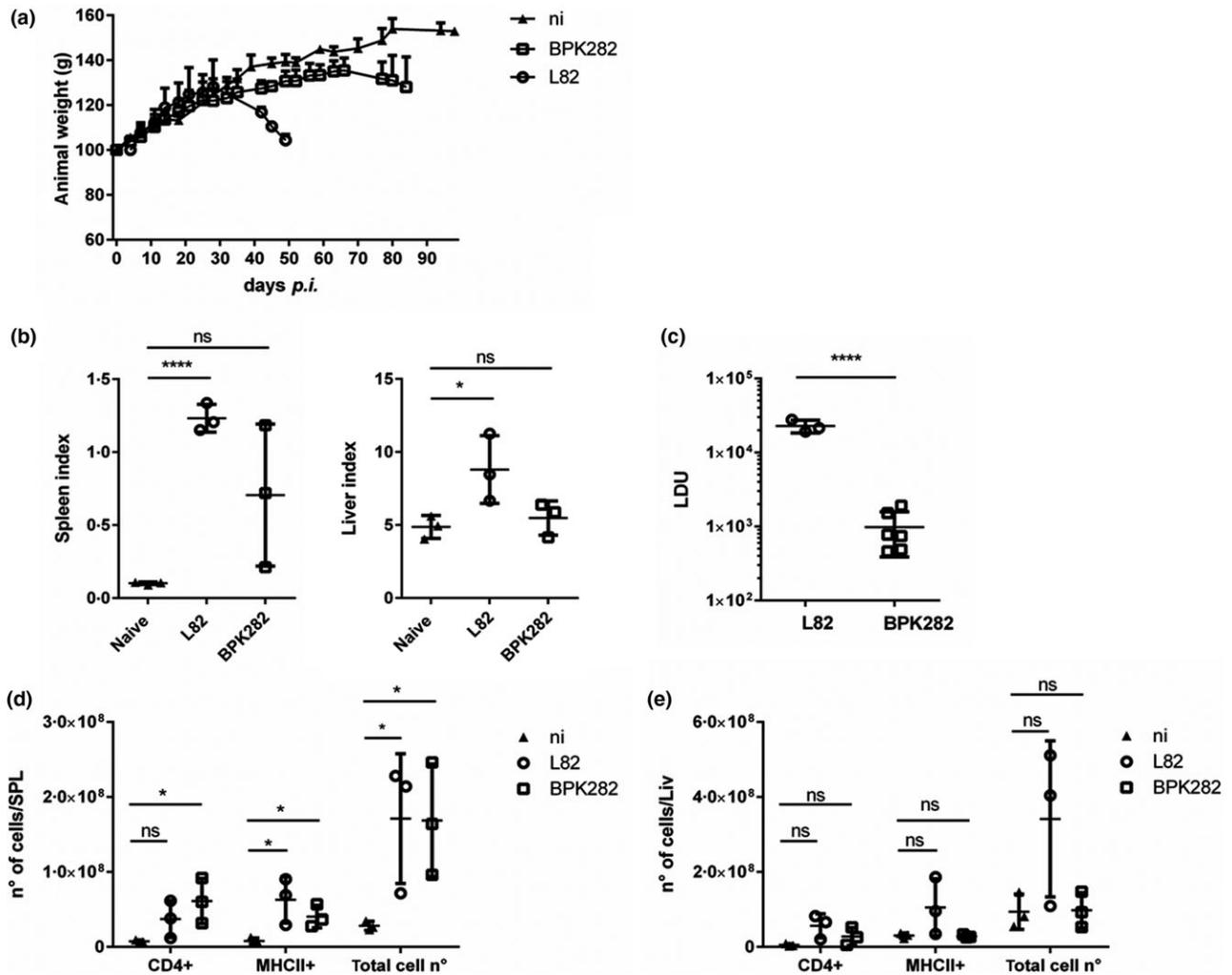


Figure 2 *L. donovani* BPK282 infection in hamsters results in lower virulence than *L. donovani* L82 infection. Syrian hamsters were infected with 2×10^7 amastigotes of the *L. donovani* L82 strain or the *L. donovani* BPK282 intracardiacally (*i.c.*). (a) Naive and infected animals were weighed over the course of infection and their weights were normalized compared to their initial weight at the day of infection. (b) The level of splenomegaly (left) and hepatomegaly (right) were measured and expressed as the relative organ weight to the animal's body weight. (c) Parasitaemia levels in hamster spleen was assessed and expressed as Leishman Donovan Units (LDU). Flow cytometry analysis of the spleen of naive and infected hamsters with 2×10^7 amastigotes of the *L. donovani* BPK282 strain or the *L. donovani* L82 strain after 8 weeks of infection. Spleen cells were pre-gated according to size, granularity and livability. Hereafter, the cells were gated on their expression of CD4 and MHCII. Percentages of CD4⁺ T cells, MHCII⁺ cells and total cell numbers in the spleen (d) and liver (e) of naive and infected hamsters after 8 weeks of infection. Data represent three independent experiments with at least three hamsters per group. Error bars are shown as standard deviations (SD). * $P < 0.05$, **** $P < 0.0001$.

