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Leishmania infantum transfected with toxic plasmid induces protection in mice infected with wild type *L. infantum* or *L. amazonensis*

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ABSTRACT

Leishmania infantum infection may cause visceral leishmaniasis (VL), a fatal disease having worldwide distribution, that may be silent or asymptomatic. The latter indicates that immunity is naturally developed in some individuals, and, therefore, a vaccine against VL would be possible. Molecular mechanisms of gene expression are being understood in *Leishmania*, and this knowledge may be useful for vaccine development. The aim of this study was developing an attenuated strain by regulating the expression of toxic proteins in a stage specific manner. For that purpose, the 3' UTR of an amastin gene, known by its increased expression in the amastigote phase, was selected for direct the expression of exogenous proteins. This construct (pFL-AMA), firstly, was proved effective for the expression of mCherry specifically in the intracellular form of *L. infantum*, as demonstrated by fluorescence microscopy, flow cytometry and Western blotting. Afterwards, mCherry coding sequence was replaced, in the pFL-AMA plasmid, by either egg avidin or the active form of bovine trypsin. Viability of transfected parasites was evaluated in promastigote axenic cultures and in *in vitro* infection of macrophages. Both lines of transfected parasites showed a limited capacity to multiply inside macrophages. BALB/c mice were inoculated intraperitoneally (i.p.) with a single dose consisting of 2×10^6 *L. infantum* promastigotes transfected with plasmids bearing the toxic genes. After 10 weeks post-inoculation, no parasites were recovered by limiting dilution in either liver or spleen, but a specific immunological response was detected. The immunization with transfected parasites induced cellular and humoral immune responses with activation of $TCD4^+$, $TCD8^+$ and B cells, having a TH1-type response with increased levels of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-6. In parallel groups of mice, a challenge consisting on 1×10^6 virulent parasites of either *L. infantum* (inoculated i.p.) or *L. amazonensis* subcutaneously (s.c.) was performed. Vaccinated mice, challenged with *L. infantum*, showed lower parasite burdens in liver, spleen and bone marrow than infected mice with *WT L. infantum* (non-vaccinated); similarly, vaccinated mice developed smaller footpad inflammation than control group. These data support this strategy as an efficient immunization system aimed to the development of vaccines against different forms of leishmaniasis.

1. Introduction

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, which is transmitted to the mammalian host through the bite of infected sandflies. Currently, this disease affects between 12 and 15 million people worldwide (Torres -Guerreiro et al., 2017). At least 20 species of *Leishmania* are pathogenic for humans, and depending on the species and the host immunity status, three main clinical manifestations

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[1] All authors importantly contributed to this work. It has been supervised by Dr. Daniel R. Abánades and Dr. Selma Giorgio in the Brazil, and by Dr. José María Requena in Spain.

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of the disease, cutaneous (CL), mucocutaneous (MCL) and visceral (VL), have been described (Desjeux, 2004). The VL is caused by *L. donovani* in the Indian subcontinent and eastern Africa and by *L. infantum* in the whole Mediterranean area, the Middle East, and Latin America. The VL is the most severe form of the disease, in which parasites migrate and multiply in lymphoid organs, including spleen, liver and bone marrow, leading to organ malfunction and fatality in absence of treatment (Alvar et al., 2012). There are few available drugs and sometimes they have toxic effects, moreover, the appearance of drug-resistant parasites is another factor that limits their use (Kumar et al., 2011).

There is no currently vaccine available for human leishmaniasis, even though it is considered a feasible goal since natural infections in some individuals generate immunity, a successful vaccine will depend on the generation of memory T cells that can be maintained without the continuous presence of parasites (Glennie and Scott, 2016; Peters et al., 2014). Host resistance to *Leishmania* infection is mediated by cellular immune response involving mainly a Th1 response, characterized by the production of IL-12 (Interleukin-12) and IFN-γ (Interferon-gamma) (Messlinger et al., 2018).

