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The impact of IL-10 dynamic modulation on host immune response against visceral leishmaniasis

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ABSTRACT

Leishmaniasis is a vector-borne disease caused by protozoan parasites from the genus *Leishmania*. The most severe form of disease is visceral leishmaniasis (VL), which is fatal if left untreated. It has been demonstrated that interleukin (IL)-10, is associated with disease progression and susceptibility. In this work, we took advantage of a transgenic mouse model that expresses high levels of IL-10 upon zinc sulfate administration (pMT-10). We addressed the role of IL-10 during the initial stages of *L. donovani* infection by analyzing the parasite burden in the spleen and liver of the infected pMT-10 and WT mice as well as the histopathological alterations upon IL-10 induction. Furthermore, the profile of cytokines expressed by T cells was assessed. Our results demonstrate that an increase in IL-10 production has an impact early but not later after infection. This specific temporal role for IL-10-mediated susceptibility to VL is of interest.

1. Introduction (1896)

Leishmaniasis is a group of tropical neglected diseases caused by protozoan parasites from the genus *Leishmania*, which range from self-healing cutaneous lesions to fatal visceral disease. *Leishmania donovani* is one of the causative agents of visceral leishmaniasis (VL) responsible for high morbidity and mortality in East Africa and South Asia [13]. Immunity to VL has long been known to depend on the development of type I immune responses characterized by initial production of interleukin-12 (IL-12) by antigen-presenting cells (APCs) that induce interferon-γ (IFN-γ)-secreting Th1 T cells [11]. These, in turn, will activate and prime host innate cells for the production of microbicidal molecules as reactive nitrogen and oxygen species (RNS and ROS, respectively). However, the presence of a Th1 response does not immediately correlate with protection, whose action is neutralized by immune regulatory factors [11]. Among those, the role of IL-10 as a major mediator in the suppression of a leishmanicidal immune response has gained a particular attention in the past [1,4,7,8,9],. IL-10 is an anti-inflammatory cytokine induced during experimental and human infections [7], whose levels correlate with disease severity [5,14]. IL-10 has the potential to disable host anti-leishmanial defense and foster visceral infection by compromising Th1 responses and deactivating host phagocytes [4,8],. In contrary, mice deficient in IL-10 or in which IL-10 signaling is blocked are highly resistant to *Leishmania* infection [11]. Yet, the relevance of IL-10 production during the initial innate immune response or upon the establishment of an adaptive immune response is still unknown. Since a coordinated inflammatory response is the hallmark of a protective immune response to infection, we evaluate the early impact of IL-10 expression before or after the onset of a *Leishmania*-specific adaptive immune response. To address this issue, we took advantage of

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the pMT-10 mouse model, in which IL-10 was induced by zinc sulfate administration. Herein, we show that overexpression of IL-10 during the initial steps of infection underlies susceptibility to *L. donovani* infection, by modulating the expansion of multifunctional CD4 T cells.

2. Material and methods

2.1. Animals

pMT-10 on a C57BL/6 background and C57BL/6 mice were maintained under specific pathogen-free at the Life and Health Sciences Research Institute (ICVS, Braga, Portugal) and allowed food and water *ad libitum*. Animals with eight to twelve weeks were used in the experiments. Experimental animal procedures agreed with the European Council Directive (2010/63/EU) guidelines that where transposed into Portuguese law (Decree-Law no. 113/2013, August 7th). Additionally, the experiments were conducted with the approval of the UMinho Ethical Committee (process no. SECVS 074/2016) and complied with the guidelines of the Committee and National Council of Ethics for the Life Sciences (CNECV). RS has an accreditation for animal research given from Portuguese Veterinary Direction (Ministerial Directive 1005/92).

2.2. Parasites

A cloned line of virulent *L. donovani* (MHOM/IN/82/Patra1) was maintained by weekly subpassages at 26 °C in RPMI 1640 medium (Lonza, Swtzerland) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Lonza, Switzerland), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 20 mM HEPES buffer (BioWhittaker, Walkersville, MD). Only *L. donovani* promastigotes under four to ten passages were used in the experiments as previously defined [6]. To prepare soluble *Leishmania donovani* antigens (SLA), 50×10^6 parasites were resuspended in 1 ml of sterile PBS and 10 cycles of freeze/thaw were performed. Total protein content was assessed through BCA method.

