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

Received Date : 24-Jan-2016

Revised Date : 16-Mar-2016

Accepted Date : 19-Mar-2016

Article type : Original Paper

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

Florence Kauffmann[1,2], Franck Dumetz[3], Sarah Hendrickx[4], Eric Muraille[5], Jean-Claude Dujardin[3], Louis Maes[5], Stefan Magez[1,2], Carl De Trez[1,2].

1. Laboratory of Cellular and Molecular Immunology (CMIM), Vrije Universiteit Brussel (VUB), Brussels, Belgium

2. Department of Structural Biology Research Center, Vlaams Instituut voor Biotechnologie (VIB), Brussels, Belgium

3. Molecular Parasitology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerpen, Belgium.

4. Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Antwerpen, Belgium

5. Laboratory of Parasitology, Université Libre de Bruxelles, Brussels, Belgium

Corresponding author: fkauffma@vub.ac.be

Key words: *Leishmania spp,* Rodent, Flow cytometry, Immunopathology, Leishmaniasis

Disclosures: The authors report no conflict of interest.

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

Abstract

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 immunopathologic 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 parasitemia 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.

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 500000 new people contract VL and about 10000 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 behavior 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 clinicopathologic 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, hypergammaglobulinemia 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 post infection (*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 tumor 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 immunopathologic behavior 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 BPK282/0cl4) 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 $2x10^7$

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 non-infected 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 $2x10^7$ 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% fetal calf serum (FCS), 100 U/ml penicillin (GIBCO), 100 µg/ml streptomycin (GIBCO), 4 mM sodium $NaHCO_3$ (Merck), 0.0005% hemin (Sigma-Aldrich) and 0.1 mM adenosine (Sigma-Aldrich). A part of the suspension (200 µl) was plated in 96-well plates in triplicates at a 2-fold 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 1,000 host cell nuclei × 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) × 100.

Flow cytometry

Spleen and liver were harvested from CO_2 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 µm pore filter).

Hepatic non-parenchymal cells were prepared from the liver of naive or infected mice digested with 100 U/ml collagenase III (Worthington Biochemical Corporation, Lakewood, New Jersey, USA) and 50 U/ml DNAse I (Worthington Biochemical Corporation, Lakewood, New Jersey, USA) in RPMI medium. The digestion was blocked with 20ml of 2.5 mM EDTA in RPMI medium and the suspension filtered (70 µm pore filter). The non-parenchymal 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 NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2-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).

Isolated cells were labeled 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), 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.). 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 n° 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). 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) before digesting 500ng of the amplification product by restriction enzyme *HaeIII* (Thermo Fisher Scientific) for 4h at 37°C. Digestion product was migrated on a 3% small fragment agarose gel (Eurogentec) for 5h 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). Values are expressed as mean ± 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 1A) and digestion with *HaeIII* restriction enzyme indicated the presence of three distinct *L. donovani* kinetoplast minicircle genotypes (Figure 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 (Figure 2A). In contrast, BPK282-infected hamsters continuously gained weight until 12 weeks *p.i.* (Figure 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 (Figure 2B). Moreover, the spleen of L82-infected hamsters contained significantly higher parasite loads than the one from BPK282-infected hamsters (Figure 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 (Figure 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 compared to naive hamsters (Figure 2D). BPK282-infected hamsters had higher numbers of splenic $CD4^+$ T cells and $MHCII^+$ B cells at week *8 p.i.* than naive mice (Figure 2D). The livers of L82- and BPK282-infected hamsters had similar amounts of non-parenchymal cells as found in naive hamsters (Figure 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 (Figure 2E).

The BPK282 strain induces lower levels of parasitemia 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-pathologic 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 $2x10^7$ amastigotes, we observed that the *L. donovani* L82 strain developed a peak parasitemia 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 (Figure 3A and 3B). In contrast, the BPK282 strain displayed lower parasitemia 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 (Figures 3A and 3B). 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) (Figure 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 (Figure 3C). In a similar way, the liver of BPK282-infected mice did not gain weight as much as the liver of L82-infected mice (Figure 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 (Figure 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 all three time points investigated, significantly higher liver cell numbers could be found in L82-infected mice compared to BPK282-infected mice (Figure 4C). During infection, the increase in CD4$^+$ T cell percentages compared to uninfected mice was higher during L82 infection (3-fold 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.*) (Figure 4B). CD8$^+$ T cell percentages in liver increased compared to naive situation but did not significantly differ between L82 and BPK282 infection (Figure 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 (Figure 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.* (Figure 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 (Figure 5A and 5B). Nevertheless, L82-infected mice had significantly higher numbers of CD4$^+$ and CD8$^+$ T cell numbers as of week 3 *p.i.* compared to naive mice (Figure 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 (Figure 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 (Figure 6A). In the spleen however, mature B cell decreased in numbers during L82 infection, but increased during BPK282 infection (Figure 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 (Figure 6C and 6D).

