

Human hepatic stellate cells in primary culture are safe targets for *Leishmania donovani*

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SUMMARY

Leishmania parasites can escape the immune response by invading cell types lacking leishmanicidal mechanisms. Silent persistence of *Leishmania* parasites in the host organism is responsible for asymptomatic carriage and relapses after cured leishmaniasis. Here, we studied the interaction between Hepatic Stellate Cells (HSC) and *Leishmania*. An original model of human HSC in primary culture infected with *L. donovani* was developed. The presence of intracellular parasites was studied and quantified using optical and confocal microscopy. HSC characteristics were studied using microscopy, methylene blue assay, long-term cultures and qPCR. We showed for the first time that human HSC are permissive to *L. donovani* infection, with no modification of HSC survival, growth rate and proinflammatory and fibrogenic characteristics. Intracellular parasites did not replicate but HSC had no effect on their survival. Indeed, after a 40-day culture, infected HSC cultures transferred on NNN medium yielded new promastigotes that were able to proliferate and efficiently infect new cells. HSC are permissive to *L. donovani*, with neither parasite killing nor apparent cell damage. Thus, HSC could act as potent sanctuary cells for *Leishmania* in the liver, which could partially explain parasite reactivation after an asymptomatic carriage or a cured visceral leishmaniasis.

Key words: *Leishmania donovani*, visceral leishmaniasis, liver, hepatic stellate cells, sanctuary cells, asymptomatic carriage, relapses.

INTRODUCTION

Visceral leishmaniasis (VL) is a vector-borne systemic disease, caused by *Leishmania* protozoans, especially *L. donovani* and *L. infantum* (*L. chagasi*). More than 500 000 VL cases per year are reported worldwide (Desjeux, 2004). Following promastigote inoculation by sandflies, parasites are passively phagocytosed by competent cells and disseminate through the blood flow towards target organs, i.e. bone marrow, spleen and liver. The main symptoms of VL are anarctic fever, hepatosplenomegaly, substantial weight loss, and finally death if left untreated. Efficient chemotherapy usually leads to a life-long immune response and allows clearance of *Leishmania* intracellular amastigotes and patient recovery. However, relapse or reactivation episodes frequently occur in seriously immunocompromised patients such as HIV-positive patients (Alvar *et al.* 1997, 2008), but also in organ transplant patients

(Basset *et al.* 2005) and occasionally in other patients who are apparently immunocompetent (Dereure *et al.* 2003; Gorski *et al.* 2010; Kajaia *et al.* 2011). Reactivation can occur after several years of residence in a non-endemic area and despite a well conducted therapy. Moreover, after *L. infantum* (*L. chagasi*) infection, it is now admitted that a great number of infections can remain asymptomatic and the disease can develop later on (Michel *et al.* 2011).

Leishmania parasites have developed various strategies to escape from the host immune system, which have been extensively reviewed (Zambrano-Villa *et al.* 2002; Antoine *et al.* 2004; Olivier *et al.* 2005; Kima, 2007; Bogdan, 2008). Intracellular amastigotes are characterized by having a high resistance against low pH, hydrogen peroxide (H₂O₂), nitric oxide (NO) and lysosomal enzymes (Mauel, 1996; Miller *et al.* 2000). Then, amastigotes are able to initiate an inappropriate immune response with reduced interleukin (IL-12) that allows parasite survival (Reiner *et al.* 1994). Finally, *Leishmania* parasites can evade the immune response, by invading cell types lacking leishmanicidal effector mechanisms, using them as sanctuary cells where they can persist in a latent stage until their further release

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(Rittig and Bogdan, 2000). Limited data are available on potential sanctuary cells in deep sites during VL, such as the spleen or the liver.

During VL, Kupffer cells (KC) are target cells for *Leishmania* in the liver and allow the formation of inflammatory granulomas that lead to parasite clearance (Murray, 2001; Kaye *et al.* 2004). Hepatocytes which are not professional phagocytic cells, were shown to be permissive to *L. donovani in vitro* (Gangneux *et al.* 2005). Hepatic stellate cells (HSC) are another important cell type in the liver. They are localized in the Disse's spaces and account for approximately one third of the non-parenchymal cell population (Friedman, 2008b). HSC are at a quiescent stage in the healthy liver but can become activated in the setting of chronic hepatic inflammation. Once activated, HSC proliferate and acquire a myofibroblast-like phenotype (Friedman, 2008a). They can secrete pro-fibrotic cytokines such as TGF- β , IL-6 and IL-33 (Gressner *et al.* 2002; Thirunavukkarasu *et al.* 2006; Marvie *et al.* 2010), and contribute to hepatic fibrosis through production of collagen fibres and structural glycoproteins (Tsukada *et al.* 2006), and inhibition of matrix degradation (Arthur, 2000). Interestingly, an increase of the number of HSC, the deposition of collagen fibres and the enlargement of the Disse's spaces were demonstrated in 35 out of 47 biopsies or necropsies from VL patients (Duarte and Corbett, 1987). Whereas skin and lymph node fibroblasts have been shown to be permissive to *L. major*, no data are available about the interaction of HSC and *Leishmania donovani*. Skin fibroblasts were proposed to be safe targets for *L. major* during cutaneous leishmaniasis (CL), which could explain life-long parasite persistence and patient relapses after efficient chemotherapy (Bogdan *et al.* 2000). In this work, using human HSC in primary culture infected with *L. donovani*, we analysed the complex interplay between this particular cell type and *L. donovani*. We showed that quiescent, as well as activated HSC, are permissive to *L. donovani*, with no apparent impact on HSC functional characteristics following the cell exposure to parasites. Conversely, HSC had no impact on intracellular parasite survival and infection capability, indicating that these cells could be another sanctuary cell type for the parasite.