2.3. In vivo infection

WT and pMT-10 mice were infected intraperitoneally with 20×10^6 stationary-phase *L. donovani* promastigotes resuspended in apyrogenic sterile PBS. IL-10 was induced by the administration of 2% sucrose solution containing 50 mM of zinc sulphate (Sigma) in the drinking water as previously described [2]. WT mice received water with 2% of sucrose, as a control. IL-10 induction in pMT-10 mice was performed at early (0–12 days) and late (35–47 days) time points of infection (Fig. 1A). Sixty days post-infection, the animals were euthanized and the spleen, liver and blood were recovered for further biochemical and histopathological analysis.

2.4. Parasite burden

DNA was extracted from spleen and liver homogenates using the phenol-chloroform-isoamyl alcohol method. Parasite burden was assessed as previously described [10], using a TaqMan-based qPCR assay for detection and quantification of *L. donovani* kinetoplastid DNA.

2.5. Flow cytometry analysis

Splenocytes were stimulated with SLA (10 µg/ml) during 18 h to allow the proliferation and functional response of specific T cells. Brefeldin A (10 µg/ml) was added during the last 3 h of SLA stimulation. The anti-mouse monoclonal antibodies used to perform this study were all purchased to BioLegend (CA, USA). Abs used include: FITC

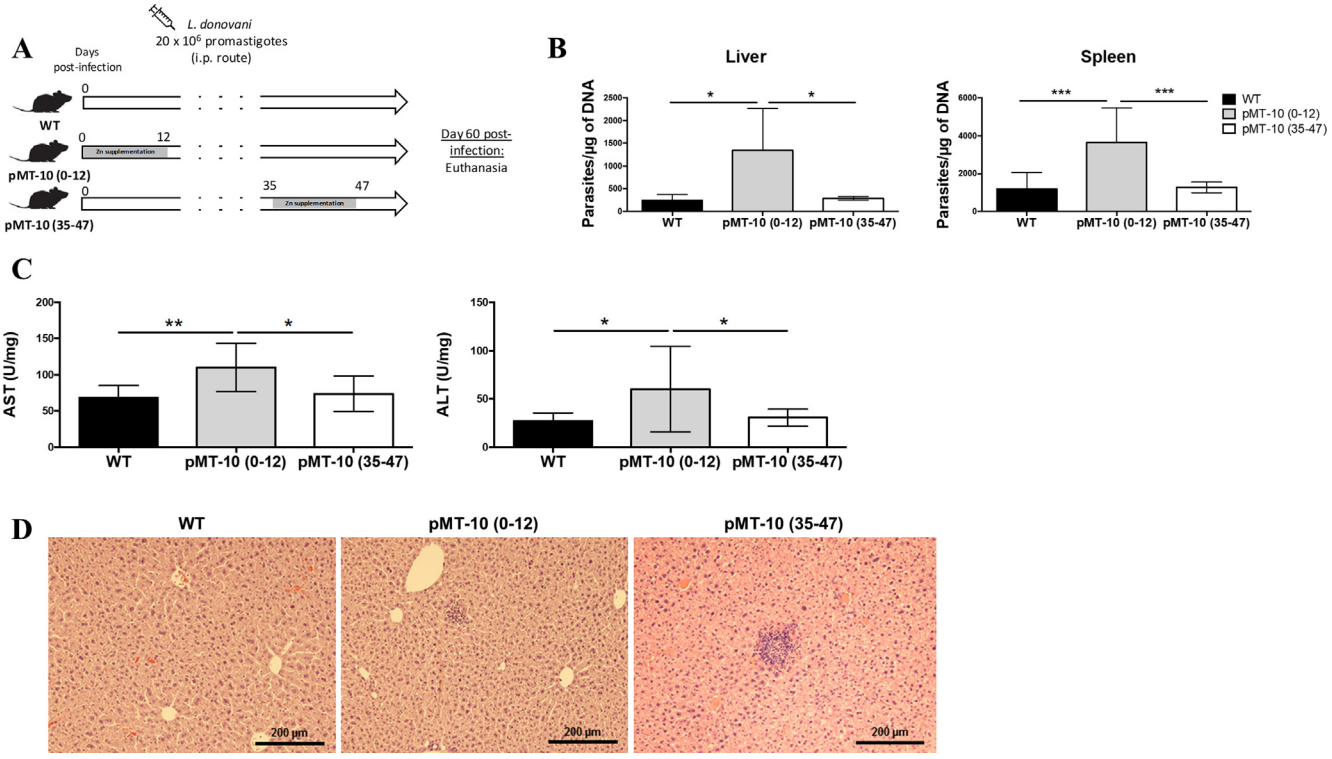

Fig. 1. IL-10 overexpression at distinct time points of *Leishmania donovani* infection associates with distinct clinical outcomes. (A) pMT-10 and WT mice were intraperitoneally infected with 20×10^6 *L. donovani* promastigotes. The pMT-10 (0–12) group received zinc supplementation from day 0 to 12 post-infection while the pMT-10 (35–47) received from day 35 to 47 post-infection. WT mice were not supplemented with zinc through the course of infection. (B) All animals were sacrificed at day 60 post-infection. Parasite burden in spleen and liver homogenates. (C) ALT and AST levels in the serum of infected animals. (D) H&E staining of liver sections (5 µm). Data is shown as mean ± SD; n = 4–6 mice/group. One representative experiment is shown out of two. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