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 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.* (Figure 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 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 immunopathologic 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 parasitemia 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 parasitemia 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 parasitemia 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 parasitemia 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 pathologic 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 non-parenchymal 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 parasitemia 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 pathologic 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 1S strains are concerned (69). Therefore, we speculate to observe a similar immunopathologic 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 repsonses to drug treatment, these strains are appropriate for first-line testing of new drug compounds against experimental visceral leishmaniasis.

Acknowledgements

This work was supported by the Fonds voor Wetenschappelijk Onderzoek (FWO) Project fwoTM639, the Interuniverity Attraction Pole program (PAI-IAP N. P7/41) and the VUB PhD grant (DP/AP/JV/dbd/BEURS/451). Kauffmann F. and Dumetz F. performed the research. Muraille E., De Trez C. and Magez S. designed the research study. Dujardin J.C., Maes L. and Hendrickx S. contributed to the parasites and tools. Kauffmann F. and Dumetz F. analysed the data. Kauffmann F., Magez S. and De Trez C. wrote the paper.

References

1. Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 2004; **27**: 305–18.

2. Alvar J, Vélez ID, Bern C, *et al.* Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 2012; **7**: e35671.

3. Croft SL, Sundar S, Fairlamb AH. Drug Resistance in Leishmaniasis. *Clin Microbiol Rev* 2006; **19**: 111–126.

4. Chappuis F, Sundar S, Hailu A, *et al.* Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 2007; **5**: 873–82.

5. Maes L, Germonprez N, Quirijnen L, Van Puyvelde L, Cos P, Vanden Berghe D. Comparative activities of the triterpene saponin maesabalide III and liposomal amphotericin B (AmBisome) against Leishmania donovani in hamsters. *Antimicrob Agents Chemother* 2004; **48**: 2056–60.

6. Vermeersch M, da Luz RI, Toté K, Timmermans J-P, Cos P, Maes L. In vitro susceptibilities of Leishmania donovani promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrob Agents Chemother* 2009; **53**: 3855–9.

7. Berg M, García-Hernández R, Cuypers B, *et al.* Experimental resistance to drug combinations in Leishmania donovani: metabolic and phenotypic adaptations. *Antimicrob Agents Chemother* 2015; **59**: 2242–55.

8. Berg M, Vanaerschot M, Jankevics A, *et al.* Metabolic adaptations of Leishmania donovani in relation to differentiation, drug resistance, and drug pressure. *Mol Microbiol* 2013; **90**: 428–42.

9. Bhandari V, Sundar S, Dujardin JC, Salotra P. Elucidation of cellular mechanisms involved in experimental paromomycin resistance in Leishmania donovani. *Antimicrob Agents Chemother* 2014.

10. Mukherjee B, Mukhopadhyay R, Bannerjee B, *et al.* Antimony-resistant but not

antimony-sensitive Leishmania donovani up-regulates host IL-10 to overexpress multidrug-resistant protein 1. *Proc Natl Acad Sci U S A* 2013; **110**: E575–82.

11. Mukherjee B, Paul J, Mukherjee S, *et al.* Antimony-Resistant Leishmania donovani Exploits miR-466i To Deactivate Host MyD88 for Regulating IL-10/IL-12 Levels during Early Hours of Infection. *J Immunol* 2015; **195**: 2731–42.

12. Guha R, Das S, Ghosh J, Sundar S, Dujardin JC, Roy S. Antimony resistant Leishmania donovani but not sensitive ones drives greater frequency of potent T-regulatory cells upon interaction with human PBMCs: role of IL-10 and TGF-β in early immune response. *PLoS Negl Trop Dis* 2014; **8**: e2995.

