IJP: Drugs and Drug Resistance 9 (2019) 1–7

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Immunosuppression of Syrian golden hamsters accelerates relapse but not the emergence of resistance in *Leishmania infantum* following recurrent miltefosine pressure

S. Hendrickx[1], D. Bulté[1], M. Van den Kerkhof, P. Cos, P. Delputte, L. Maes, G. Caljon*

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

ARTICLE INFO

Keywords:
immunosuppression
Hamster VL model
Resistance
Miltefosine

ABSTRACT

Although miltefosine (MIL) has only been approved for the treatment of visceral leishmaniasis (VL) in 2002, its application in monotherapy already led to the development of two confirmed MIL-resistant isolates by 2009. Although liposomal amphotericin B is recommended as first-line treatment in Europe, MIL is still occasionally used in HIV co-infected patients. Since their immune system is incapable of controlling the infection, high parasite burdens and post-treatment relapses are common. Linked to the particular pharmacokinetic profile of MIL, successive treatment of recurrent relapses could in principle facilitate the emergence of drug resistance.

This study evaluated the effect of immunosuppression (cyclophosphamide 150 mg/kg once weekly) on the development of MIL-resistance in Syrian golden hamsters infected with *Leishmania infantum*. The hamsters were treated with MIL (20 mg/kg orally for 5 days) whenever clinical signs of infection or relapse were observed. The immunosuppression resulted in a significant depletion of $CD4^+$ lymphocytes and MHCII-expressing cells in peripheral blood, and a concomitant increase in tissue parasite burdens and shorter time to relapse, but the strain's susceptibility upon repeated MIL exposure remained unaltered. This study demonstrates that immunosuppression accelerates the occurrence of relapse without expediting MIL resistance development.

1. Introduction

The extensive use of miltefosine (MIL) monotherapy as first-line treatment for visceral leishmaniasis (VL) in the Indian subcontinent has become severely compromised by the increasing treatment failure rates over the past decade (Rijal et al., 2013). Although these failures could originally not be linked to a decreased parasite drug susceptibility, the first MIL-resistant *Leishmania donovani* isolates have recently been described in India (Srivastava et al., 2017). In the Mediterranean region, liposomal amphotericin B remains the treatment of choice for the large population of HIV co-infected patients, however, MIL is also still used given its acceptable efficacy and safety profile (Monge-Maillo et al., 2014b; van Griensven et al., 2014). Despite its lower overall use in Europe, the first reports on European MIL-resistant *L. infantum* isolates already date back to 2009 (Cojean et al., 2012; Hendrickx et al., 2014). These isolates were derived from HIV co-infected patients who had received multiple (possibly incomplete) courses of MIL. As parasite elimination in HIV co-infection is virtually impossible given the high parasite burdens and defective immunity, frequent relapses requiring recurrent drug exposure are not exceptional (Monge-Maillo and Lopez-Velez, 2016; van Griensven et al., 2014). Although several studies reported the generation of MIL-resistant promastigotes in the past (Perez-Victoria et al., 2003; Seifert et al., 2003, 2007), previous work from our group already showed that the selection of MIL resistance on intracellular parasites both *in vitro* and *in vivo* was very difficult (Hendrickx et al., 2014, 2015a). However, both experimental selection on promastigote level and amastigote level and clinical resistance could be linked to defects in the inward *Leishmania donovani* putative MIL-transporter (LdMT) (Cojean et al., 2012; Mondelaers et al., 2016; Perez-Victoria et al., 2003).

This study specifically evaluated whether prolonged immunosuppression could expedite the development of MIL-resistance in the Syrian VL hamster model. While selection in immunocompetent hamsters was successful in generating resistance against paromomycin, recurrent MIL-exposure did not result in reduced drug susceptibility within five successive selection cycles (Hendrickx et al., 2015a). In

* Corresponding author. University of Antwerp, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Universiteitsplein 1 – building S, B-2610 Antwerp, Wilrijk, Belgium.
E-mail address: guy.caljon@uantwerpen.be (G. Caljon).
[1] Equal contribution.

https://doi.org/10.1016/j.ijpddr.2018.12.001
Received 23 October 2018; Received in revised form 8 December 2018; Accepted 10 December 2018
Available online 11 December 2018

analogy, hamsters were now infected with a *L. infantum* laboratory reference strain, immunosuppressed with cyclophosphamide (CPA) and treated for five successive dosing cycles of MIL covering four post-treatment relapse episodes. Next to evaluating MIL susceptibility, the effects of immunosuppression on the overall condition of the animals, the immune cell subsets in the peripheral blood, the tissue parasite loads and the time-to-relapse were assessed.

2. Materials and methods

2.1. Ethics statement

The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its latest version) and was approved by the Ethical Committee of the University of Antwerp, Belgium (UA-ECD 2011–77/17-02-2012).

2.2. Animals

Female Swiss mice (6–8 weeks old; body weight 20 g) for the collection of peritoneal macrophages and female golden hamsters (8–10 weeks old; body weight 100–120 g) for *Leishmania* infection were purchased from Janvier (France). Food for laboratory rodents (Carfil, Arendonk, Belgium) and drinking water were available *ad libitum*. Hamsters were kept in quarantine at least 5 days before infection. For the development and validation of the immunosuppression model, hamsters were randomly allocated to groups of 3 animals each.

2.3. Leishmania parasites

The *L. infantum* laboratory reference strain (MHOM/MA/67/ITMAP263) was maintained by passage in Syrian golden hamsters. To infect naive hamsters, e*x vivo* amastigotes were purified from the spleen of heavily infected donor hamsters, as described previously (Hendrickx et al., 2015a). An infection inoculum containing 2×10^7 amastigotes/100 µL phosphate buffered saline (PBS) was administered to anaesthetized hamsters (isoflurane inhalation) by intracardial injection. The general condition and body weight of infected animals was monitored twice weekly to evaluate the course of infection.