compared to naive hamsters (Fig. 2d). BPK282-infected hamsters had higher numbers of splenic CD4⁺ T cells and MHCII⁺ B cells at week 8 *p.i.* than naive mice (Fig. 2d). The livers of L82- and BPK282-infected hamsters had similar amounts of nonparenchymal cells as found in naive hamsters (Fig. 2e). As far as the amounts of CD4⁺ T cells and MHCII⁺ B cells were concerned, there were no significant differences observed between L82- or BPK282-infected and naive hamsters (Fig. 2e).

The BPK282 strain induces lower levels of parasitaemia and hepatosplenomegaly than the L82 strain in C57BL/6 mice

From the above, it was noted that the *L. donovani* L82 strain scored higher for clinico-pathological signs of visceral leishmaniasis in the hamster model as compared to the BPK282 strain. Whether these results could be translated to the C57BL/6 mouse model was investigated further. Upon intravenous infection with 2×10^7 amastigotes, we

observed that the *L. donovani* L82 strain developed a peak parasitaemia in the liver and in the spleen around week 3 *p.i.*, and that this parasite level was maintained in the spleen and lowered in the liver over the course of infection (Fig. 3a and b). In contrast, the BPK282 strain displayed lower parasitaemia levels both in the spleen and liver of C57BL/6 mice compared to infection with the L82 strain, with a peak at week 6 *p.i.* in the spleen and at week 3 *p.i.* in the liver (Fig. 3a and b). In terms of spleen and liver enlargement, the L82 strain induced gradually increasing splenomegaly upon infection, with a maximum reached after 6 weeks *p.i.* (spleen indexes of 2.7 ± 0.8 in L82-infected compared to 0.3 ± 0.1 in naive mice, $n = 4$) (Fig. 3c). Although the BPK282 strain also induced increasing levels of splenomegaly until week 6 *p.i.*, this happened to a significantly lesser extent (spleen indexes of 1.5 ± 0.6 in BPK282-infected compared to 0.3 ± 0.1 in naive mice, $n = 4$) than the L82 strain (Fig. 3c). In a similar way, the liver of BPK282-infected mice did not gain weight as much as the liver of L82-infected mice (Fig. 3d).

Livers and spleens of BPK282-infected C57BL/6 mice accumulate less CD4⁺ T cells and CD8⁺ T cells compared to L82-infected mice

As mentioned earlier, CD4⁺ T cells are important for the establishment and maintenance of anti-parasitic immunity in the liver and in the spleen in the murine model of VL (45). CD8⁺ T cells are important for the control of *L. donovani* infections in the liver through their ability to produce IFN- γ and/or their cytolytic activity (25, 47). The *L. donovani* L82 and BPK282 strains generated a different pathologic response in the spleen and liver of C57BL/6 mice. The amounts of T lymphocytes present in the spleen and liver of naive and L82- or BPK282-infected C57BL/6 mice after 3, 6 and 9 weeks *p.i.* were investigated by flow cytometry.

In accordance with the enlarged liver (Fig. 3d) during both L82 and BPK282 infection, the total cell number purified from the liver after percoll gradient increased upon infection and was the highest after 3 weeks *p.i.* At