13. Stauber LA. Characterization of strains of Leishmania donovani. *Exp Parasitol* 1966; **18**: 1–11.

14. Yardley V, Gamarro F, Croft SL. Antileishmanial and antitrypanosomal activities of the 8-aminoquinoline tafenoquine. *Antimicrob Agents Chemother* 2010; **54**: 5356–8.

15. Lunagariya NA, Gohil VM, Kushwah V, *et al.* Design, synthesis and biological evaluation of 1,3,6-trisubstituted β-carboline derivatives for cytotoxic and anti-leishmanial potential. *Bioorg Med Chem Lett* 2016; **26**: 789–94.

16. Gupta S, Yardley V, Vishwakarma P, *et al.* Nitroimidazo-oxazole compound dndi-vl-2098: An orally effective preclinical drug candidate for the treatment of visceral leishmaniasis. *J Antimicrob Chemother* 2015; **70**: 518–527.

17. Mallick S, Dutta A, Ghosh J, *et al.* Protective therapy with novel chromone derivative against Leishmania donovani infection induces Th1 response in vivo. *Chemotherapy* 2011; **57**: 388–93.

18. Bhaumik SK, Naskar K, De T. Complete protection against experimental visceral leishmaniasis with complete soluble antigen from attenuated Leishmania donovani promastigotes involves Th1-immunity and down-regulation of IL-10. *Eur J Immunol* 2009; **39**: 2146–60.

19. Downing T, Imamura H, Decuypere S, *et al.* Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome Res* 2011; **21**: 2143–56.

20. Vanaerschot M, Maes I, Ouakad M, *et al.* Linking in vitro and in vivo survival of clinical Leishmania donovani strains. *PLoS One* 2010; **5**: e12211.

21. Zijlstra EE, Musa AM, Khalil EAG, el-Hassan IM, el-Hassan AM. Post-kala-azar dermal leishmaniasis. *Lancet Infect Dis* 2003; **3**: 87–98.

22. Sundar S, More DK, Singh MK, *et al.* Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis* 2000; **31**: 1104–7.

23. Rijal S, Chappuis F, Singh R, *et al.* Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit further decline. *Trans R Soc Trop Med Hyg* **97**: 350–4.

24. Loría-Cervera EN, Andrade-Narváez FJ. Animal models for the study of leishmaniasis immunology. *Rev Inst Med Trop Sao Paulo* **56**: 1–11.

25. Stern JJ, Oca MJ, Rubin BY, Sylvia L, Murray HW. ROLE OF L3T4 + AND LYT-2 + CELLS IN EXPERIMENTAL VISCERAL LEISHMANIASIS ' by the abil-. 1988.

26. Kaye PM, Curry a J, Blackwell JM. Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. *J Immunol* 1991; **146**: 2763–2770.

27. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. *Front Immunol* 2012; **3**: 251.

28. Tumang MC, Keogh C, Moldawer LL, *et al.* Role and effect of TNF-alpha in experimental visceral leishmaniasis. *J Immunol* 1994; **153**: 768–75.

29. Murray HW, Xiang Z, Ma X. Responses to Leishmania donovani in mice deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Am J Trop Med Hyg* 2006; **74**: 1013–1015.

30. Bankoti R, Stäger S. Differential Regulation of the Immune Response in the Spleen and Liver of Mice Infected with Leishmania donovani. *J Trop Med* 2012; **2012**: 639304.

31. Melby PC, Tabares a, Restrepo BI, Cardona a E, McGuff HS, Teale JM. Leishmania donovani: evolution and architecture of the splenic cellular immune response related to control of infection. *Exp Parasitol* 2001; **99**: 17–25.

32. Titus RG, Marchand M, Boon T, Louis JA. A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. *Parasite Immunol* 1985; **7**: 545–55.

33. Liu H, Alder JD, Steiner BM, Stein-Streilein J, Lim L, Schell RF. Role of L3T4+ and 38+ T-cell subsets in resistance against infection with Treponema pallidum subsp. pertenue in hamsters. *Infect Immun* 1991; **59**: 529–36.

34. Witte PL, Streilein JW. Monoclonal antibodies to hamster class II MHC molecules distinguish T and B cells. *J Immunol* 1983; **130**: 2282–6.