During the *in vivo* selection for resistance, a promastigote back-transformation assay was performed after each selection cycle to expand the parasite population and to allow MIL susceptibility testing. Promastigotes were cultured in HOMEM medium supplemented with 10% inactivated fetal calf serum (iFCS) (Invitrogen). To stimulate initiation of promastigote back-transformation from infected tissue, 20% spent promastigote medium was added to the medium and iFCS was increased to 20% (Hendrickx et al., 2015a).

2.4. Development and validation of an immunosuppression model in Syrian golden hamsters

Based on previous immunomodulatory studies in mice, a weekly dose of 150 mg/kg cyclophosphamide (CPA, Endoxan®) was administered intraperitoneally starting on the day of infection (Vanhoutte et al., 2017; Xu and Zhang, 2015). The CPA solution was prepared freshly in MilliQ water at 40 mg/mL. To evaluate the impact of repeated CPA administration in infected hamsters in comparison with immunocompetent controls, body weight was monitored over time as a general parameter for disease severity. Additionally, the dynamics of the percentages of $CD4^+$ and MHC-II + cells were compared by flow cytometry. Starting from the first day of immunosuppression until 21 days post-infection (dpi), blood from infected/immunosuppressed and infected/immunocompetent animals was collected twice weekly via sublingual vein puncture. After two cycles of lysis with erythrocyte lysis (EL) buffer (QIAamp RNA Blood Mini kit), white blood cells (WBCs) were suspended in RPMI-1640 without phenol-red, supplemented with 10% iFCS. After Fc-receptor blocking, the cell suspension was transferred into a flow cytometer tube, stained with the cell viability solution 7-AAD (BD Pharmingen™), fluorescein-labelled anti-CD4 (clone GK1.5) and phycoerythrin-labelled anti-MHC-II (clone 14-4-45) monoclonal mouse antibodies (mAbs) both showing cross-reactivity towards hamster antigens (eBioscience, Thermo Fisher Scientific) (Kauffmann et al., 2016). Hamsters were followed-up until 45 dpi after which parasite burdens in the main target organs (liver, spleen and bone marrow) were determined on Giemsa-stained tissue imprints.

2.5. Drug formulations and preparation

Miltefosine (MW = 407.57) was purchased from Sigma (Diegem, Belgium). An *in vitro* stock solution of 20 mM was prepared in PBS. For *the in vivo* treatment, MIL was formulated in distilled water at 20 mg/mL.

2.6. In vivo selection of resistance

Subsequent treatment/relapse rounds in the immunosuppressed hamster model were conducted as described earlier (Hendrickx et al., 2015a). In brief, infected hamsters were repeatedly exposed to sub-curative schedules of MIL (*i.e.* oral dosing at 20 mg/kg for 5 consecutive days, resulting in about 60% reduction in the spleen, 39% in the bone marrow and almost no reduction in the liver) (Fortin et al., 2012). Higher dosing schemes would most likely hamper repeated treatment cycles of a same animal. Infected animals were treated starting from 21 dpi and closely monitored for the occurrence of treatment relapses. Upon suspicion of treatment relapse (based on body weight decrease and clinical appearance), a liver biopsy was taken to quantify the parasite burden. When the presence of parasites was confirmed, the same treatment schedule was repeated. To minimize animal suffering, a maximum of two treatment rounds were performed in the same animal. After the second relapse, *ex vivo* amastigotes were harvested from the spleen and transferred to a naive animal, which was then treated 21 dpi. Five successive treatment/relapse cycles were run in total, after which *ex vivo* spleen amastigotes were back-transformed to enable MIL susceptibility evaluation. Amastigote burdens were expressed as Leishman Donovan units (LDU) which corresponds to the number of Leishmania amastigotes per 1000 nucleated cells multiplied by the organ weight (g).

2.7. Promastigote and amastigote susceptibility determination in vitro

In vitro promastigote and amastigote susceptibilities were determined as previously described (Vermeersch et al., 2009). In brief, procyclic promastigotes were exposed to two-fold drug dilutions for 72 h and their viability was assessed by the resazurin assay. To determine drug susceptibility of intracellular amastigotes, primary peritoneal macrophages were harvested from starch-stimulated Swiss mice and infected with metacyclic promastigotes at an infection ratio of 15:1. After 24 h, the medium of the infected cells was discarded to remove residual extracellular promastigotes. The plates were incubated at 37 °C and 5% CO_2 for 5 days in presence of two-fold drug dilutions. The inhibitory concentration killing 50% of the parasites (IC_{50}) was determined microscopically after Giemsa-staining.

2.8. Statistical analysis

Statistical differences between groups were analyzed using two-way ANOVA. Results were considered statistically significant if $p < 0.05$.