anti-mouse TNF Ab, clone MP6-XT22; PerCP/Cy5.5 anti-mouse CD3, clone 145-2C11; PeCy7 anti-mouse IFN-γ, clone XMG1.2; APC anti-mouse IL-10; clone JES5-16E3; APC/Cy7 anti-mouse CD4, clone GK1.5; BV421 anti-mouse IL-2, clone JES6-5H4; BV711 anti-mouse CD8, clone 53–6.7. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and data analysed using FlowJo software (TreeStar). Supplementary figure 1 depicts the identification criteria used for selecting the cellular populations of interest.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.07.001.

2.6. Histology

After mice euthanasia, the liver was perfused with saline solution and fixed with 10% (w/v) formalin. The sections were further dehydrated and embedded in paraffin for further histopathological analysis. Samples were then sectioned for haematoxylin and eosin staining. Bright field images were acquired on a BX6 microscope.

2.7. ALT/AST quantification

ALT and AST levels were quantified in the serum using an AutoAnalyzer (Prestige 24i, PZ Cormay S.A) as previously described [10].

2.8. IL-10 quantification

IL-10 levels were quantified on blood, spleen and liver homogenates by ELISA and the transcriptional levels of *Il10* gene were assessed by real-Time quantitative PCR (qRT-PCR). Briefly, spleen and liver were homogenized and stored for IL-10 quantification using IL-10 mouse uncoated ELISA kit (ThermoScientific) or in TRIreagent (Sigma-Aldrich) for RNA extraction. Total RNA was extracted and the synthesis of cDNA was made with SensiFAST™ cDNA Synthesis Kit (Bioline). qRT-PCR reactions were run for each sample on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler (Bio-rad) using SensiFAST SYBR Hi-ROX Kit (Bioline). Primer sequences were obtained from Alfagene (Portugal) and thoroughly tested. The results were normalized to the expression of the housekeeping gene *ubiquitin*. After amplification, cycle threshold-values (Ct-values) were calculated for all samples and gene expression changes were analyzed in the CFX Manager Software (Bio-Rad). The serum, splenic and liver IL-10 transcript and expression levels are shown on Supplementary figure 2A and B respectively.

2.9. Statistical analysis

Statistical analyses were performed with the GraphPad Prism 6 software. A one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test was employed for multiple group comparisons. Statistically significant values are as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