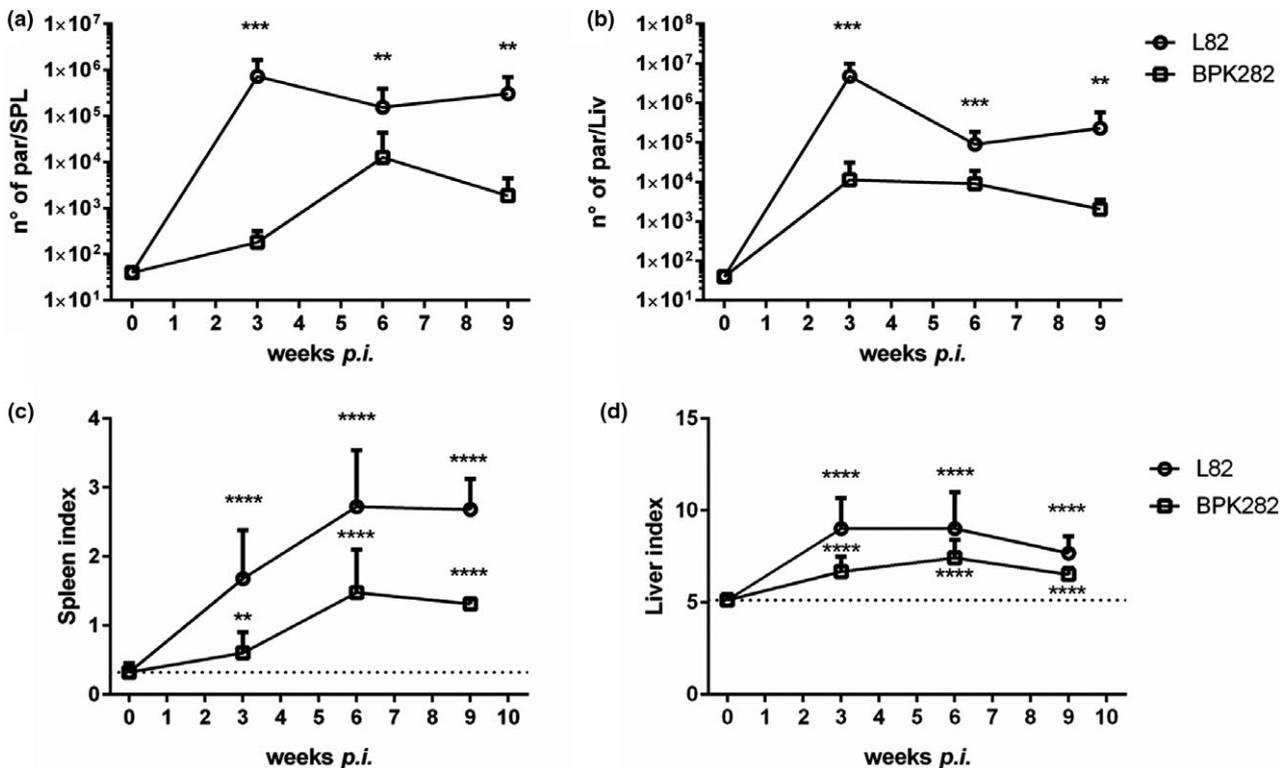


Figure 3 *L. donovani* BPK282 strain displays lower virulence compared to the L82 strain in C57BL/6 mice. C57BL/6 mice were injected *i.v.* with 2×10^7 amastigotes from the *L. donovani* L82 strain or the *L. donovani* BPK282 strain. Parasitaemia in spleen (a) and liver (b) was assessed by limiting dilution technique. The level of splenomegaly (c) and hepatomegaly (d) was measured and expressed as the relative organ weight to the animal's body weight. Data represent three independent experiments with at least four mice per group. Error bars are shown as standard deviations (SD). Student *t*-test was performed between infected and naive mice. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

all three time points investigated, significantly higher liver cell numbers could be found in L82-infected mice compared to BPK282-infected mice (Fig. 4c). During infection, the increase in CD4⁺ T cell percentages compared to uninfected mice was higher during L82 infection (threefold at week 3 and week 6 *p.i.*, 2-fold at 9 weeks *p.i.*) compared to during BPK282 infection (1.5-fold at week 3 and week 9 *p.i.*, 2.5-fold at week 6 *p.i.*) (Fig. 4b). CD8⁺ T cell percentages in liver increased compared to naive situation but did not significantly differ between L82 and BPK282 infection (Fig. 4b). These observations could also be found in total CD4⁺ T and CD8⁺ T cell numbers in the livers of L82- and BPK282-infected mice. As such, livers

of L82-infected mice had relatively more CD4⁺ T and CD8⁺ T cells than BPK282-infected mice (Fig. 4d).