These two cytokines control the effector functions of macrophages that are crucial for destruction of the internalized parasites (Peruhype-Magalhães et al., 2005). To date, leishmanization remains as the sole vaccination strategy that results against leishmaniasis effectively (Iborra et al., 2018). This strategy, used during centuries for preventing CL, consists in the inoculation of live virulent parasites on specific skin sites with the aim of inducing an immune protective response capable of preventing leishmaniasis in posterior natural infection (Saljoughian et al., 2014). In addition, leishmanization has been found to induce cross-protection, for instance, protection against VL in murine models results after initial *L. major* infection (Romano et al., 2015). However, the use of leishmanization has been discontinued in many countries due to biosecurity and bioethical concerns. In order to improve vaccine safety, different strategies have been developed, such as killed parasite-based vaccines (Vélez et al., 2000, 2005), protein extracts from both vector saliva (Kamhawi et al., 2014) and parasite (Rivier et al., 1999), DNA (Abdian et al., 2011) or recombinant proteins (Araújo et al., 2011) vaccines. However, vaccines based on dead parasites, protein extracts or recombinants are generally ineffective as they induce short-term immunity and usually improve immune responses when associated with adjuvants, which can present problems with vaccine licensing to human (Okwor and Uzonna, 2008).

The possibility of creating genetically modified parasites, through either the elimination of essential genes or the inclusion of detrimental genes, is considered a powerful alternative in developing effective leishmaniasis vaccines (Silvestre et al., 2008; Saljoughian et al., 2014; Anand and Madhubala, 2015; Fiuza et al., 2015). Vaccination using genetically modified live parasites can mimic natural infections and thus would be better candidates for leishmanization. These modified parasites display the entire antigenic repertoire of the parasite and their use alleviates the need of using adjuvants (Silvestre et al., 2008).

Attenuation by the directed expression of suicidal genes in the intracellular stage has been explored in previous works. For example, Ma et al. (2015) designed a strategy for the development of *Trypanosoma cruzi* strains expressing the alpha-toxin DDDHA and Cecropin A-DDDHA genes in order to induce self-destruction in the process of differentiation in the intracellular phase. In *Leishmania*, suicide metabolic gene has been described such as Herpes Simplex type-1 Virus Thymidine Kinase (HSV-1 TK) and transfected *Leishmania* with this gene has shown reduced viability in macrophage infections when treated with ganciclovir (Muyombwe et al., 1997). In this work, we describe the development of transgenic *L. infantum* strains that express detrimental genes specifically in the intracellular form of the parasite. The expression of these genes promoted the parasite death after differentiation into amastigote, and, therefore, this molecular system would represent a new method of "attenuation". Parasites transfected with these constructs might constitute valuable vaccine candidates against leishmaniasis.