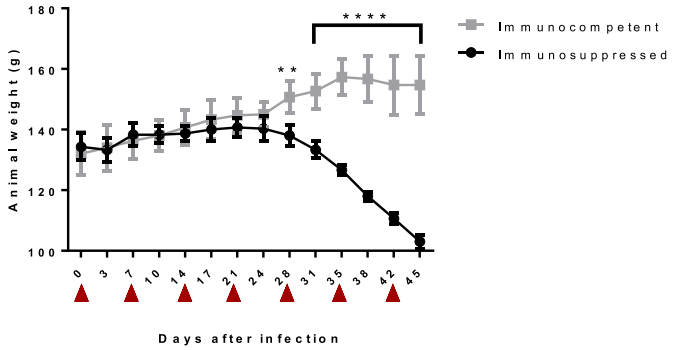

Fig. 1. Body weight of infected/immunocompetent and infected/immunosuppressed animals over time. The general condition of the animals was checked twice weekly and followed-up for a total of 45 days, at which the animals were autopsied. After 21 days of infection, the body weight of immunosuppressed animals started to decline significantly. Weekly CPA administrations are indicated with arrowheads. Data on the graph represent the average body weight of 3 animals ± the standard error of the mean and are representative of two independent experiments (SEM) (**p < 0.01; ****p < 0.0001).

3. Results

3.1. Development and validation of an immunosuppression model in Syrian golden hamsters

To evaluate the impact of immunosuppression in the *L. infantum* VL hamster model, the general condition of the animals was monitored twice weekly using body weight and overall appearance as read-outs. A clear reduction in body weight was observed starting after 2–3 weeks of infection and immunosuppression (Fig. 1).

At autopsy (45 dpi), organ weights and tissue parasite burdens in liver, spleen and bone marrow were compared between immunocompetent and immunocompromised animals. Next to a significantly reduced liver weight, the parasite burdens were increased in all target organs of the immunocompromised animals, although only being statistically significant for liver and bone marrow (Fig. 2).

To evaluate the immunological consequences of CPA immunosuppression, the WBC composition in peripheral blood was characterized. Given the lack of hamster-specific immunological reagents and antibodies, the cross-reactivity of anti-mouse antibodies targeting WBC markers was evaluated and two antibodies could be identified, namely an anti-CD4 (clone GK1.5) and an anti-MHC-II antibody (clone 14-4-45). Consequently, flow cytometric analysis of hamster blood allowed identification of diverse WBC subsets [$CD4^+$ and $CD4^-$ lymphocytes, monocytes and granulocytes] based on the light scattering properties and the two cellular markers (Fig. 3).

When comparing the basic WBC profiles in the peripheral blood between infected and uninfected hamsters, no clear immunological impact of *Leishmania* infection could be observed at 21 dpi, with only a significant increase in the proportion of lymphocytes (from 35.5 ± 1.07% to 51.2 ± 2.8%) and a relative drop in the gate II fraction (from 35.7 ± 1.7% to 20.4 ± 1.8%) (Fig. 4).

To evaluate the effect of the weekly CPA administrations in infected hamsters, the blood profiles were compared at 21 dpi between infected/immunocompetent animals and infected/immunosuppressed animals. After three doses of CPA, a significant alteration of the WBC composition with a complete disruption of the normal FSC/SCC profiles was observed. Analysis of $CD4^-$ and MHCII-expression within the total WBC fraction showed a significant depletion of $CD4^+$ lymphocytes and $MHCII^+$ cells as a result of CPA administration (Fig. 5).

3.2. Promastigote and amastigote susceptibility is not altered upon repeated MIL exposure

The differences in drug susceptibility profile of the parasites was evaluated after each selection cycle. No significant differences in drug susceptibility could be observed for all tested antileishmanial reference drugs [miltefosine, paromomycin, antimonials and amphotericin B (Fungizone®)] at the intracellular amastigote level (Table 1). As previous *in vitro* resistance selection on amastigotes resulted in a slightly decreased promastigote susceptibility to MIL (Hendrickx et al., 2014), promastigote IC_{50} values were compared as well, but revealed no significant differences between parent and selected parasites (Table 2).

Although no changes in MIL susceptibility could be observed after repeated resistance selection cycles, CPA administration did reduce the time until post-treatment relapse by > 2-fold (from 5.5 ± 0.5 weeks to 2.5 ± 0.5 weeks), and must most likely be linked to the higher organ burdens (Fig. 2).

4. Discussion

Although the number of MIL-treatment relapses in the Indian subcontinent has been expanding over the past decade, treatment failures could generally not be correlated with drug resistance (Rijal et al., 2013). Although some studies on relapse isolates did suggest a (marginal) decrease in *in vitro* drug susceptibility (Bhandari et al., 2012), so far only two MIL-resistant Indian clinical isolates have recently been reported (Srivastava et al., 2017), despite MIL's unfavorable pharmacokinetic profile and its widespread use as recommended first-line therapy in the frame of the Kala-azar elimination programme (Dhillon et al., 2008; Dorlo et al., 2012). On the other hand in the Mediterranean region, the use of MIL in VL was mostly restricted to HIV co-infected patients (Monge-Maillo et al., 2014a) and for the treatment of canine leishmaniasis [17]. Since absolute parasite clearance in both populations is virtually impossible, continuous use of the same antileishmanial drugs remains the sole option to combat the infection (Noli and