MATERIALS AND METHODS

Parasite culture

Leishmania donovani strain MHOM/SD/97/LEM3427 Zym MON-18 was isolated from a patient with VL and cryopreserved at the WHO collaborative centre of Montpellier (French Reference Center for *Leishmania*). This strain is regularly passed in mouse to recover promastigotes from the amastigotes of the spleen or the liver, and then it is grown *in vitro*

on Novy-McNeal-Nicolle (NNN) blood Agar. Prior to infection, amplification of promastigotes was carried out by culture in Schneider's *Drosophila* medium (Gibco, Invitrogen) supplemented with 10% FCS, penicillin 200 IU/ml and streptomycin 200 μ g/ml for 6 days, until they reached the infective stationary phase. The infectivity of *L. donovani* promastigotes was assessed by efficient infection of THP-1 driven macrophages and mice.

Cell culture

Human HSC were isolated as previously described from histologically normal biopsy specimens after partial hepatectomy in adult patients from both sexes undergoing hepatic resection for liver metastases (Le Pabic *et al.* 2005; Bourd-Boittin *et al.* 2008; Marvie *et al.* 2010). Access to the biopsy material was in agreement with French laws and satisfied the requirements of the Ethics Committee of the institution. Dissociation of the liver was performed by perfusion with a pronase and collagenase solution by the company Biopredic International (Rennes, France). HSC purification was performed from the first supernatant of liver perfusion enriched in non-parenchymal cells after hepatocyte isolation. It relies on particular properties of HSC naturally rich in vitamin-A, which allow their purification by a single-step density-gradient centrifugation with Nycodenz (Sigma-Aldrich[®]). For this, the supernatant was centrifuged and the pellet was re-suspended with 7 ml of final volume of cold Gey's balanced salt solution (GBSS), and added with 5 ml of calcium-free GBSS Nycodenz 29%. After mixing of the tube, 1 ml of calcium-free GBSS BSA 0.3% was gently placed on the cell suspension surface. The tube was then centrifuged for 15 min at 1400 *g*, to allow HSC collection at the interface between Nycodenz and BSA, whereas other residual cell types fell to the bottom of the tube. Finally, HSC were washed once in GBSS and seeded on tissue-culture dishes treated by vacuum gas plasma (BD Falcon[™], USA) in DMEM GlutaMAX[™] containing glucose 1 g/l and completed with 10% FCS, penicillin 200 IU/ml and streptomycin 200 μ g/ml. HSC primary cultures were maintained at 37 °C with 5% CO₂ with a passage every 5–10 days. Purification and culture on plastic dishes is known to spontaneously initiate HSC activation (Friedman, 2008b), so that quiescent HSC are only available for a few days. HSC purity was assessed using a Cy3 mouse monoclonal antibody that specifically targets human α -Smooth Muscle Actin (α SMA) (Sigma-Aldrich[®]). Labelling of the HSC cytoskeleton showed that activated HSC obtained after the first passage and maintained until passage 8 were 100% α SMA positive.

THP-1 macrophage cells were propagated in RPMI medium containing 10% FCS, penicillin 200 IU/ml and streptomycin 200 μ g/ml.

Cell infection

Freshly purified quiescent HSC were seeded the day before their exposure to *L. donovani*. Activated HSC were seeded in similar conditions. Prior to infection, THP-1 cells were plated in the bottom of culture wells in RPMI supplemented with 2 μ M phorbol 12-myristate-13-acetate (Sigma-Aldrich®). In all cases, the medium was removed after overnight exposure to *L. donovani* (ratio cell/parasites: 1/10) and the wells were thoroughly washed to remove free promastigotes. At the end-point of each experiment, cells were stained with May-Grünwald Giemsa (MGG). The percentage of infected cells and the mean number of parasites per infected cell were recorded after microscopic examination of at least 100 cells per condition with a Zeiss Primostar microscope (magnification:1000X). Pictures were obtained with a Nikon 80i optical microscope (magnification: 600X) equipped with a numerical camera. Each experiment was repeated 3–4 times.

Fluorescent staining and confocal microscopy

Approximately 10^7 *L. donovani* promastigotes were labelled with 2 μ M of PKH-26 (Sigma-Aldrich®) or carboxy-fluorescein diacetate succinimidyl Ester (CFSE) (Invitrogen) during 5 min following the manufacturer's instructions. The labelled promastigotes were incubated overnight with activated HSC, directly seeded on a FlexiPERM® glass slide (Sigma-Aldrich®) (ratio cell/parasites: 1/10). The slide was vigorously washed 3 times in PBS, fixed with PBS4% formaldehyde, washed and dried. Cells were permeabilized using PBS and 0.5% Triton X-100. Then, we used the fluorescent membrane and Golgi apparatus marker AlexaFluor488-labelled wheat germ agglutinin (WGA) (Molecular Probe®, Invitrogen) or a Cy3 mouse monoclonal antibody anti-human α SMA (Sigma-Aldrich) to highlight HSC shapes, in the presence of a 100-fold excess of mouse immunoglobulins to avoid non-specific background. The cell and parasite nuclei were stained with DRAQ5 (Alexis® Biochemicals, Enzo Life Sciences). Cell slides were submitted to confocal analysis in 200 nm thick planes using a Leica TCS SP5 microscope (Leica Microsystems). Images were processed using LAS-AF software (Leica Microsystems) and images analyses were performed using ImageJ software.

HSC viability test

The viability of HSC exposed to *L. donovani* promastigotes was assessed by a methylene blue colourimetric assay as previously described (Meurette *et al.* 2005). Briefly, activated HSC were seeded in 96-well flat-bottomed plates at a density of 10^5 cells per well and exposed overnight to

L. donovani promastigotes at different cell/parasites ratios (1/5, 1/10 or 1/20). Cells were then thoroughly washed to remove promastigotes and maintained during 1, 3 or 7 days. At each endpoint, cells were washed twice with PBS, and fixed with absolute ethanol for 30 min. Following removal of ethanol, fixed cells were dried and stained for 5 min in methylene blue. After 3 washes with tap water, 100 μ l of 0.1 M HCl per well were added. Plates were analysed using a spectrophotometer (VersaMAX™, Molecular devices) at 620 nm. The percentage of viable cells was calculated by the following formula: (optical density values of HSC exposed to *L. donovani*/optical density values of control HSC) \times 100. HSC treated overnight with H₂O₂ 30% or staurosporine 1 μ M in complete DMEM medium were used as the cell death positive control. Statistical analysis was performed using the Mann-Whitney test to compare the viability of HSC exposed to parasites or toxic treatments compared to control uninfected and untreated HSC at each endpoint. A *P*-value <0.05 was considered significant and annotated *.