Upon infection, the total number of cells in the spleen after 3 weeks of L82 infection was significantly increased compared to naive mice and remained stable until 9 weeks *p.i.* In contrast, spleens of BPK282-infected mice only had significantly higher cell numbers starting at week 6 *p.i.* and reached similar cell numbers as L82-infected mice after 9 weeks *p.i.* (Fig. 5c). The percentages of CD4⁺ and CD8⁺ T cells in the spleen of L82- and BPK282-infected mice were not significantly different compared to naive mice (Fig. 5a and b). Nevertheless, L82-infected mice had significantly higher numbers of CD4⁺ and CD8⁺ T cell

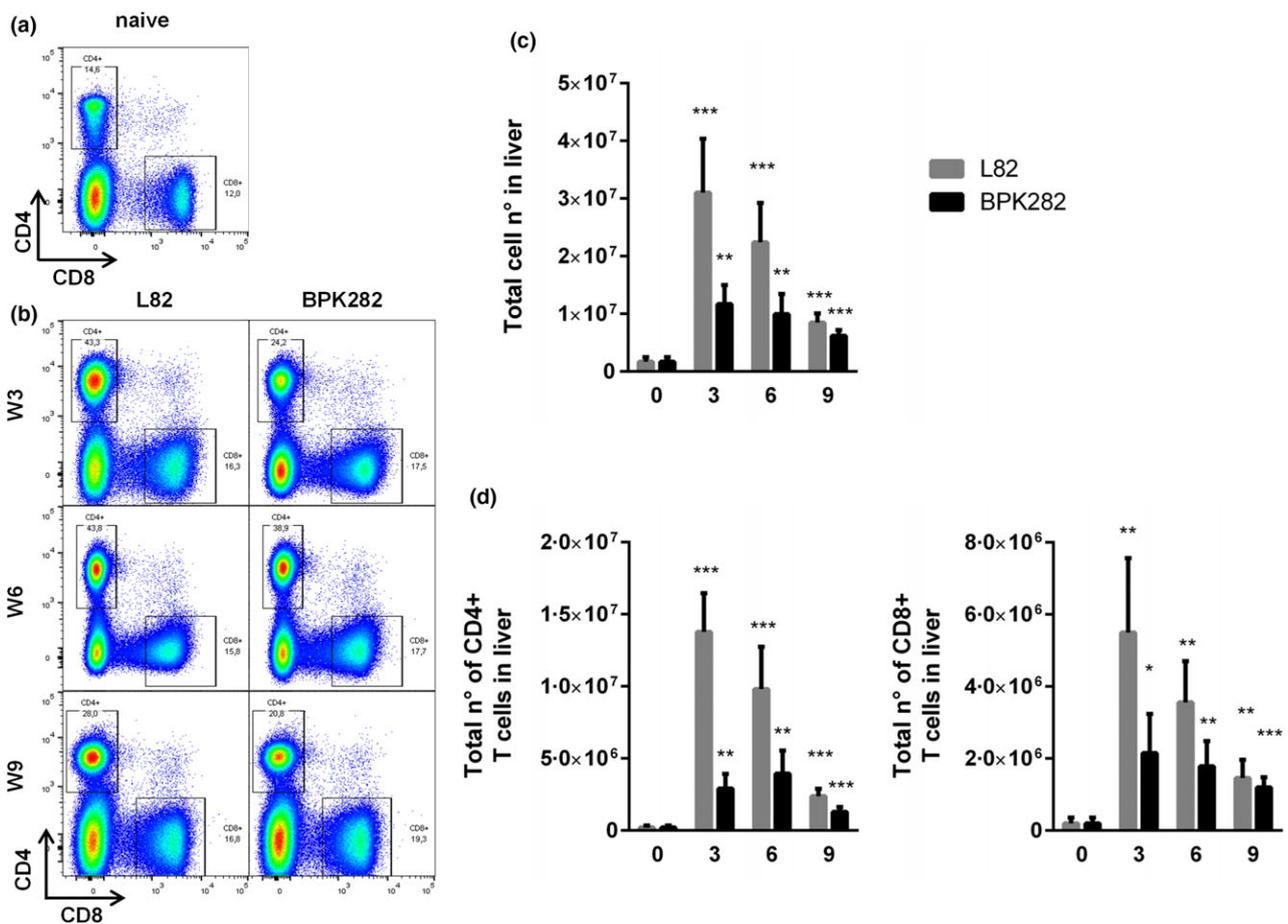


Figure 4 Livers of BPK282-infected mice accumulate less CD4⁺ T and CD8⁺ T cells as compared to L82-infected mice. Flow cytometry analysis of the liver of infected C57BL/6 mice with either 2×10^7 amastigotes of the *L. donovani* BPK282 strain or the *L. donovani* L82 strain. Nonparenchymal liver cells were pre-gated according to size, granularity and livability. Hereafter, the cells were gated on their expression of CD4 and CD8. (a) Liver cells of naive mice. (b) Liver cells from L82-infected (left panels) and BPK282-infected (right panels) mice over the course of infection (3 weeks, w3; 6 weeks, w6; 9 weeks, w9). (c) Total cell number of naive and infected livers after percoll gradient. (d) Total cell numbers of CD4⁺ T cells (left panel) and CD8⁺ T cells (right panel) in the liver of naive and infected mice (grey, L82; black, BPK282). over the course of infection. Data represent three independent experiments with at least four mice per group. Error bars are shown as standard deviations (SD). Student *t*-test was performed between infected and naive mice. **P* < 0.05, ***P* < 0.01 ****P* < 0.001.