35. Bhattarai NR, Dujardin JC, Rijal S, De Doncker S, Boelaert M, Van der Auwera G. Development and evaluation of different PCR-based typing methods for discrimination of Leishmania donovani isolates from Nepal. *Parasitology* 2010; **137**: 947–57.

36. Debrabant A, Joshi MB, Pimenta PF., Dwyer DM. Generation of Leishmania donovani axenic amastigotes: their growth and biological characteristics. *Int J Parasitol* 2004;

34: 205–217.

37. Zelazny AM, Fedorko DP, Li LI, Neva FA, Fischer SH. EVALUATION OF 7SL RNA GENE SEQUENCES FOR THE IDENTIFICATION OF LEISHMANIA SPP . 2005; **72**: 415–420.

38. García-Hernández R, Manzano JI, Castanys S, Gamarro F. Leishmania donovani develops resistance to drug combinations. *PLoS Negl Trop Dis* 2012; **6**: e1974.

39. Satoskar A, Bluethmann H, Alexander J. Disruption of the murine interleukin-4 gene inhibits disease progression during Leishmania mexicana infection but does not increase control of Leishmania donovani infection. *Infect Immun* 1995; **63**: 4894–9.

40. Bradley DJ& KJ. Regulation of Leishmania populations within the host. 1977: 119–129.

41. Hendrickx S, Mondelaers A, Eberhardt E, Delputte P, Cos P, Maes L. In vivo selection of paromomycin and miltefosine resistance in Leishmania donovani and L. infantum in the Syrian hamster model. *Antimicrob Agents Chemother* 2015.

42. Hendrickx S, Leemans A, Mondelaers A, *et al.* Comparative Fitness of a Parent Leishmania donovani Clinical Isolate and Its Experimentally Derived Paromomycin-Resistant Strain. *PLoS One* 2015; **10**: e0140139.

43. Vanaerschot M, Doncker S De, Rijal S, *et al.* Antimonial resistance in Leishmania donovani is associated with increased in vivo parasite burden. *PLoS One* 2011; **6**: 1–5.

44. Pandey K, Yanagi T, Pandey BD, Mallik AK, Sherchand JB, Kanbara H. Characterization of Leishmania isolates from Nepalese patients with visceral leishmaniasis. *Parasitol Res* 2007; **100**: 1361–9.

45. Bunn PT, Stanley AC, de Labastida Rivera F, *et al.* Tissue Requirements for Establishing Long-Term CD4+ T Cell-Mediated Immunity following Leishmania donovani Infection. *J Immunol* 2014.

46. Smelt SC, Cotterell SE, Engwerda CR, Kaye PM. B cell-deficient mice are highly resistant to Leishmania donovani infection, but develop neutrophil-mediated tissue pathology. *J Immunol* 2000; **164**: 3681–8.

47. Tsagozis P, Karagouni E, Dotsika E. CD8(+) T cells with parasite-specific cytotoxic activity and a Tc1 profile of cytokine and chemokine secretion develop in experimental visceral leishmaniasis. *Parasite Immunol* **25**: 569–79.

48. Bankoti R, Gupta K, Levchenko A, Stäger S. Marginal zone B cells regulate antigen-specific T cell responses during infection. *J Immunol* 2012; **188**: 3961–71.

49. Cervia JS, Rosen H, Murray HW. Effector role of blood monocytes in experimental visceral leishmaniasis. *Infect Immun* 1993; **61**: 1330–1333.

50. Murray HW, Stern JJ, Welte K, Rubin BY, Carriero SM, Nathan CF. Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin 2 and interferon-gamma. *J Immunol* 1987; **138**: 2290–7.

51. Engwerda CR, Ato M, Cotterell SEJ, *et al.* A Role for Tumor Necrosis Factor- ␣ in Remodeling the Splenic Marginal Zone during Leishmania donovani Infection. 2002; **161**: 429–437.

52. Yurdakul P, Dalton J, Beattie L, Brown N, Erguven S. Compartment-Specific Remodeling of Splenic Micro- Architecture during Experimental Visceral Leishmaniasis. 2011; **179**: 23–29.

53. Moore JWJ, Beattie L, Dalton JE, *et al.* B cell: T cell interactions occur within hepatic granulomas during experimental visceral leishmaniasis. *PLoS One* 2012; **7**: e34143.