Long-term follow-up of infected HSC

Approximately 10^5 HSC from 3 different donor patients were exposed to *L. donovani* promastigotes (ratio cell/parasites: 1/10). The following day, the medium was changed and cells were washed with Ca²⁺- and Mg²⁺-free PBS to remove free promastigotes. The 3 cell cultures were maintained in parallel with their non-infected controls during 40 days. The medium was changed every 2 or 3 days and a passage with cell counting was performed every 10 days to quantify cell growth. A detailed observation under an inverted microscope showed the absence of free residual promastigotes in the medium before the first passage. After the fourth passage, cells were centrifuged. The cell pellet obtained from each culture was seeded in NNN blood agar, a culture medium usually used for parasite isolation from human specimens, which favours the propagation of amastigotes and their differentiation into mobile promastigotes. After several passages on NNN medium, promastigotes were recovered and amplified in Schneider's *Drosophila* medium and used to infect THP-1 cells as described above (Fig. 1).

Quantification of gene expression by real-time quantitative PCR (qPCR)

Total cellular RNA was extracted and purified using a NucleoSpin®RNAII Kit (Macherey-Nagel) and reverse transcribed with a High Capacity cDNA Reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time qPCR was performed in 96-well optical plates with

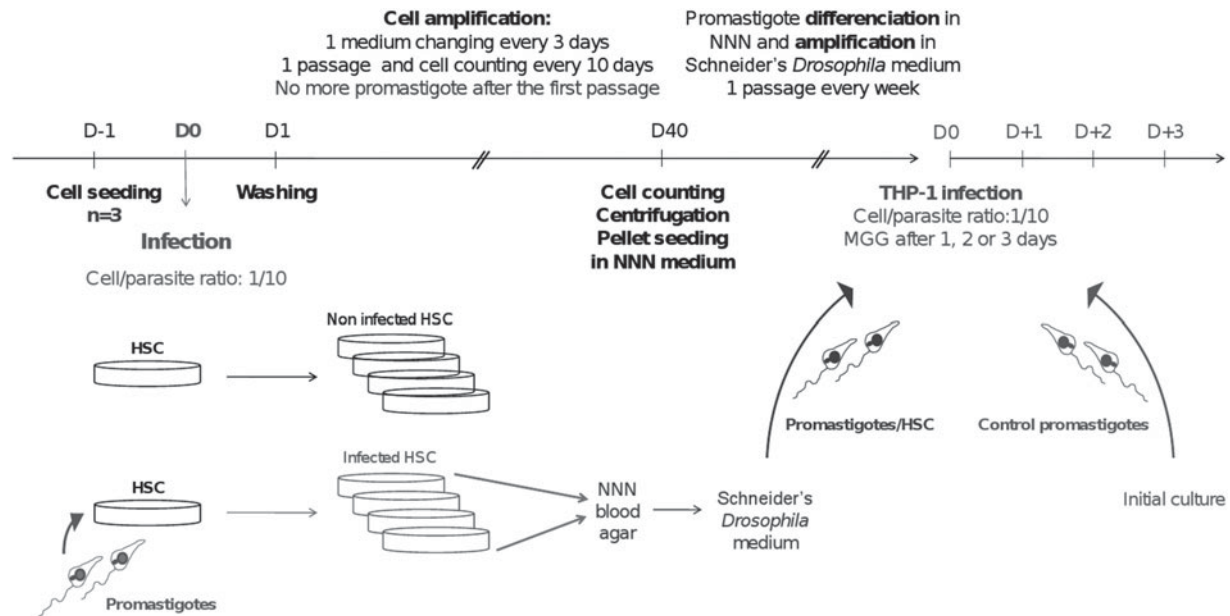


Fig. 1. Long-term monitoring of hepatic stellate cells (HSC) after overnight exposure to *Leishmania donovani*. HSC from 3 different patients were infected with *L. donovani* promastigotes. The following day, the medium was changed and cells were washed to remove free promastigotes. The 3 cell cultures were maintained in parallel with their non-infected controls until 40 days post-infection (D40), with a medium change every 2 or 3 days and a passage and total cell count performed every 10 days. At D40, the cells were centrifuged. The pellet obtained from each culture was seeded in NNN blood agar in order to reactivate intracellular parasites. After 1 month, the mobile promastigotes obtained were amplified in Schneider's *Drosophila* medium and used to infect THP-1 cells for 1, 2 or 3 days, in parallel to control promastigotes from the initial culture. Infection rates were quantified after MGG colouration.

Table 1. Primer sequences for qPCR analyses

Genes	Forward primers	Reverse primers
TGF- β	TGCGCTTGAGATCTTCAAACA	GGGCTAGTCGCACAGACCTC
TNF- α	AACCTCCTCTGCCATCAA	AACCTCCTCTGCCATCAA
IL-6	AGGCACTGGCAGAAAACAAC	TTTTACCAGGCAAGTCTCC
IL-33	AATCAGGTGACGCTGTTG	ACACTCCAGGATCAGTCTTG
IL-1 β	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA
MMP-2	TACTGAGTGGCCGTGTTTGC	AGGGAGCAGAGATTCCGACTT
TIMP-1	ACCCACAGACGGCCTTCT	CTTCTGGTGTCCCCACGAACTT
iNOS	ATGCTCAGCTCATCCGCTAT	CGATGCACAGCTGAGTGAAT
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTACG

Power SYBR[®] green PCR MasterMix (Applied Biosystems), 300 nM primers and cDNA corresponding to 30 ng of total RNA input in a final volume of 20 μ l using the 7300 sequence detection system ABIPrism (Applied Biosystems). Each sample was tested in duplicate in at least 3 independent experiments, to analyse different genes using primers whose sequences are presented in Table 1. The 18S mRNA level was used for normalization of gene expression and induction was calculated by comparison to a 1-day culture of non-infected HSC. The use of 18S as a housekeeping gene for data normalization was validated using SDHA and GAPDH as other control genes (data not shown). A Mann-Whitney statistical analysis was performed to compare the gene expression between

HSC exposed to parasites and control HSC at each endpoint.