numbers as of week 3 *p.i.* compared to naive mice (Fig. 5d). CD4⁺ and CD8⁺ T cell number in spleen of BPK282-infected mice were significantly higher as of week 6 *p.i.* and reached similar values as in L82-infected mice (Fig. 5d).

Other main immune cell types which are involved in the spleen and liver immunopathology of *L. donovani* infections were investigated. B cells are involved in susceptibility to EVL, suppress protective T cell responses and dampen neutrophil-mediated pathology (46, 48). Inflammatory monocytes are important for parasite control of *L. donovani* infection in the liver via the formation of hepatic granulomas (49). The number of mature B cells (B220⁺ AA4.1⁺) increased significantly to a similar extent in the liver of L82- and BPK282-infected mice compared

to naive mice (Fig. 6a). In the spleen however, mature B cell decreased in numbers during L82 infection, but increased during BPK282 infection (Fig. 6b). The amount of inflammatory monocytes (CD11b⁺ Ly6C⁺) did not significantly increase upon L82 or BPK282 infection compared to naive mice in both the spleen and liver (Fig. 6c and d).

Infection with *L. donovani* L82 or BPK282 strain induces the production of IFN- γ

IFN- γ is a key effector cytokine generating a protective immune response upon *L. donovani* infection by activating monocytes and macrophages to exhibit enhanced anti-leishmanial activity (50). We hypothesized that the lower