54. Smelt SC, Engwerda CR, McCrossen M, Kaye PM. Destruction of follicular dendritic cells during chronic visceral leishmaniasis. *J Immunol* 1997; **158**: 3813–3821.

55. Melby PC, Yang Y-Z, Cheng J, Zhao W. Regional Differences in the Cellular Immune Response to Experimental Cutaneous or Visceral Infection with Leishmania donovani. *Infect Immun* 1998; **66**: 18–27.

56. Osorio EY, Travi BL, da Cruz AM, Saldarriaga OA, Medina AA, Melby PC. Growth factor and Th2 cytokine signaling pathways converge at STAT6 to promote arginase expression in progressive experimental visceral leishmaniasis. *PLoS Pathog* 2014; **10**: e1004165.

57. Osorio Y, Travi BL, Renslo AR, Peniche AG, Melby PC. Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system. *PLoS Negl Trop Dis* 2011; **5**: e962.

58. Decuypere S, Rijal S, Yardley V, *et al.* Gene expression analysis of the mechanism of natural Sb(V) resistance in Leishmania donovani isolates from Nepal. *Antimicrob Agents Chemother* 2005; **49**: 4616–21.

59. Sadlova J, Yeo M, Seblova V, *et al.* Visualisation of Leishmania donovani fluorescent hybrids during early stage development in the sand fly vector. *PLoS One* 2011; **6**: e19851.

60. Mannaert A, Downing T, Imamura H, Dujardin J-C. Adaptive mechanisms in pathogens: universal aneuploidy in Leishmania. *Trends Parasitol* 2012; **28**: 370–6.

61. Aslan H, Dey R, Meneses C, *et al.* A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. *J Infect Dis* 2013; **207**: 1328–38.

62. Lafuse WP, Story R, Mahylis J, *et al.* Leishmania donovani infection induces anemia

in hamsters by differentially altering erythropoiesis in bone marrow and spleen. *PLoS One* 2013; **8**: e59509.

63. Melby PC, Chandrasekar B, Zhao W, Coe JE. The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. *J Immunol* 2001; **166**: 1912–20.

64. Ato M, Stäger S, Engwerda CR, Kaye PM. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat Immunol* 2002; **3**: 1185–91.

65. Stanley AC, de Labastida Rivera F, Haque A, *et al.* Critical roles for LIGHT and its receptors in generating T cell-mediated immunity during Leishmania donovani infection. *PLoS Pathog* 2011; **7**: e1002279.

66. Owens BMJ, Beattie L, Moore JWJ, *et al.* IL-10-producing Th1 cells and disease progression are regulated by distinct $CD11c^+$ cell populations during visceral leishmaniasis. *PLoS Pathog* 2012; **8**: e1002827.

67. Joshi T, Rodriguez S, Perovic V, Cockburn I a, Stäger S. B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against Leishmania donovani infections. *PLoS Pathog* 2009; **5**: e1000431.

68. Cd I-, Stäger S, Maroof A, *et al.* Distinct roles for IL-6 and IL-12p40 in mediating protection against Leishmania donovani and the expansion of IL-10+ CD4+ T Cells. 2009; **36**: 1764–1771.

69. Murray HW. Tissue granuloma structure-function in experimental visceral leishmaniasis. 2001.

Legends to figures

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.

Figure 2: *L. donovani* BPK282 infection in hamsters results in lower virulence than *L. donovani* L82 infection. Syrian hamsters were infected with $2x10^7$ 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) Parasitemia 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 $2x10^7$ 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 3 hamsters per group. Error bars are shown as standard deviations (SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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 $2x10^7$ amastigotes from the *L. donovani* L82 strain or the *L. donovani* BPK282 strain. Parasitemia 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 4 mice per group. Error bars are shown as standard deviations (SD). Student T test was performed between infected and naive mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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 $2x10^7$ amastigotes of the *L. donovani* BPK282 strain or the *L. donovani* L82 strain. Non-parenchymal 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 (gray, L82; black, BPK282). over the course of infection. Data represent three independent experiments with at least 4 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 5: Spleens 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 $2x10^7$ 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 (gray, L82; black, BPK282). Data represent three independent experiments with at least 4 mice per group. Error bars are shown as standard deviations (SD). Student T test was performed between infected and naive mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 6: Spleens 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 $2x10^7$ 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 4 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. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.