RESULT

HSC are permissive to Leishmania donovani

Human activated HSC from 4 different patients were plated on slides and infected overnight with *L. donovani* promastigotes. Then, the medium was removed to eliminate free promastigotes, and the culture was stopped (Day 1) or fresh medium was added for 1 or 2 more day(s) (Day 2 and Day 3). After MGG staining, the presence of intracellular amastigotes was easily recognizable by their 2–5 μ m flagellum-free ovoid shape, from the first day after

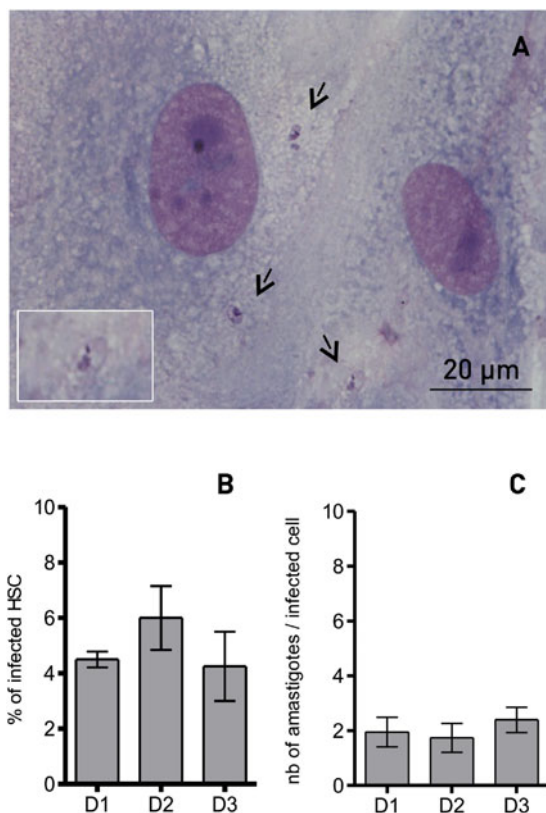


Fig. 2. Analysis of activated hepatic stellate cell (HSC) permissivity to *Leishmania donovani* using optical microscopy. Activated HSC were infected with *L. donovani* promastigotes overnight. After a 1-, 2- or 3-day culture, slides were stained with MGG and examined under optical microscopy. (A) Representative slide of infected HSC (magnification: 600X). (B) Percentage of infected cells over time. (C) Mean number of parasites per infected cell. Arrows indicate intracellular parasites. Results are expressed as mean \pm S.E.M. of at least 3 independent experiments.

cell exposure to *L. donovani* (Fig. 2A). The mean percentage of amastigote-carrying cells was $4.6 \pm 0.3\%$, $6.0 \pm 1.2\%$, and $4.3 \pm 1.3\%$ at Day 1, Day 2 and Day 3, respectively (Fig. 2B). The mean number of parasites per cell was low and stable during the experiment and reached 1.9 ± 0.5 at Day 1, 1.7 ± 0.5 at Day 2 and 2.1 ± 0.6 at Day 3 (Fig. 2C). It was also unchanged after 1 week (data not shown), suggesting that intracellular *Leishmania* were not able to significantly proliferate inside activated HSC.

In order to demonstrate that human HSC are definitely permissive to *L. donovani*, we performed a confocal microscopy analysis. Activated HSC were exposed overnight to PKH-26- labelled *L. donovani* promastigotes and analysed by confocal microscopy in a 200 nm focal plane, after a DRAQ5 nucleus staining and an Alexa488 WGA staining (Fig. 3A–D). We observed that *Leishmania* parasites (red label) were actually inside HSC (green label). To ascertain this observation despite the HSC autofluorescence level, HSC were then infected with promastigotes labelled with CFSE (green label) and stained with a

Cy3 antibody that target α SMA (red label) which is a specific HSC activation marker. This second staining protocol confirmed the presence of amastigotes inside the cells (Fig. 3E–H). Moreover, specific labelling also indicated that infected cells were indeed HSC and not potential residual macrophages, since the Cy3 anti- α SMA antibody did not stain the cytoskeleton of human macrophages in primary culture (data not shown).

To assess to what the extent the HSC are in a quiescent state in a normal liver before parasite infection *in vivo*, we verified whether quiescent human HSC were also permissive to *L. donovani* (Fig. 4A). Using freshly purified quiescent HSC, we quantified approximately 5% of infected cells after MGG colouration, with no obvious parasite proliferation (data not shown). Interestingly, the presence of intracellular parasites in quiescent HSC does not prevent their progressive activation, which is morphologically characterized by cell spreading with loss of lipidic droplets and cytoskeleton densification the week following their purification (Fig. 4A–C).

Leishmania donovani does not affect HSC functional characteristics

In order to determine whether *L. donovani* can affect HSC viability, a methylene blue assay was performed on activated HSC isolated from 4 liver biopsy donors, at different time-points following overnight exposure to promastigotes and at different cell/parasite ratios. No cell toxicity due to *L. donovani* was observed during the 7-day period of observation, whatever the cell/parasite ratio, indicating that human activated HSC are able to survive despite cell interactions or infection with *L. donovani*, whereas control HSC treated with H_2O_2 or staurosporine displayed approximately 70% mortality (Fig. 5A–C).

Because VL is a chronic disease characterized by life-long parasite persistence, we performed a prolonged follow-up of HSC growth after serial passages during 40 days (Fig. 1). No difference in terms of cellular proliferation was observed between infected and non-infected HSC cultures (Fig. 5D).

During chronic liver injury, HSC become progressively activated, then proliferate and acquire a myofibroblast-like fibrogenic phenotype. To better understand the impact of *L. donovani* on HSC differentiation and functions, we quantified by real-time qPCR the expression of pro-inflammatory and fibrogenic genes known to be induced in HSC and/or to be involved in the hepatic immune response during VL. No statistically significant difference was observed in the expression of the important pro-inflammatory and/or pro-fibrotic cytokines TGF- β , IL-6, TNF- α , IL-33 and IL-1 β , as well as of matrix degradation regulators MMP-2 and TIMP-1 between activated HSC exposed or not to *L. donovani* (Table 2).