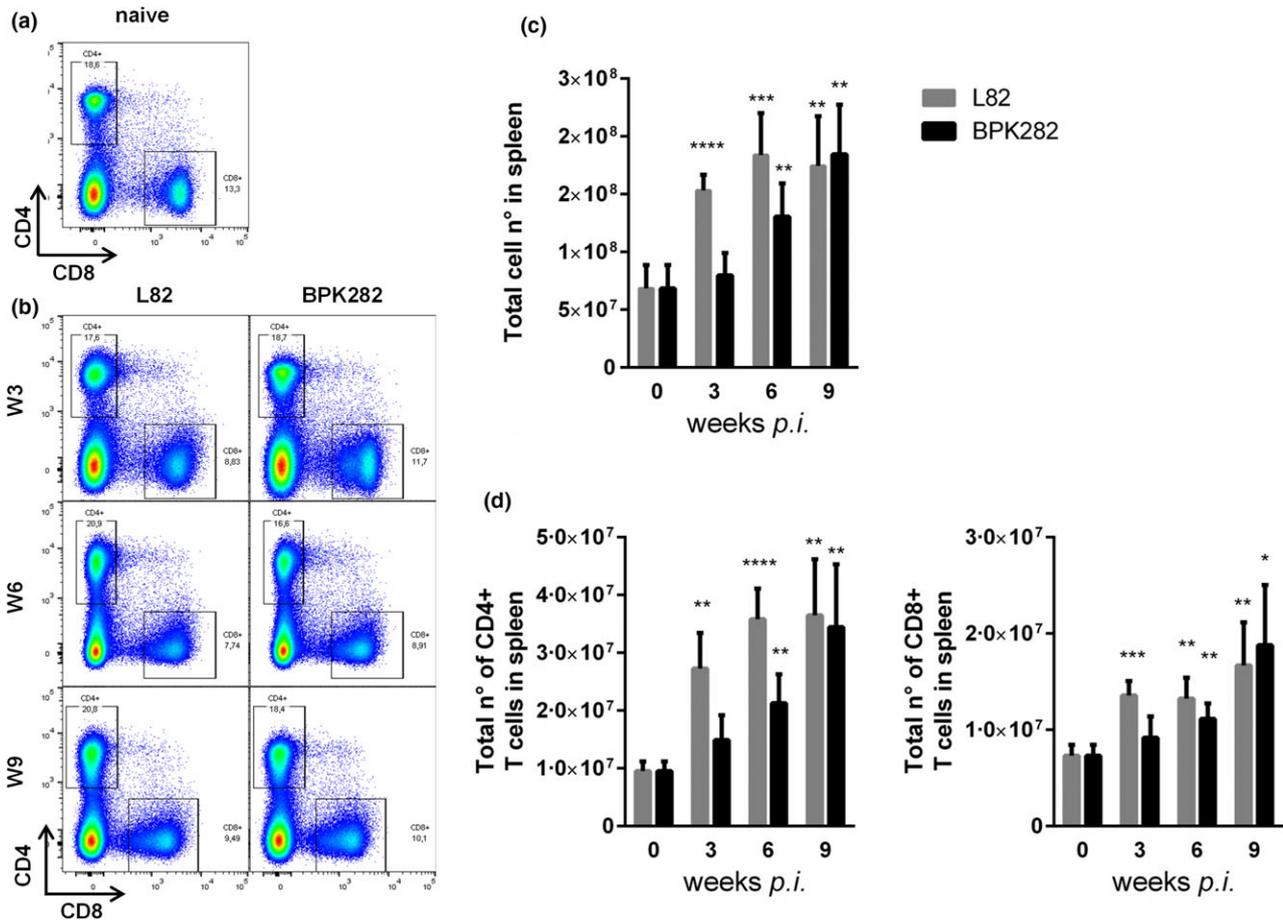


Figure 5 Splenes of BPK282-infected mice accumulate less CD4⁺ T and CD8⁺ T cells as compared to L82-infected mice. Flow cytometry analysis of the spleen of infected C57BL/6 mice with either 2×10^7 amastigotes of the *L. donovani* BPK282 strain or the *L. donovani* L82 strain. Spleen cells were pre-gated according to size, granularity and livability. Hereafter, the cells were gated on their expression of CD4 and CD8. (a) Spleen cells of naive mice. (b) Spleen cells from L82-infected (left panels) and BPK282-infected (right panels) mice over the course of infection (3 weeks, w3; 6 weeks, w6; 9 weeks, w9). (c) Total cell number of naive and infected spleens. (d) Total cell numbers of CD4⁺ T cells (left panel) and CD8⁺ T cells (right panel) in the spleen of naive and infected mice over the course of infection (grey, L82; black, BPK282). Data represent three independent experiments with at least four mice per group. Error bars are shown as standard deviations (SD). Student *t*-test was performed between infected and naive mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *****P* < 0.0001.

virulence of the BPK282 strain as opposed to the L82 strain could be due to a lower activation of the immune system towards a Th1 response generated by the former. IFN- γ analysis in the serum of L82- and BPK282-infected C57BL/6 mice indicated a similar IFN- γ response upon L82 infection as upon BPK282 infection after 4 and 6 weeks *p.i.* (Fig. 7).

DISCUSSION

Until now, most *in vivo* studies investigating the immunopathology of *L. donovani* infections were performed with the LV9 strain (51–54) and the 1S Sudan strain (31, 55–57), both originating from East Africa. *L. donovani* L82 strain has mostly been described *in vitro* (6, 36–38) and was poorly studied in *in vivo* mouse models (39, 40) or in the Syrian hamster model (5, 41). Very few studies on *L. donovani* clinical isolate strains exist due to the difficulty to adapt those strains to the animal host (20, 42–44). Here, we described two other *L. donovani* strains, one Ethiopian strain (L82) first described by R.S. Bray in 1969, and one Nepalese strain (BPK282), first described by Decuypere *et al.* in 2005 (58) both in the C57BL/6 mouse model and the Syrian hamster model. Not only do

L. donovani strains from the Indian subcontinent differ strongly from East Africa genomically (19), but the diseases they induce are also quite different. For yet unknown reasons, the proportion of post-kala-azar dermal leishmaniasis (PKDL) is much higher in East Africa (50–60%) compared to in the Indian continent (5–15%) (21). The response to therapy is different; for instance sodium stibogluconate (SSG) does not work anymore in the Indian subcontinent, while it works perfectly in East Africa (3, 22, 23). Thus, besides the need for additional *in vivo* strains, it is essential to have models for visceral leishmaniasis of both regions.

First, a RFLP assay was performed to determine to what extent these strains were different from each other. Moreover, the *L. donovani* L82 strain is originally from Ethiopia and much confusion exists as to whether this strain is inherently different from the LV9 strain, as observed in the different nomenclature used for this strain in literature (36, 38, 59). Based on the technique developed by Bhattarai *et al.* (35) for the discrimination of Nepalese *L. donovani* strains, three observations could be made. Firstly, by including the *L. donovani* BPK282 strain which was continuously passaged *in vitro*, it was observed that *in vitro* culturing did not drastically modify the BPK282 strain on a genetic