2. Materials and methods

2.1. Plasmids construction

The complete 844bp sequence between two alpha tubulin coding regions (LinJ.13.1450 and LinJ.13.1460) was amplified from total *L. infantum* DNA by PCR using three sets of primers pairs: UtrTub1XbaI: GCTCTAGATAAGGTACACTCGTGCCGCG and UtrTub1BamHI: CGGGATCCGTTTTGTGTTCGCCAGGAGG; UtrTub2EcoRIf: CGGAATTCTAAGGTACACTCGTGCCGCG and UtrTub2EcoRVr: CGGATATCGTTTTGTGTTCGCCAGGAGG; UtrTub3HindIIIf: CGAAGCTTTAAGGTACACTCGTGCCGCG and UtrTub3ClaIr: CGATCGATGTTTTGTGTTCGCCAGGAGG. The complete 2.1Kb sequence between two amastin-like surface protein coding regions (LINF_340024300 and LINF_340024200) was amplified from total *L. infantum* DNA by PCR using the following primers: UtrAmaHindIIId: CCCAAGCTTTAGGATAGAGGTAGGACAGG and UtrAmaClaIr: CCATCGATCATCGTCACAAAAAGGAGCGAC. The 600bp Puromycine-*N*-acetyltransferase coding region was amplified from the pGeneClip™ Puromycin Vector (Clontech®) by PCR using the following primers: PuroBamHId: CGGTGGATCCATGACCGAGTACAAGCCCAC and PuroEcoRIr: CGGAATTCTCAGGCACCGGGCTTGCGGG, and the 711pb mCherry coding sequence was amplified from pmCherry vector (Clontech®) using the following primers: mCherryEcoRVf: CGGATATCATGGTGAGCAAGGGCGAGG and mCherryHindIIIr: CCAAGCTTTTACTTGTACAGCTCGTCC. Restriction sites are indicated in primer name and are underlined in the sequences. For amplification, DNA was denatured at 94 °C, 5 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and a final step of 72 °C for 5 min in a MJ Research PCR thermal cycler. The PCR products were visualized on a 0.8 % agarose gel and expected size fragments were purified (QIAquick PCR purification Kit, Qiagen®); after digestion with the corresponding pair of restriction endonucleases, the inserts were ligated into the similarly digested pBluescript SK (-) (Stratagene) cloning vector. After confirmation of selected clones by restriction digestion and DNA sequencing, fragments were combined following a five-step cloning procedure. Firstly, the plasmid pBls-IRTub1 was digested with *Xba*I and *Bam*HI and the obtained fragment was introduced into pBls-Puro to obtain pBls-Tub1-Puro. Afterwards, the plasmid pBls-Tub1-Puro was digested with *Xba*I and *Eco*RI and the fragment was introduced into pBls-Tub2 to obtain pBls-Tub1-Puro-Tub2. Thirdly, the plasmid pBls-mCherry was digested with *Eco*RV and *Hin*dIII and the fragment was introduced into pBls- Tub1-Puro-Tub2 to obtain pBls-Tub1-Puro-Tub2-mCherry. Finally, we cloned the pBls-IRama or the pBls-IRtub3, digesting these plasmids with *Hin*dIII and *Cla*I and the obtained fragment was introduced into pBls-Tub1-Puro-Tub2-mCherry to obtain the two final constructions: pBls-Tub1-Puro-Tub2-CherryAma (pFL-AMA) and pBls-Tub1-Puro-Tub2-Cherry-Tub3 (pFL-TUB) (Fig. 1). By double digestion with *Eco*RV and *Hind*III of pFL- AMA plasmid, mCherry coding region was substituted by egg avidin or activated form of trypsin coding sequences, obtained pFL-AMA-AviTox or –TrypTox plasmids respectively. Avidin and Trypsin coding sequences were synthetized including 6xHis tag coding after ATG initiation codon (Genone S.L.) and submitted to codon optimization process, based in previously published data (Sinha et al., 2011) (see Supplementary file 1 for further details).

Aiming at analyzing a whether the stage-specific expression is maintained in plasmid pFL-AMA, the open reading frame (ORF) encoding mCherry was cloned upstream of the amastin 3'-UTR, generating the plasmid pFL-AMA-mCherry. In parallel, a similar construct was generated using the alpha-tubulin 3'-regulatory region instead of the amastin one; this plasmid, named pFL-TUB-mCherry, was used as control.

2.2. Parasites culture and transfection

L. infantum promastigotes (MHOM/BR/1972/LD) were grown in Schneider medium supplemented with 10 % fetal bovine serum (FBS – Sigma-Aldrich®), 5% filtered human urine, and gentamicin (Sigma-