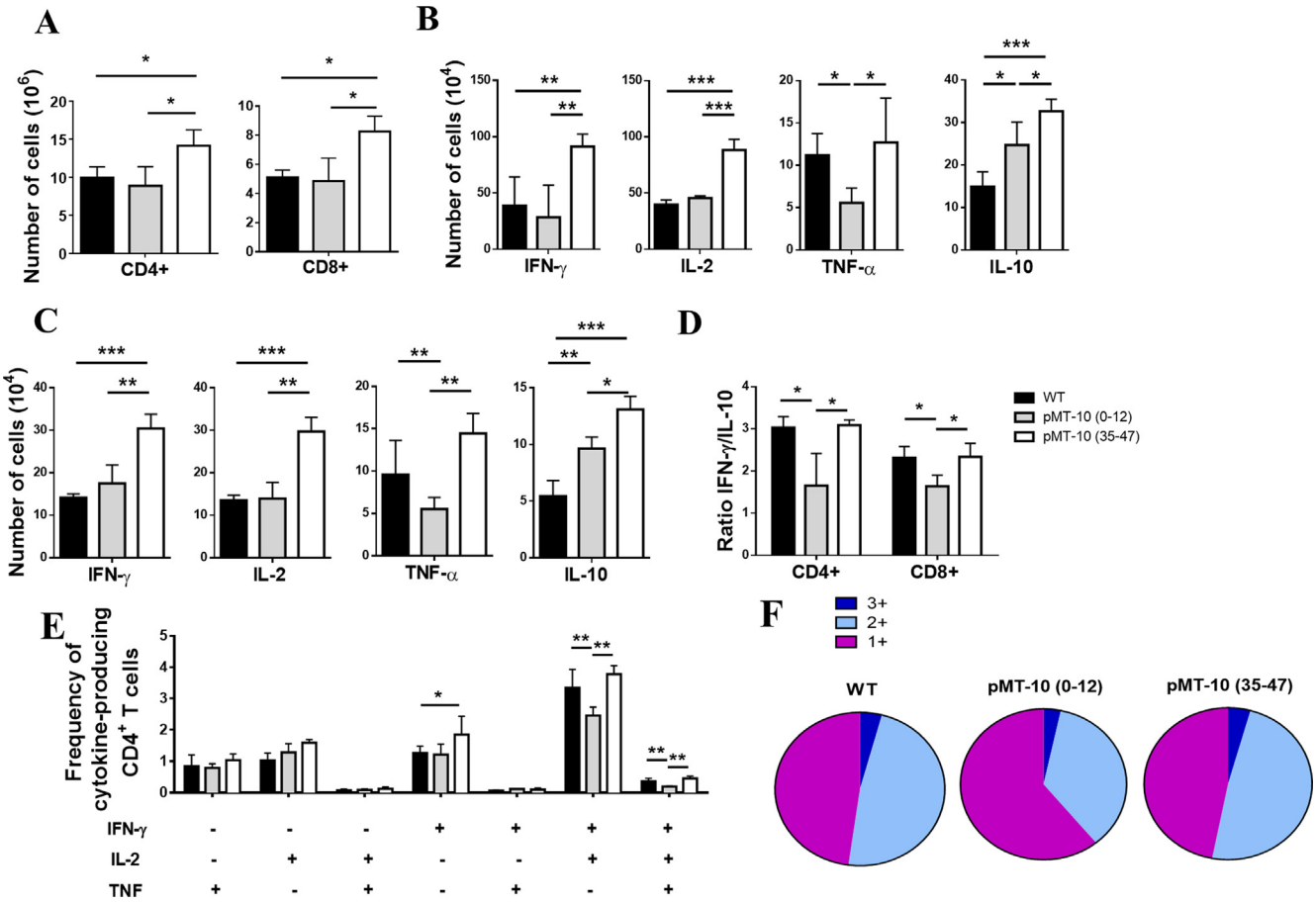

Fig. 2. Early IL-10 overexpression leads to a loss of multifunctional CD4 T cells and lower IFN-γ/IL-10 ratios that associates with susceptibility. (A) Total CD4 and CD8 T cells number in the spleen of infected mice. Number of IFN-γ-, IL-2-, TNF- and IL-10-producing CD4 T cells (B) and CD8 T cells (C). (D) IFN-γ/IL-10 ratio in CD4 and CD8 T cells. (E) Frequency of cytokine-producing CD4 T cells expressing each of the seven possible combinations of IFN-γ, IL-2 and TNF. (F) Fraction of the total response of CD4 T cells comprising cells expressing all three (3+), two (2+) or one cytokine (1+). Data is shown as mean ± SD; n = 4–6 mice/group. One representative experiment is shown out of two. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results and discussion