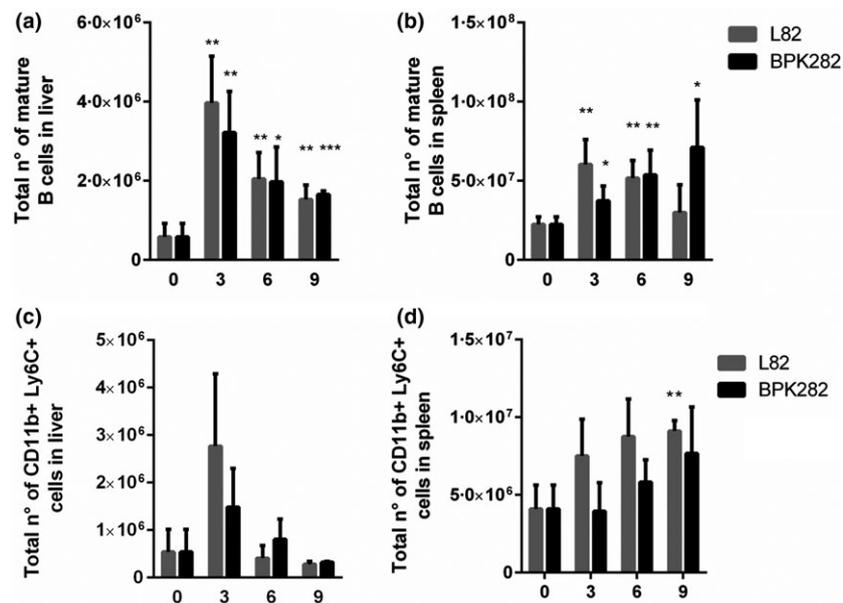


Figure 6 Splens and livers of BPK282-infected mice accumulate mature B cells and inflammatory monocytes to the same extent as L82-infected mice. Flow cytometry analysis of the spleen and liver of infected C57BL/6 mice with either 2×10^7 amastigotes of the *L. donovani* BPK282 strain or the *L. donovani* L82 strain. Spleen and liver cells were pre-gated according to size, granularity and livability. Hereafter, the cells were gated on their expression of AA4-1⁻ B220⁺ (B cells) and CD11b⁺ Ly6C⁺ (inflammatory monocytes). Total number of mature B cells in the liver (a) and the spleen (b) of naive and infected mice over the course of infection (3 weeks, w3; 6 weeks, w6; 9 weeks, w9). Total number of inflammatory monocytes in the liver (c) and the spleen (d) of naive and infected mice over the course of infection (3 weeks, w3; 6 weeks, w6; 9 weeks, w9). Data represent three independent experiments with at least four mice per group. Error bars are shown as standard deviations (SD). Student *t*-test was performed between infected and naive mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

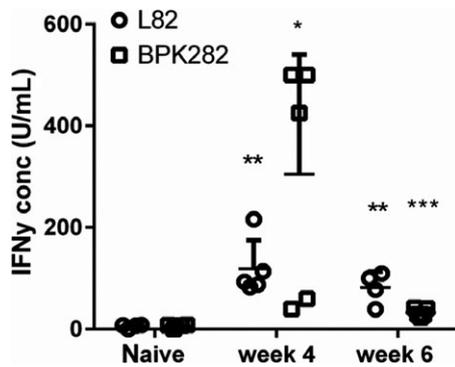


Figure 7 Murine infection with *L. donovani* L82 or BPK282 strain induces the production of IFN- γ . ELISA analysis for the detection of IFN- γ in the serum of naive, L82- or BPK282-infected C57BL/6 mice after 4 and 6 weeks of infection. Error bars are shown as standard deviations (SD). Student *t*-test was performed between infected and naive mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

level and it still could be identified by RFLP. Nevertheless, fluctuations outside the kDNA minicircle region targeted by this assay cannot be excluded. Secondly, the RFLP showed that the *L. donovani* L82 strain significantly differed from the *L. donovani* LV9 strain, indicating a genotypic difference between L82 and LV9. The nomenclatures L82, Hu3 (also known as LV9) have been used interchangeably to describe a *L. donovani* strain, which was originally documented by R.S. Bray in 1967 and taken up into the bank of strains by the WHO. To our understanding, these three names initially referred to the same *L. donovani* strain. Considering the high plasticity of the genome of *L. donovani* (60), this strain could have diverged genomically over time and generate distinct genetic strains, as observed in this report by RFLP assay. Thirdly, the RFLP assay demonstrated that the *L. donovani* BPK282 strain was different from both the L82 and the LV9 strain. This RFLP assay could be implemented as a routine quality control to ensure the authenticity of the *L. donovani* strains that are exchanged between research laboratories for *in vivo* studies. Infections in hamsters revealed how different these two *L. donovani* strains were at inducing the clinical immunopathological symptoms of VL. The *L. donovani* L82 strain generated a virulent form of the disease in hamsters, characterized by an important weight loss as of week 6 *p.i.*, high parasitaemia and hepatosplenomegaly, comparable to LV9 or 1S infections (61–63). In contrast, the *L. donovani* BPK282 strain induced a milder form of the disease, characterized by minimal weight loss over the course of infection, lower parasitaemia levels and no significant increase in spleen and liver weights in hamsters.