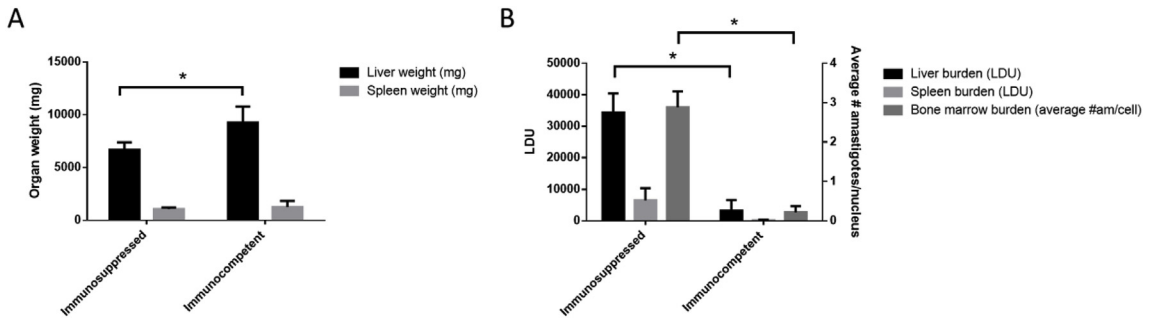

Fig. 2. Comparison of organ weights (A) and parasite burdens in liver, spleen and bone marrow (B) between immunocompromised and immunocompetent hamsters at autopsy (45 dpi). Immunosuppression is associated with a significantly decreased liver weight and significantly elevated parasite burdens in liver and bone marrow. Results are expressed as average organ weights or parasite burdens determined on 3 animals ± SEM (*p < 0.05) (LDU: Leishman Donovan Units).

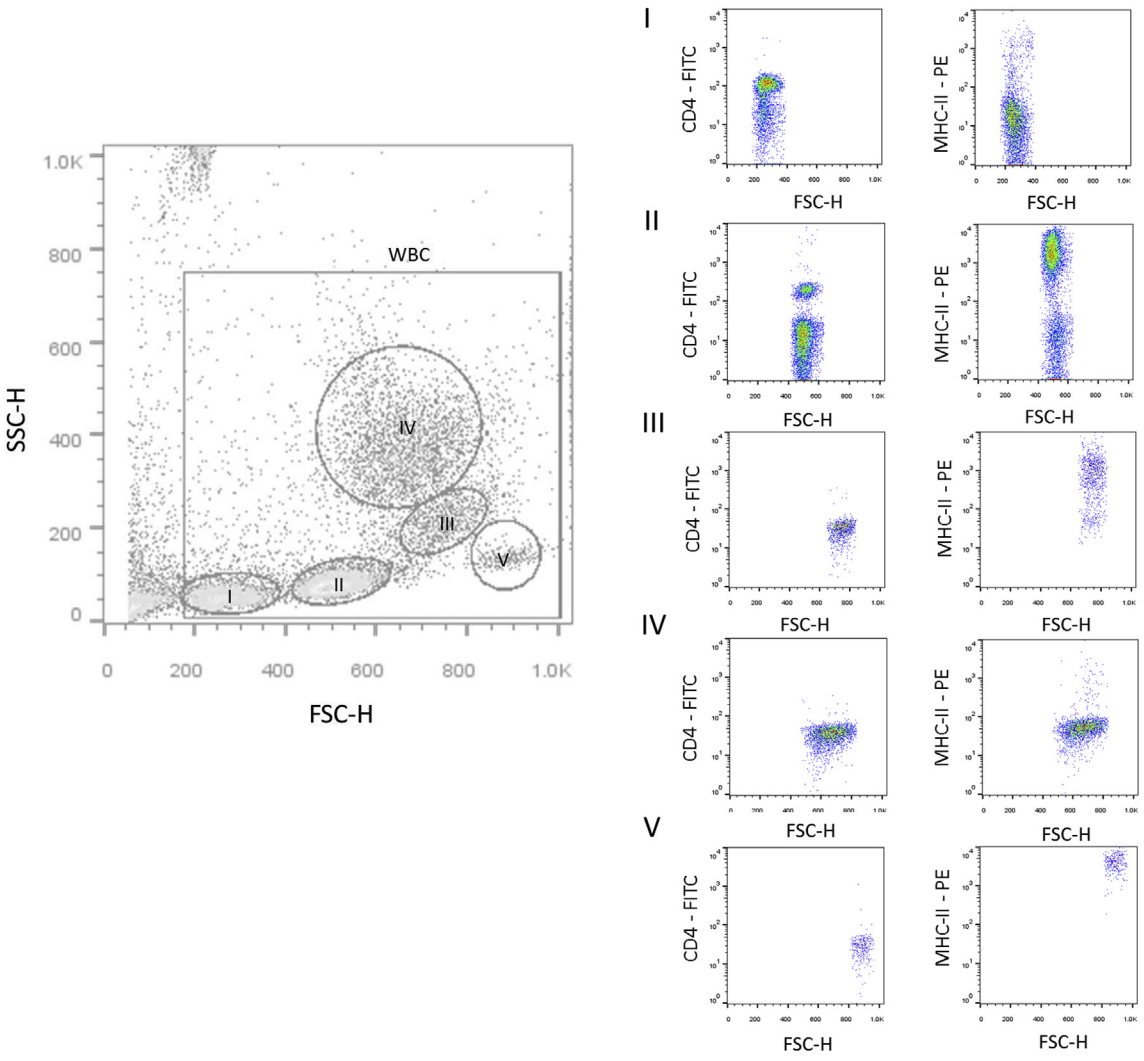

Fig. 3. Flow cytometry analysis of hamster white blood cells (WBC). The major immune cell subsets (lymphocytes, monocytes and granulocytes) were gated according to their light-scattering characteristics. The WBC fraction was incubated with fluorescein-labelled anti-CD4 (left) and phycoerythrin-labelled anti-MHC-II mouse mAbs (right) and subjected to a live/dead 7-AAD staining. The cells in gate I are primarily $CD4^+$/$MHC\text{-}II^-$ lymphocytes, while gate II contains $CD4^+$ lymphocytes as well as cells with a B lymphocyte profile ($CD4^-$ and $MHC\text{-}II^+$). Gate III contains primarily $CD4^-$ and $MHC\text{-}II^+$ monocytes. Cells in gate IV are predominantly $CD4^-$ and $MHC\text{-}II^{-/dim}$ granulocytes (neutrophils) with a high side scatter. The minor gated cell population V was $CD4^-$ and $MHC\text{-}II^{Hi}$.