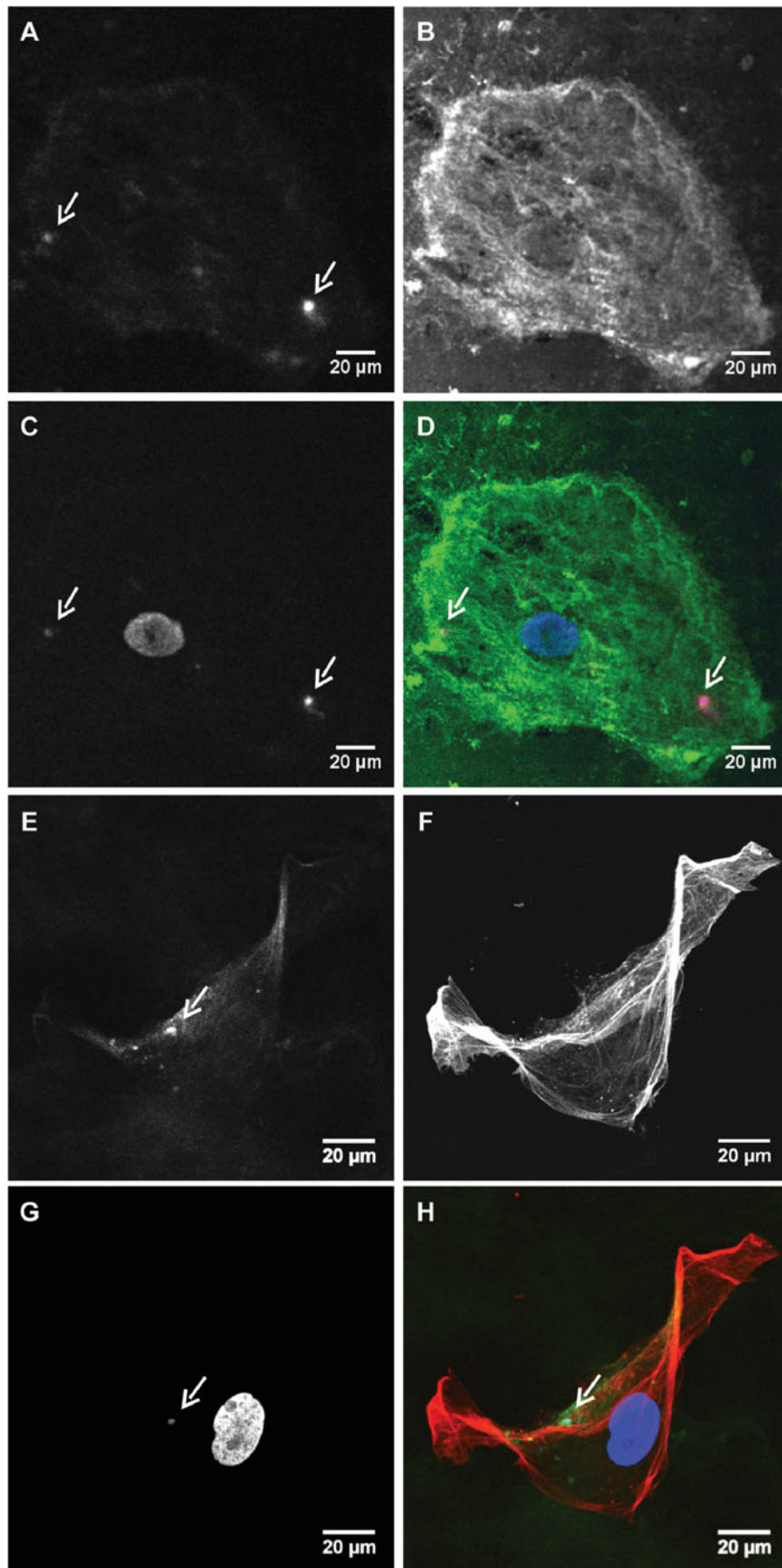


Fig. 3. Analysis of hepatic stellate cell (HSC) permissivity to *Leishmania donovani* using confocal microscopy. PKH-26 or CFSE labelled promastigotes were used to infect activated HSC. One day after infection, the cell shapes were

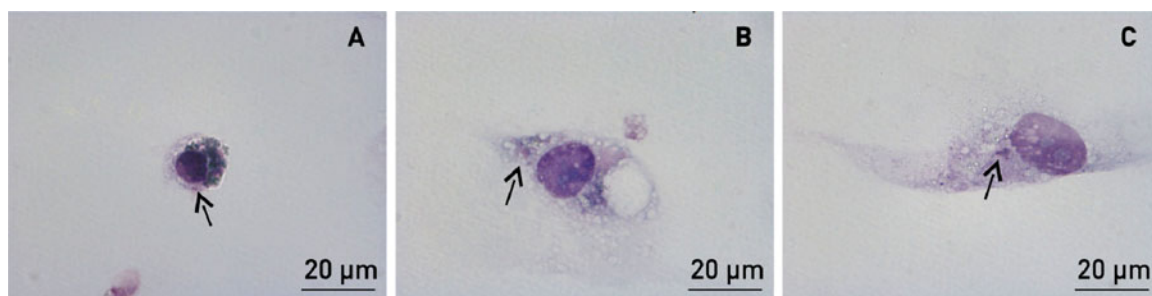


Fig. 4. Observation of hepatic stellate cells (HSC) activation after *Leishmania donovani* infection using optical microscopy. Freshly purified quiescent HSC were infected with *L. donovani* promastigotes overnight. After a 1-day (A), 3-day (B) or 5-day (C) culture, slides were stained with MGG and examined under optical microscopy (magnification: 600X). Arrows show intracellular parasites.