Similarly to infections in the hamster, murine infections in C57BL/6 mice with the *L. donovani* L82 strain lead to

high parasite levels in the spleen and liver with concomitant hepatosplenomegaly, similarly to what is documented for *L. donovani* LV9 and 1S strains (39, 64–67). The *L. donovani* BPK282 strain also induced increasing parasitaemia levels in spleen and liver and hepatosplenomegaly but the kinetics seemed shifted later and never reached the same levels as with the L82 strain. This difference in inducing pathology between L82 and BPK282 indicated an inherently lower virulence of the *L. donovani* BPK282 strain as compared to the *L. donovani* L82 strain. However, as opposed to murine infections with *L. donovani* LV9 and 1S strains which clear liver parasitaemia after 6 weeks *p.i.*, livers of C57BL/6 mice infected with either L82 or BPK282 were only cleared from parasites after 12 weeks *p.i.* (data not shown). This again shows the variation that exists between different *L. donovani* strains as to the pathologic response they evoke in the mammalian host. The spectrum of pathological symptoms that can arise during visceral leishmaniasis should be broadened far beyond those observed with LV9 and 1S strains.

As expected from the increases in liver and spleen weights during L82 and BPK282 infection, the numbers of nonparenchymal liver cells after percoll gradient and total hematopoietic spleen cells were significantly higher for L82 infection than for BPK282 infection as compared to naive mice. Infection with L82 induced a faster and higher increase in the amount of CD4⁺ and CD8⁺ T cells in spleen and liver of mice as compared to BPK282 infection, which was not correlated to a better control of parasitaemia in L82-infected mice. These results stress the fact that functional information about the activation status of these CD4⁺ and CD8⁺ T cells is necessary to draw any conclusion. Besides their role in generating anti-parasitic immune responses, CD4⁺ T cells have also been shown have pro-leishmanial properties during *L. donovani* infections via the production of IL-10 (68). The higher relative virulence of *L. donovani* L82 strain compared to the BPK282 strain could not be observed by measuring the IFN- γ levels in the serum, as L82- and BPK282-infected mice displayed similar IFN- γ levels.

In summary, this report describes the pathological response upon murine and hamster infections of two *L. donovani* strains, which originated from two distinct geographical locations (*i.e.* L82 from Ethiopia and BPK282 from Nepal) and have been isolated from the original patient with about 50 years difference (*i.e.* L82 in 1967 and BPK282 in 2005). This work demonstrated that the L82 strain generated a pathologic response in hamsters and C57BL/6 mice, comparable to what has been seen for other confirmed laboratory strains, such as LV9 or 1S. However, the BPK282 strain displayed limited pathology both in hamster and C57BL/6 mice. Both BALB/c and

C57BL/6 mice and develop visceral leishmaniasis in a similar way as far as infections with LV9 and IS strains are concerned (69). Therefore, we speculate to observe a similar immunopathological response after L82 or BPK282 infection in BALB/c mice as reported here for C57BL/6 mice, being an overall higher virulence of the L82 strain as opposed to the BPK282 strain. Our results also suggest that the *L. donovani* BPK282 strain might not yet been fully adapted to the *in vivo* models or that these differences in virulence are region-specific (East Africa vs. Indian continent). This should be taken into account when using the *L. donovani* BPK282 strain in future investigations. Depending on the research question, the choice here lies between using a *L. donovani* strain (*i.e.* L82) with high experimental virulence but is further away from the original isolated strain or a *L. donovani* strain (*i.e.* BPK282) with lower experimental virulence, but is closer to the original isolated strain. Further investigations on other *L. donovani* strains from the Indian continent are required to elucidate whether these pathologic differences described

here are region-specific and can constitute a good model to explain the differences observed in the clinical practice. Furthermore, given that the *L. donovani* L82 and BPK282 strains come from two separate geographic regions, with distinct responses to drug treatment, these strains are appropriate for first-line testing of new drug compounds against experimental visceral leishmaniasis.

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