Saridomichelakis, 2014; van Griensven et al., 2014). Hence, repeated exposure of a same parasite population to the drug may trigger more rapid selection towards drug resistance. In Europe, the first MIL-resistant clinical isolates were obtained in 2009 from French HIV patients who had undergone several (possibly incomplete) courses of MIL (Cojean et al., 2012; Hendrickx et al., 2014), suggesting an involvement of immunosuppression in the emergence of MIL-resistance.

By implementing the earlier established drug resistance selection procedure in the Syrian golden hamster VL model (Hendrickx et al., 2015a), the present study specifically aimed to evaluate whether immunosuppression could indeed trigger a more rapid emergence/selection of MIL-resistance. Five successive selection rounds were used on the basis of ethical/animal welfare considerations and were inspired by other successful resistance selection schemes for other drugs. Own experience with *Leishmania* indicated that drug resistance can be obtained against paromomycin within 3 selection cycles (Hendrickx et al., 2015a). For *Trypanosoma*, immunosuppression of the host lead to a rapid development of drug resistance that was reached within even fewer selection cycles (Pyana Pati et al., 2014). The immunosuppression principle using CPA was based on prior literature reports of both mice and hamster experiments (Vanhoutte et al., 2017; Xu and Zhang, 2015). The application of hamster cross-reactive mouse monoclonal antibodies enabled the quantitative follow-up of the major WBC subsets during immunosuppression, enabling a more detailed validation of the model. As earlier reports already demonstrated that *Leishmania* infection itself can cause neutropenia in dogs and hamsters (Freitas et al., 2012; Moreira et al., 2016), our primary goal was to evaluate the effect of infection. No trend towards reduced granulocyte numbers could be observed within 21 days of infection. When immunosuppressed and immunocompetent hamsters were compared to evaluate the overall impact of immunosuppression, a profound distortion of the WBC composition was recorded with declining numbers of $CD4^-$ and MHCII-expressing cells, clearly endorsing the immunosuppressive effect of the weekly CPA administrations. Immunosuppression was accompanied by elevated parasite burdens in all target organs, although only being statistically significant for liver and bone marrow. Liver weights were also significantly elevated, whereas the absence of an increased spleen weight following CPA-treatment might be linked to suppression of lymphoproliferative responses. Given that almost no immunological work has been performed in the hamster model, to our knowledge this is the first report extracting immunological information on WBC composition of hamster blood by exploiting cross-reactivity of mouse monoclonal antibodies (Kauffmann et al., 2016).

While a quick development of drug resistance in *Trypanosoma evansi*

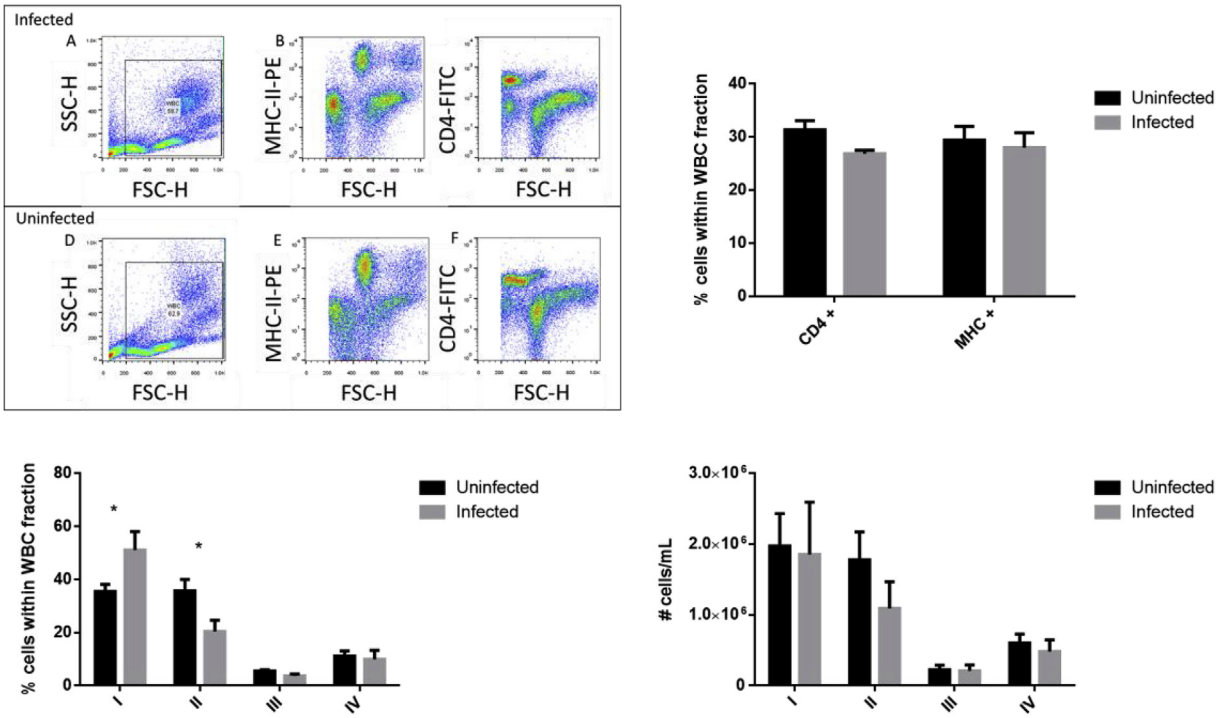

Fig. 4. Differences in cellular composition of the WBC fraction between infected (21 dpi) and uninfected animals. WBCs of infected (**A**) and uninfected hamsters (**D**) were collected at 21 dpi and compared for the amount of MHC-II$^+$ (**B** and **E**) and CD4$^+$ cells (**C** and **F**). The proportion of lymphocytes (gate I) increases upon infection whereas a significant decrease of monocytes (gate II) within the WBC fraction can be observed (bottom left). Absolute cell numbers did not change significantly (bottom right). Results are presented as the average percentage of cells within the WBC fraction ± SEM and are based on 3 replicates. Results are representative of two independent experiments. (*p < 0.05).