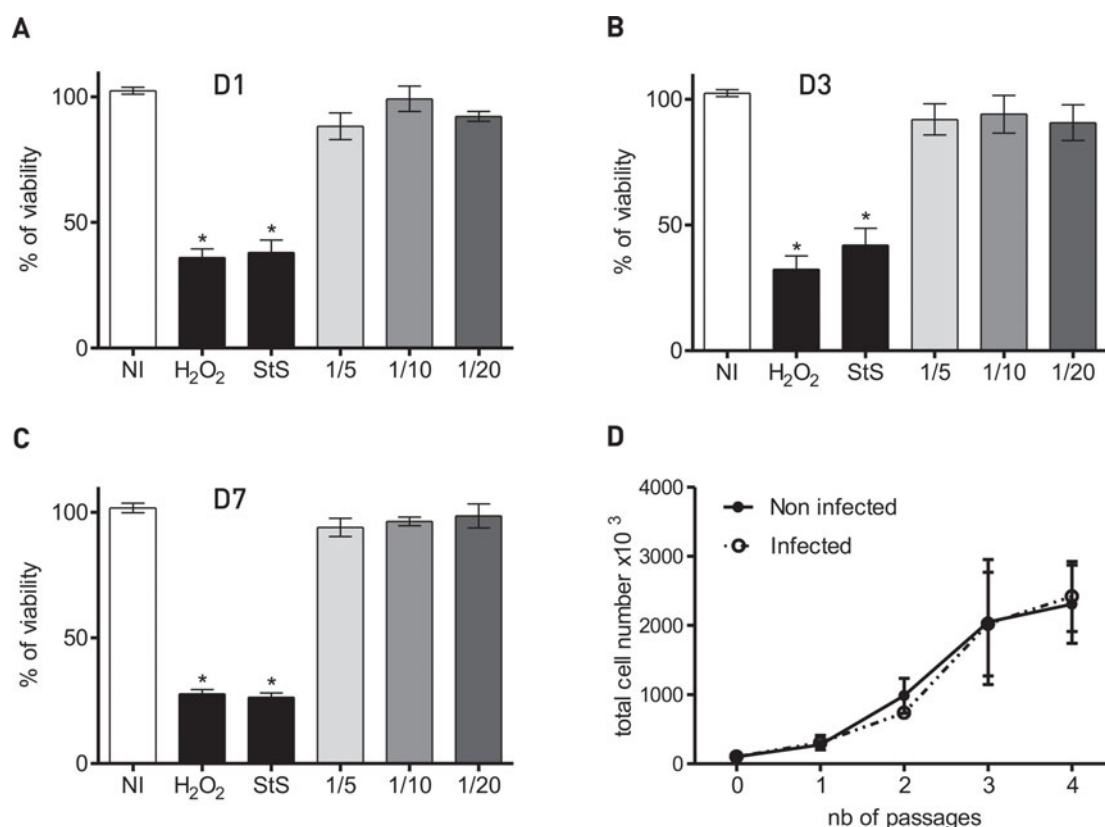


Fig. 5. Quantification of viability and proliferation of hepatic stellate cells (HSC) exposed to *Leishmania donovani*. Human HSC were exposed overnight to *L. donovani* promastigotes with different cell/parasites ratio (1/5, 1/10 and 1/20). Viability was assessed after 1 day (A), 3 days (B) or 7 days (C) using a methylene blue viability test. Non-infected cells were used as the 100% viability control, and overnight treatments with H₂O₂ 30% or 1 µM staurosporine (StS) were used as positive cell death controls. Results are expressed as mean ± s.e.m. of HSC viability from 4 different patients (**P* < 0.05). Human activated HSC from 3 different patients were infected overnight with *L. donovani* promastigotes and maintained in parallel with non-infected cells during 40 days, with a passage and cell count performed every 10 days, as described in Fig. 6. (D) Growth curve of infected and non-infected HSC cultures. Results are expressed as mean ± s.e.m.

highlighted using Alexa488 WGA or a Texas red anti- α SMA antibody, respectively. Nuclei were stained using DRAQ5. Examination was performed by confocal microscopy in a 200 nm thick plane. (A) PKH-26 amastigote labelling. (B) Alexa488 WGA cell staining. (C) DRAQ5 nucleus staining. (D) Overlay of PKH-26 (red), Alexa488 WGA (green) and DRAQ5 (blue) labelling. (E) CFSE parasite labelling. (F) Cy3 α SMA labelling. (G) DRAQ5 nucleus staining. (H) Overlay of CFSE (green), Cy3- α SMA (red) and DRAQ5 (blue) labelling. White arrows show fluorescent parasites.

Table 2. Quantification of pro-inflammatory and fibrogenic gene expression of hepatic stellate cells (HSC) after overnight exposure to *Leishmania donovani*

(Human activated HSC from at least 3 different patients were infected with *L. donovani* promastigotes, then culture was continued until 1, 2 or 3 days (D1, D2 and D3), before real-time qPCR analysis of pro-inflammatory and fibrogenic genes expression. Relative induction level of each gene was normalized with 18S mRNA and compared to its expression in D1 non-infected control cells. Results are expressed as mean \pm s.e.m. The *P*-value represents the result of statistical analysis using the Mann-Whitney test to compare infected and non-infected conditions at each time-point.)

Genes	Days	Exposure to parasites	No exposure	<i>P</i> -value
TGF- β	D1	1.00 \pm 0.00	0.85 \pm 0.28	0.75
	D2	1.56 \pm 1.04	1.01 \pm 0.43	0.80
	D3	0.63 \pm 0.51	0.30 \pm 0.18	0.80
TNF- α	D1	1.00 \pm 0.00	1.94 \pm 1.78	1.00
	D2	0.56 \pm 0.07	1.50 \pm 1.35	1.00
	D3	0.86 \pm 0.30	1.11 \pm 0.94	1.00
IL-6	D1	1.00 \pm 0.00	4.02 \pm 2.64	0.50
	D2	4.10 \pm 1.63	4.79 \pm 3.41	1.00
	D3	3.42 \pm 1.55	3.97 \pm 2.46	1.00
IL-33	D1	1.00 \pm 0.00	1.26 \pm 0.70	1.00
	D2	1.04 \pm 0.40	1.08 \pm 0.35	1.00
	D3	0.56 \pm 0.17	0.78 \pm 0.15	0.70
IL-1 β	D1	1.00 \pm 0.00	4.33 \pm 3.20	1.00
	D2	1.94 \pm 0.26	2.07 \pm 1.07	1.00
	D3	0.89 \pm 0.33	1.08 \pm 0.13	1.00
MMP-2	D1	1.00 \pm 0.00	1.37 \pm 0.87	1.00
	D2	1.17 \pm 0.31	1.54 \pm 0.70	1.00
	D3	1.37 \pm 0.40	1.82 \pm 0.40	0.70
TIMP-1	D1	1.00 \pm 0.00	1.34 \pm 0.87	1.00
	D2	0.67 \pm 0.36	0.99 \pm 0.53	1.00
	D3	0.63 \pm 0.30	0.81 \pm 0.27	1.00