To understand the temporal impact of IL-10 during the immune response towards *L. donovani*, we used the pMT-10 mouse model infected with *L. donovani*. IL-10 expression induced by the administration of zinc supplementation in the water was controlled and induced at different time points during the infection. The IL-10 was overexpressed from day 0 to 12 [pMT-10 (0–12)] or from day 35 to 47 [pMT-10 (35–47)] post-infection (Fig. 1A). We demonstrated that overexpression of IL-10 during the initial 12 days of infection led to a significantly increased parasite burden at day 60 post-infection in the major parasitized organs, the spleen and the liver, when compared to WT and pMT-10 (35–47) counterparts (Fig. 1B). In the presence or absence of zinc supplementation in the drinking water, the parasite burdens of C57BL/6 wt were similar disregarding any potential impact of zinc supplementation on the immune response to *Leishmania* infection (data not shown). The increased susceptibility displayed by pMT-10 (0–12) animals was associated with increased serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) (Fig. 1C), which are biomarkers associated with hepatic toxicity [3]. Surprisingly, the overexpression of IL-10 upon the 35th day of infection had no impact on the visceral parasite burden nor on induced liver damage. Nevertheless, a larger number of complex inflammatory infiltrates were found in the liver of infected pMT-10 (35–47) (Fig. 1D). The formation of hepatic granulomas during *Leishmania* infection has been extensively studied. It is currently known that at one week post-infection, T cells are recruited to the granuloma to confine parasitized resident Kupffer cells and prevent parasite dissemination [11]. Overexpression of IL-10 in infected pMT-10 (35–47) displayed larger and more numerous infiltrates than those observed in infected pMT-10 (0–12) mice, suggesting that these might be associated with a lower parasite load when compared to pMT-10 (0–12). Altogether, our results suggest that IL-10 overexpression later after infection, at a moment where the T cell response is established, leads to an increased cellular mobilization to achieve the same control as seen in WT mice. In opposition, overexpression of IL-10 during the initial steps of the infection impacts host ability to control *L. donovani* infection by limiting the development of a protective adaptive immune response. To further address this issue, a qualitative and quantitative analysis of splenic parasite-specific T cells was performed at day 60 post-infection. Our results indicated that the pMT-10 (35–47) group displayed a higher number of both CD4 and CD8 T cells in the spleen when compared to pMT-10 (0–12) and WT mice (Fig. 2A), which is consistent with the inflammatory infiltrates observed in the liver (Fig. 1D). Moreover, the infected pMT-10 (35–47) mice displayed increased *Leishmania*-specific IFN-γ- and IL-2-producing CD4 (Fig. 2B) and CD8 T cells (Fig. 2C). Yet, this was accompanied by a significant increase on the number of IL-10-producing CD4 (Fig. 2B) and CD8 T cells (Fig. 2C). In accordance with the observed susceptibility to infection (Fig. 1B), the *L. donovani*-infected the pMT-10 (0–12) group displayed lower number of TNF-α-producing CD4 and CD8 T cells and higher number of IL-10-producing CD4 and CD8 T cells (Fig. 2B-C). We and others have previously shown that increased IFN-γ/IL-10 ratios are associated with protection against *Leishmania* infection [12,9],. We observed that pMT-10 (0–12) with increased susceptibility to *L. donovani* infection (Fig. 1B) are associated to a lower IFN-γ/IL-10 ratio compared to WT and pMT-10 (35–47) mice (Fig. 2D). We next extended the analyses to multifunctional CD4 T cells. Strikingly, we found a loss of CD4 T cells expressing both IFN-γ and IL-2 (2+) as well of those expressing the three cytokines (3+ - IFN-γ^+IL-2$^+$TNF-α^+) in infected pMT-10 (0–12) compared to infected WT and pMT-10 (35–47) mice (Fig. 2E). Thus, our results are consistent with the notion that protection against distinct *Leishmania* species is accompanied by the induction of *Leishmania*-specific multifunctional T cell response, shifting qualitatively from single to triple producers Darrah et al. [15], Selvapandiyan et al. (2009). Quantifying the fraction of the total cytokine response comprising three (3+), any two (2+) or any one (1+) cytokine, we found that over half of the response in the pMT-10 (0–12) was in single-producers, whereas the response in the other groups were dominated by double producer T cells (Fig. 2F). Overall, the early up-regulation of IL-10 seems to be clearly associated with a decrease of IFN-γ/IL-10 ratio in T cells dampening multifunctional CD4 T cells. Such immunologic landscape contributes therefore for the establishment of a successful infection.