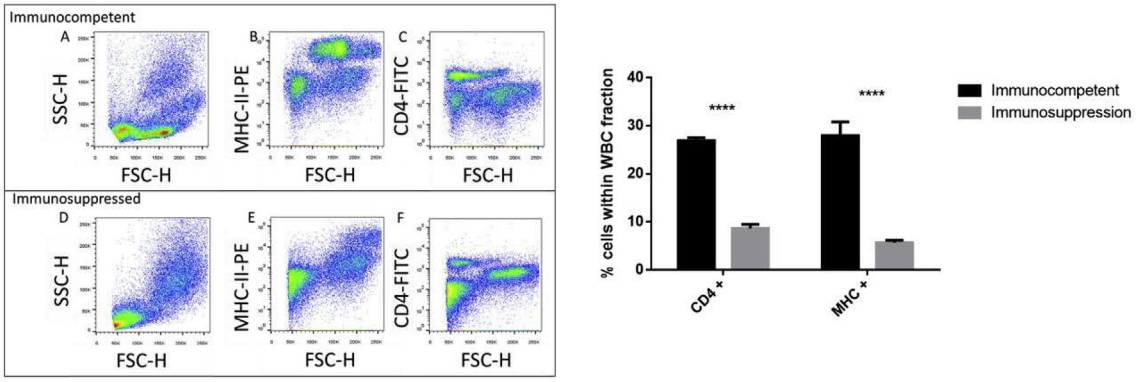

Fig. 5. Flow cytometry analysis of the WBC fraction to evaluate the effect of weekly CPA administration in infected hamsters. WBCs of immunocompetent (**A**) and immunosuppressed hamsters (**D**) were collected at 21 dpi and compared for the amount of MHC-II$^+$ (**B** and **E**) and CD4$^+$ cells (**C** and **F**). Upon immunosuppression, a complete disruption of the FSC/SCC profile was observed. Immunosuppression with CPA resulted in a significant decrease of CD4$^-$ and MHCII-expressing cells. Results are presented as the average percentage of cells within the WBC fraction ± SEM and are based on 3 replicates. Results are representative of two independent experiments. (*p < 0.05) (*p < 0.05; **p < 0.01; ****p < 0.0001).

Table 1
Amastigote susceptibility to the antileishmanial reference drugs miltefosine (MIL), paromomycin (PMM), antimonials (Sb^{III}) and amphotericin B (Fungizone®, AmB) of the *L. infantum* parent strain and the lines obtained after 5 subsequent MIL selection rounds under immunosuppression. No significant differences could be demonstrated between the parent (WT) and MIL-exposed parasites. Drug susceptibility values are expressed as the average IC_{50} value (μM) ± SEM and are the result of three independent assays run in duplicate.

	MIL (μM)			PMM (μM)			Sb^{III} (eq.)			AmB (μM)		
	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
WT	5.9	±	1.7	162.7	±	14.06	2.9	±	0.9	0.05	±	0.001
MIL	7.2	±	1.5	164.6	±	12.2	1.1	±	0.5	0.04	±	0.001

Table 2
Promastigote susceptibility of the *L. infantum* parent strain and lines obtained after 5 subsequent MIL selection rounds under immunosuppression. No significant biologically relevant differences could be demonstrated between the parent (WT) and MIL-exposed parasites. Drug susceptibility values are expressed as the average IC_{50} value (μM) ± SEM and are the result of three independent assays run in duplicate.

Species *L. infantum*	Promastigote susceptibility		
	MIL (μM)		
	Mean	±	SEM
WT	3.9	±	0.3
MIL	2.5	±	0.2

and *T. brucei gambiense* was obtained in immunosuppressed mouse models (Osman et al., 1992; Pyana Pati et al., 2014), such approach did not expedite MIL-resistance acquisition in *Leishmania*. Although the mode-of-action of MIL also involves immunomodulatory effects (Correa et al., 1992; Murray and Delph-Etienne, 2000), experimental modification of hamster immunity did not directly impact on drug efficacy. Despite the profound impact of CPA immunosuppression, no effects on MIL-susceptibility upon successive selection cycles could be observed, which is in accordance with previous *in vitro* work (Hendrickx et al., 2015b) and selection experiments in immunocompetent hamsters (Hendrickx et al., 2015a), both indicating that MIL-resistance is extremely difficult to generate. Acquisition of resistance related to a defective Miltefosine Transporter (MT) was indeed shown to have a severe impact on fitness even in immunosuppressed BALB/c mice (Eberhardt et al., 2018), which highlights the functional importance of the MT for survival in the host. The observed reduction in relapse time in immunosuppressed hamsters in principle will impose a faster recurrence of drug treatment, which can also represent a risk for alterations in epidemiological parasite behavior (Deep et al., 2017; Rai et al., 2013).