HSC do not affect *L. donovani* viability and cell infection capability

In order to determine whether intracellular *L. donovani* were able to survive in HSC, HSC from 3 different donor patients were exposed overnight to promastigotes and maintained in culture for 40 days. After inoculation in NNN blood agar medium maintained at 27 °C for 4 weeks (Fig. 1), mobile promastigotes in 2 of 3 cultures were observed after 1 and 2 weekly passages, respectively.

To analyse whether HSC could exert phagocytic cell functions, inducible NO synthase (iNOS) expression was analysed by real-time qPCR. A 2-fold lower expression of iNOS was observed in activated HSC compared to plated THP-1. Overnight HSC exposure to *L. donovani* had no effect on iNOS expression level (Fig. 6A).

Finally, to determine whether HSC can alter the virulence of *L. donovani* promastigotes collected from long-term cultured HSC, parasites were amplified by

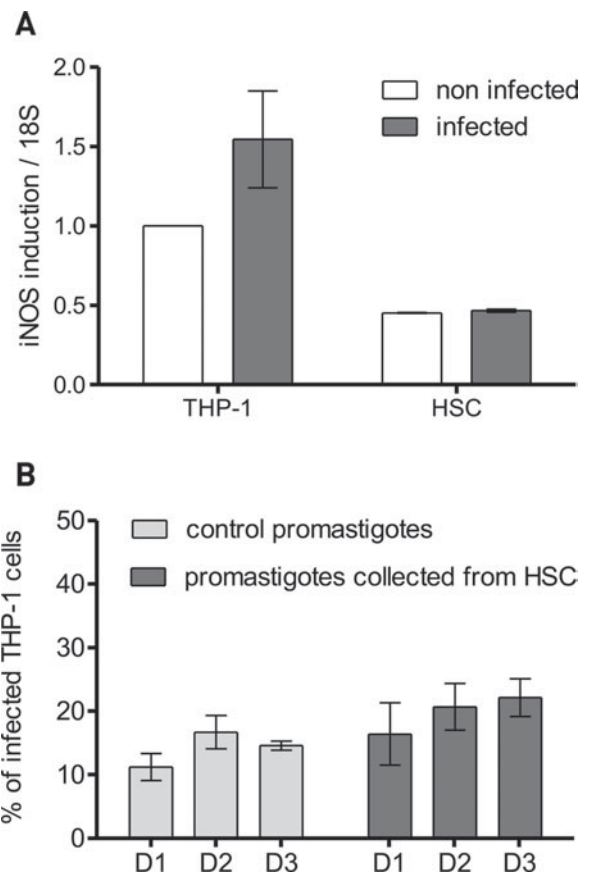


Fig. 6. Lack of leishmanicidal effects of human hepatic stellate cells (HSC). (A) Induction rates of iNOS mRNA in HSC, compared to non-infected control THP-1. Human activated HSC from 3 donors and THP-1 cells were infected overnight with *Leishmania donovani* promastigotes. Real-time qPCR analysis of iNOS mRNA expression was performed after 1, 2 or 3 days and normalized with 18S mRNA expression. Results are expressed as mean \pm s.e.m. (B) Infection rate of THP-1 cells by promastigotes recovered from long-term cultured of human activated HSC transferred on NNN medium. THP-1 cells infected with promastigotes from the initial culture were used as controls. Results are expressed as the percentage of infected cells after microscopic examination of slides stained with MGG and are mean \pm s.e.m. of 3 independent experiments.

subculture in Schneider's *Drosophila* medium and inoculated onto plated THP-1 cells. The infection rates of THP-1 cells were quantified after 1, 2 or 3 days and compared to that obtained with control promastigotes. The infection rate of THP-1 infected with control promastigotes was 11.2 \pm 2.1% at Day 1, 19.1 \pm 2.2% at Day 2 and 13.9 \pm 0.2% at Day 3, whereas it was 16.4 \pm 9% at Day 1, 20.6 \pm 3.6% at Day 2 and 19.3 \pm 1.2% for THP-1 infected with promastigotes collected from HSC (*P*=ns) (Fig. 6B)

DISCUSSION

During visceral leishmaniasis (VL), Kupffer cells (KC) are acknowledged as pivotal phagocytic cells in

the host defence against *Leishmania donovani* infection in the liver (Murray, 2001), whereas little is known on HSC, which play a major role in the fibrotic process (Friedman, 2008a,b) complicating some infectious diseases (Bartley *et al.* 2006; Chang *et al.* 2006; Tomanovic *et al.* 2009) and commonly observed in human VL (Duarte and Corbett, 1987; Corbett *et al.* 1993; el Hag *et al.* 1994). Hepatic fibrosis due to HSC activation was shown to be correlated with the progression of VL and to regress after clinical cure of the disease (Corbett *et al.* 1993; Duarte and Corbett, 1987). Similarly, canine VL is associated with collagen, fibronectin and laminin deposition in the liver, and there is a correlation between fibrosis and parasite loads or severity of the disease in dogs naturally infected with *L. chagasi* (Melo *et al.* 2009). Despite these clinical observations, the *Leishmania*-HSC interaction has never been studied.