4. Conclusions

In this work, we disclose a temporally-regulated role for IL-10 during the initial steps on infection, which impacts the pathogenesis of VL. We demonstrate that the presence of high levels of IL-10 in the initial phase of infection culminates in higher susceptibility, which is mainly associated to the development of a permissive environment characterized by a decreased frequency of multifunctional CD4 T cells. Interestingly, our results highlight the role of IL-10 in the very early phase of infection. Indeed, despite higher levels of IL-10 at the steady state (days 35) and of IL-10 expressing CD4 and CD8 T cells, IFN-γ remains elevated. As such, the overexpression of IL-10 upon the establishment of the adaptive immune response do not impact parasite burden. Overall, this work shed some light on the temporal impact of IL-10 on host immune responses against visceral leishmaniasis.

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References

[1] Y. Belkaid, et al., The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti–IL-10 receptor antibody for sterile cure, J. Exp. Med. 194 (10) (2001) 1497–1506, https://doi.org/10.1084/jem.194.10.1497.

[2] A. Cardoso, et al., The dynamics of interleukin-10-afforded protection during dextran sulfate sodium-induced colitis, Front. Immunol. 9 (MAR) (2018), https://doi.org/10.3389/fimmu.2018.00400.

[3] S. Gowda, et al., A review on laboratory liver function tests, Pan African Med. J. 3 (November) (2009) 17, https://doi.org/10.11604/pamj.2009.3.17.125.

[4] M.M. Kane, D.M. Mosser, The role of IL-10 in promoting disease progression in leishmaniasis, J. Immunol. 166 (2) (2001) 1141–1147, https://doi.org/10.4049/jimmunol.166.2.1141.

[5] C.L. Karp, et al., In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma, J. Clin. Investigat. 91 (4) (1993) 1644–1648, https://doi.org/10.1172/JCI116372.

[6] D. Moreira, et al., Impact of continuous axenic cultivation in *Leishmania* infantum virulence, PLoS Neglected Trop. Dis. 6 (1) (2012), https://doi.org/10.1371/journal.pntd.0001469.

[7] H.W. Murray, et al., Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy, Infect. Immunol. 70 (11) (2002) 6284–6293, https://doi.org/10.1128/IAI.70.11.6284-6293.2002.

[8] S. Nylén, D. Sacks, Interleukin-10 and the pathogenesis of human visceral leishmaniasis, Trends Immunol. (2007) 378–384, https://doi.org/10.1016/j.it.2007.07.004.

[9] J. Paul, S. Karmakar, T. De, TLR-mediated distinct IFN-γ/IL-10 pattern induces protective immunity against murine visceral leishmaniasis, Eur. J. Immunol. 42 (8) (2012) 2087–2099, https://doi.org/10.1002/eji.201242428.

[10] V. Rodrigues, et al., Abortive T follicular helper development is associated with a defective humoral response in *Leishmania* infantum-infected macaques, PLoS Pathogens 10 (4) (2014), https://doi.org/10.1371/journal.ppat.1004096.

[11] V. Rodrigues, et al., Regulation of immunity during visceral *Leishmania* infection, Parasites Vectors (2016), https://doi.org/10.1186/s13071-016-1412-x.

[12] R. Silvestre, et al., SIR2-deficient *Leishmania* infantum induces a defined IFN-gamma/IL-10 pattern that correlates with protection, J. Immunol. (Baltimore, Md.: 1950) 179 (5) (2007) 3161–3170 (doi: 179/5/3161 [pii]).

[13] WHO, Control of the Leishmaniases. World Health Organization, Geneva, Tech. Rep. Ser. 949 (March) (2010) 22–26.
[14] S. Saha, et al., IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of Leishmania donovani infection in India, J. Immunol. 179 (8) (2007) 5592–5603, https://doi.org/10.4049/jimmunol.179.8.5592.
[15] P.A. Darrah, et al., Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major, Nat. Med. 13 (7) (2007) 843–850, https://doi.org/10.1038/nm1592.