The features of the CPA immunosuppression model may not entirely resemble those of a HIV-infection, which is associated not only with a progressive destruction of $CD4^+$ T-cells (Lucas and Nelson, 2015), but also with a depletion of neutrophils (Pitrak, 1999; Shi et al., 2014). So far, there is no way of predicting whether the severely compromised cell-mediated immunity in HIV-infections might still directly or indirectly impact on the selection of resistance. As VL is spreading to other regions of East Africa, India, and Brazil (van Griensven et al., 2014) with high reported incidence of HIV-infection, close monitoring of drug susceptibility and well-designed resistance surveillance systems remain essential given the limited therapeutic antileishmanial options available.

Acknowledgements

The authors wish to thank the laboratory technicians Pim-Bart Feijens and Mandy Vermont for their skillful assistance with the animal work. This work was funded by the Research Fund Flanders (FWO: project G051812N and 12I0317N) and a research fund and PhD scholarship of the University of Antwerp (TT-ZAPBOF 33049 and DOCPRO4 33265). LMPH is a partner of the Antwerp Drug Discovery Network (ADDN, www.addn.be) and the Excellence Centre 'Infla-Med' (www.uantwerpen.be/infla-med).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.12.001.

References

Bhandari, V., Kulshrestha, A., Deep, D.K., Stark, O., Prajapati, V.K., Ramesh, V., Sundar, S., Schonian, G., Dujardin, J.C., Salotra, P., 2012. Drug susceptibility in Leishmania isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. PLoS Neglected Trop. Dis. 6, e1657.

Cojean, S., Houze, S., Haouchine, D., Huteau, F., Lariven, S., Hubert, V., Michard, F., Bories, C., Pratlong, F., Le, B.J., Loiseau, P.M., Matheron, S., 2012. Leishmania resistance to miltefosine associated with genetic marker. Emerg. Infect. Dis. 18, 704–706.

Correa, E.B., Cunha, J.M., Bunn-Moreno, M.M., Madeira, E.D., 1992. Cyclophosphamide affects the dynamics of granuloma formation in experimental visceral leishmaniasis. Parasitol. Res. 78, 154–160.

Deep, D.K., Singh, R., Bhandari, V., Verma, A., Sharma, V., Wajid, S., Sundar, S., Ramesh, V., Dujardin, J.C., Salotra, P., 2017. Increased miltefosine tolerance in clinical isolates of Leishmania donovani is associated with reduced drug accumulation, increased infectivity and resistance to oxidative stress. PLoS Neglected Trop. Dis. 11, e0005641.

Dhillon, G.P., Sharma, S.N., Nair, B., 2008. Kala-azar elimination programme in India. J. Indian Med. Assoc. 106, 664–668.

Dorlo, T.P., Balasegaram, M., Beijnen, J.H., de Vries, P.J., 2012. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. J. Antimicrob. Chemother. 67, 2576–2597.

Eberhardt, E., Bulté, D., Van Bockstal, L., Van den Kerkhof, M., Cos, P., Delputte, P., Hendrickx, S., Maes, L., Caljon, G., 2018 Nov 8. Miltefosine enhances the fitness of a non-virulent drug-resistant Leishmania infantum strain. J Antimicrob Chemother. https://doi.org/10.1093/jac/dky450. [Epub ahead of print].

Fortin, A., Hendrickx, S., Yardley, V., Cos, P., Jansen, H., Maes, L., 2012. Efficacy and tolerability of oleylphosphocholine (OlPC) in a laboratory model of visceral leishmaniasis. J. Antimicrob. Chemother. 67, 2707–2712.

Freitas, J.C., Nunes-Pinheiro, D.C., Lopes Neto, B.E., Santos, G.J., Abreu, C.R., Braga, R.R., Campos Rde, M., Oliveira, L.F., 2012. Clinical and laboratory alterations in dogs naturally infected by Leishmania chagasi. Rev. Soc. Bras. Med. Trop. 45, 24–29.

Hendrickx, S., Boulet, G., Mondelaers, A., Dujardin, J.C., Rijal, S., Lachaud, L., Cos, P., Delputte, P., Maes, L., 2014. Experimental selection of paromomycin and miltefosine resistance in intracellular amastigotes of Leishmania donovani and L. infantum. Parasitol. Res. 113, 1875–1881.

Hendrickx, S., Mondelaers, A., Eberhardt, E., Delputte, P., Cos, P., Maes, L., 2015a. In vivo selection of paromomycin and miltefosine resistance in Leishmania donovani and L. Infantum in a Syrian hamster model. Antimicrob. Agents Chemother. 59, 4714–4718.

Hendrickx, S., Mondelaers, A., Eberhardt, E., Lachaud, L., Delputte, P., Cos, P., Maes, L., 2015b. Intracellular amastigote replication may not be required for successful in vitro selection of miltefosine resistance in Leishmania infantum. Parasitol. Res. 114, 2561–2565.

Kauffmann, F., Dumetz, F., Hendrickx, S., Muraille, E., Dujardin, J.C., Maes, L., Magez, S., De Trez, C., 2016. In vivo characterization of two additional Leishmania donovani strains using the murine and hamster model. Parasite Immunol. 38, 290–302.

Lucas, S., Nelson, A.M., 2015. HIV and the spectrum of human disease. J. Pathol. 235, 229–241.

Mondelaers, A., Sanchez-Canete, M.P., Hendrickx, S., Eberhardt, E., Garcia-Hernandez, R., Lachaud, L., Cotton, J., Sanders, M., Cuypers, B., Imamura, H., Dujardin, J.C., Delputte, P., Cos, P., Caljon, G., Gamarro, F., Castanys, S., Maes, L., 2016. Genomic and molecular characterization of miltefosine resistance in Leishmania infantum strains with either natural or acquired resistance through experimental selection of intracellular amastigotes. PLoS One 11, e0154101.