In this work, we developed an original model of *L. donovani* infection of HSC in primary culture. For the first time, we provide evidence that human HSC in primary culture are permissive to *L. donovani* promastigotes using confocal microscopy. In order to bypass the autofluorescence level of HSC (Friedman, 2008b), 2 complementary staining protocols of parasites and cells using different fluorescent dyes were performed. In both cases, *Leishmania* parasites were localized inside the cells, clearly demonstrating their permissivity to *L. donovani*, thus raising the question of how the parasite can enter these cells. Several studies have shown that HSC can express surface markers, such as members of the HLA family (MHC-I and MHC-II), lipid presenting molecules (CD1b and CD1c), co-stimulatory proteins (CD80, CD40) and proteins involved in antigen trafficking (CD68). They can otherwise phagocytose latex bead particles, they closely interact with lymphocytes in Disse's spaces, and stimulate T-cell proliferation, suggesting that they share features of antigen presenting cells (Vinas *et al.* 2003; Muhanna *et al.* 2007). Therefore, HSC could phagocytose *Leishmania* promastigotes like professional phagocytic cells.

In our model, the cell infection rate reached 6%, both in activated HSC and in quiescent HSC freshly purified from human liver donors. No effect on activated HSC survival and proliferation was noticed after cell exposure to parasites, showing that contact or infection with promastigotes does not induce cell death or cell cycle disruption. During our study, we analysed the pro-inflammatory and fibrogenic phenotype of activated HSC exposed overnight to the parasite. Unfortunately, it was not possible to analyse separately the mRNA expression of infected cells, because of specific technical limitations: (i) strong and wide-length autofluorescence of HSC limiting phenotype analysis by flow cytometry, and (ii) lack of availability of a cell sorter operating in a negative

pressure environment adapted to sorting of living cells infected with *L. donovani*. Nevertheless, whole HSC exposure to parasites resulting both in a low percentage of infected cells but also in overall contacts and exposure to excreted/secreted antigens even without infection, did not disturb the HSC phenotype. Indeed, the expression of key genes which are the hallmark of HSC activation and hepatic fibrosis, such as TGF- β , IL-6, TNF- α , IL-33, IL-1 β , MMP-2 and TIMP-1 (Gressner *et al.* 2002; Friedman, 2008b; Marvie *et al.* 2010; Thirunavukkarasu *et al.* 2006), was not modified. Furthermore, the spontaneous activation of quiescent HSC, normally observed after several days of *ex vivo* culture (Friedman, 2008b) was never altered following infection with *L. donovani*. These data suggest that the parasite itself has neither the capacity to stimulate HSC fibrogenic phenotype, nor the ability to contribute to the expansion of extracellular matrix and fibrosis through induction of HSC proliferation. Thus, *L. donovani* itself is unlikely to be directly involved in the hepatic fibrogenesis process during VL, but could induce the onset of sustained cytokine responses favouring long-term fibrosis. Indeed, HSC are indirectly modulated by the liver micro-environment via cell-to-cell interactions or more likely via paracrine or cytokine pathways. During VL, it was shown that hypertrophic KC intensively express TGF- β that activates HSC cells for extracellular matrix production (Duarte *et al.* 2009). Thus our results spread a new light on the complex interplay between HSC and KC, which needs to be further explored to better understand the regulation of HSC proliferation and its consequences on liver injuries and hepatic fibrosis during VL.

The absence of modification in functional characteristics of infected HSC raises the hypothesis that they could be sanctuary cells for persisting *L. donovani* parasites. In this work, it was shown that HSC were able to harbour parasites during long-term primary culture (>1 month) and survive without killing parasites. This inability to get rid of *Leishmania* parasites could be explained by their low iNOS expression level compared to THP-1 cells. Indeed this key enzyme is essential for efficient phagocytic function, and is strongly induced in professional phagocytic cells (Green *et al.* 1991; James, 1995). This lack of iNOS induction probably contributes to make these cells a safe target for *L. donovani*. After a 40-day culture and serial passages of HSC, intracellular parasites were able to reactivate in Novy-Nicolle-McNeal (NNN) medium and to convert into promastigotes, for which the infectivity for THP-1 cells was not altered. This observation demonstrates that *L. donovani* can persist in a latent intracellular stage inside HSC until their further release. The sensitivity to leishmanicidal drugs of these latent intracellular parasites could be an interesting aspect to explore.

Sanctuary cells have been described as immune privileged safe targets for pathogens such as *Toxoplasma gondii*, *Leishmania major* or herpes virus, that contribute to life-long pathogen persistence even after efficient chemotherapies (Bogdan, 2008). During cutaneous leishmaniasis (CL), immature myeloid precursor cells have been described as safe targets for *L. major* (Mirkovich *et al.* 1986), as well as skin and lymph node fibroblasts (Bogdan *et al.* 2000). During VL, a few cell types were postulated to function as safe targets, especially sialoadhesin-positive stromal macrophages of the bone marrow (Leclercq *et al.* 1996; Cotterell *et al.* 2000) and hepatocytes which show a low permissivity level and limited cell damage (Gangneux *et al.* 2005). Interestingly, Bogdan and co-workers reported that persisting *L. major* parasites were largely associated with skin and lymph node fibroblasts lacking leishmanicidal effectors (Bogdan *et al.* 2000). Our results suggest that quiescent and activated myofibroblast-like HSC are able to phagocytose viscerotropic *Leishmania* parasites during VL and to behave in a similar way as fibroblasts during CL. This could be an evading mechanism for *Leishmania* parasites that may explain patient relapses after a cured VL episode. Furthermore, we observed that HSC were permissive to *L. infantum* (data not shown), suggesting that this phenomenon could occur whatever the parasite species involved and explain asymptomatic carriage frequently observed in endemic areas (Michel *et al.* 2011). As KCs regulate HSC growth during VL through TGF- β synthesis (Duarte *et al.* 2009), they could contribute to amplify the number of safe targets cells for *Leishmania* parasites.

In conclusion, human HSC in our model can harbour persisting *L. donovani* parasites and could be a sanctuary cell subset in the liver during VL, which opens new insights into the patho-physiology of immune evasion, asymptomatic carriage and clinical relapse after treatment.

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