Monge-Maillo, B., Lopez-Velez, R., 2016. Treatment options for visceral leishmaniasis and HIV coinfection. AIDS Rev. 18, 32–43.

Monge-Maillo, B., Norman, F.F., Cruz, I., Alvar, J., Lopez-Velez, R., 2014a. Visceral leishmaniasis and HIV coinfection in the Mediterranean region. PLoS Neglected Trop. Dis. 8 e3021.

Monge-Maillo, B., Norman, F.F., Cruz, I., Alvar, J., López-Vélez, R., 2014b. Visceral leishmaniasis and HIV coinfection in the mediterranean region. PLoS Neglected Trop. Dis. 8 e3021.

Moreira, N., Vitoriano-Souza, J., Roatt, B.M., Vieira, P.M., Coura-Vital, W., Cardoso, J.M., Rezende, M.T., Ker, H.G., Giunchetti, R.C., Carneiro, C.M., Reis, A.B., 2016. Clinical, hematological and biochemical alterations in hamster (Mesocricetus auratus) experimentally infected with Leishmania infantum through different routes of inoculation. Parasites Vectors 9, 181.

Murray, H.W., Delph-Etienne, S., 2000. Visceral leishmanicidal activity of hexadecylphosphocholine (miltefosine) in mice deficient in T cells and activated macrophage microbicidal mechanisms. J. Infect. Dis. 181, 795–799.

Noli, C., Saridomichelakis, M.N., 2014. An update on the diagnosis and treatment of canine leishmaniosis caused by Leishmania infantum (syn. L. chagasi). Vet. J. 202 (3), 425–435.

Osman, A.S., Jennings, F.W., Holmes, P.H., 1992. The rapid development of drug-resistance by Trypanosoma evansi in immunosuppressed mice. Acta Trop. 50, 249–257.

Perez-Victoria, F.J., Castanys, S., Gamarro, F., 2003. Leishmania donovani resistance to miltefosine involves a defective inward translocation of the drug. Antimicrob. Agents Chemother. 47, 2397–2403.

Pitrak, D.L., 1999. Neutrophil deficiency and dysfunction in HIV-infected patients. Am. J. Health Syst. Pharm. 56 (Suppl. 5), S9–S16.

Pyana Pati, P., Van Reet, N., Mumba Ngoyi, D., Ngay Lukusa, I., Karhemere Bin Shamamba, S., Buscher, P., 2014. Melarsoprol sensitivity profile of Trypanosoma brucei gambiense isolates from cured and relapsed sleeping sickness patients from the Democratic Republic of the Congo. PLoS Neglected Trop. Dis. 8 e3212.

Rai, K., Cuypers, B., Bhattarai, N.R., Uranw, S., Berg, M., Ostyn, B., Dujardin, J.C., Rijal, S., Vanaerschot, M., 2013. Relapse after treatment with miltefosine for visceral leishmaniasis is associated with increased infectivity of the infecting Leishmania donovani strain. mBio 4 e00611-e00613.

Rijal, S., Ostyn, B., Uranw, S., Rai, K., Bhattarai, N.R., Dorlo, T.P., Beijnen, J.H., Vanaerschot, M., Decuypere, S., Dhakal, S.S., Das, M.L., Karki, P., Singh, R., Boelaert, M., Dujardin, J.C., 2013. Increasing failure of miltefosine in the treatment of kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or non-compliance. Clin. Infect. Dis. 56, 1530–1538.

Seifert, K., Matu, S., Javier Perez-Victoria, F., Castanys, S., Gamarro, F., Croft, S.L., 2003. Characterisation of Leishmania donovani promastigotes resistant to hexadecylphosphocholine (miltefosine). Int. J. Antimicrob. Agents 22, 380–387.

Seifert, K., Perez-Victoria, F.J., Stettler, M., Sanchez-Canete, M.P., Castanys, S., Gamarro, F., Croft, S.L., 2007. Inactivation of the miltefosine transporter, LdMT, causes miltefosine resistance that is conferred to the amastigote stage of Leishmania donovani and persists in vivo. Int. J. Antimicrob. Agents 30, 229–235.

Shi, X., Sims, M.D., Hanna, M.M., Xie, M., Gulick, P.G., Zheng, Y.H., Basson, M.D., Zhang, P., 2014. Neutropenia during HIV infection: adverse consequences and remedies. Int. Rev. Immunol. 33, 511–536.

Srivastava, S., Mishra, J., Gupta, A.K., Singh, A., Shankar, P., Singh, S., 2017. Laboratory confirmed miltefosine resistant cases of visceral leishmaniasis from India. Parasit.Vectors 10, 49.

van Griensven, J., Carrillo, E., Lopez-Velez, R., Lynen, L., Moreno, J., 2014. Leishmaniasis in immunosuppressed individuals. Clin. Microbiol. Infect. 20, 286–299.

Vanhoutte, B., Cappoen, D., Maira, B.d.M., Cools, F., Torfs, E., Coenye, T., Martinet, W., Caljon, G., Maes, L., Delputte, P., Cos, P., 2017. Optimization and characterization of a murine lung infection model for the evaluation of novel therapeutics against Burkholderia cenocepacia. J. Microbiol. Methods s139, 181–188.

Vermeersch, M., da Luz, R.I., Tote, K., Timmermans, J.P., Cos, P., Maes, L., 2009. In vitro susceptibilities of Leishmania donovani promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. Antimicrob. Agents Chemother. 53, 3855–3859.

Xu, X., Zhang, X., 2015. Effects of cyclophosphamide on immune system and gut microbiota in mice. Microbiol. Res. 171, 97–106.