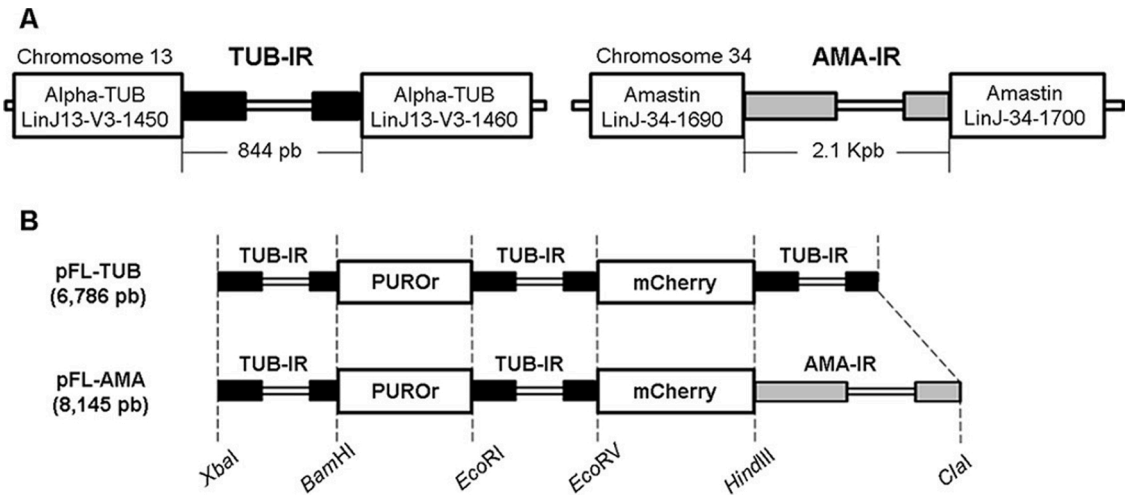

Fig. 1. pFL plasmids description. A) Schematic representation of *L. infantum* chromosomes where the Intergenic Regions (IR) of alpha-tubulin and amastin clusters used in this study are localized. White box represents the Open Reading Frame (ORF), genedb code for each gene and IR length are indicated. B) Polylinker of pBluescript plasmid showing the different IRs and ORFs included in pFL-TUB and pFL-AMA plasmids. Restriction enzymes employed to cloning (bottom) and final plasmid weight (left) are shown. PUROr (puromycin resistance gene).

Aldrich®) at 50 μg/mL (parasite medium). *L. amazonensis* promastigotes (MHOM/BR/1973/M2269) were grown in RPMI medium supplemented with 10 % FBS and gentamicin at 50 μg/mL.

L. infantum transfection procedure was conducted as described (Abanades et al., 2009), using 20 μg of plasmid DNA (pFL-TUB-mCherry, pFL-AMA-mCherry, -AviTox or -TrypTox) that were purified by the MaxiPrep® Plasmid Kit (QIAGEN®). The drug selection (Puromycin, SIGMA®) was added 24 h after the electroporation at 50 μg/mL and parasites transfected with toxic plasmids (pFL-AMA-AviTox or –TrypTox) or non-toxics (pFL-TUB-mCherry or pFL-AMA-mCherry) were maintained with presence of the drug.

2.3. Differentiation of the L. infantum promastigotes into amastigotes

For differentiation in amastigotes 1×10^7 parasites/mL of *L. infantum* transfected or un-transfected were washed twice in Schneider acidic medium (pH 5.5, adjusted by addition of succinic acid – 20 mM) and incubated at 37 °C, 21 % O_2, 5 % CO_2. For the re-differentiation to the promastigote life-stage, parasites were submitted for six or ten days to differentiation conditions and then they were washed twice in pH 7.2 parasite growth medium and incubated at 26 °C.

2.4. Immunization and infection of the BALB/c mice

Five-week-old isogenic female BALB/c mice were obtained from the Multidisciplinary Center for Biological Research (CEMIB/UNICAMP), respecting the standards and commissions required by the institution (UNICAMP Institute of Biology).The project was approved by the Committee on Ethics in the Use of Animals - CEUA (protocol 4650-1/2017). For the immunization experiments, five groups of five mice each were used and inoculated (i.p) with 2×10^6 *L. infantum* promastigotes/mouse transfected with toxic plasmids, pFL-AMA-AviTox (group 1) or pFL-AMA-TrypTox (group 2) or non-toxic ones, pFL-AMA-mCherry (group 3). For the infection experiments *L. infantum* promastigotes WT (wild type – group 4) were washed in PBS and resuspended in sterile saline with 200 μL/2×10^6 parasites and inoculated by i.p. As a control, the animals received 200 μL of sterile saline (group 5) by i.p. For infection with *L. amazonensis* promastigotes, the parasites were washed with PBS, resuspended in 40 μL/1×10^6 parasites and inoculated subcutaneously (s.c.) in the left footpad.

2.5. Fluorescence microscopy and flow cytometry

The mCherry detection in transfected parasites with pFL-TUB-mCherry and pFL-AMA-mCherry were analyzed by fluorescence microscopy and flow cytometry after axenic differentiation. For fluorescence microscopy observation of parasites was fixed with 4% formaldehyde in PBS and the images were taken in an AXIO ImagerA2 (Zeiss®). Flow cytometry analysis of mCherry-producing parasite promastigotes were performed in FACs-Galious® (Beckmann Coulter). For this, the parasites were washed with PBS and resuspended in 300 μL of cold PBS with 2% BSA for analysis.

2.6. Protein analysis and western blot

Transfected promastigotes and axenic amastigotes with pFL-AMA-mCherry, after 4 and 24 h of differentiation, were collected and mixed in *Laemmli* buffer to a final concentration of 2×10^5 parasites/μL. Briefly, *L. infantum* parasites were centrifuged 5 min at 600 *g* and washed twice with cold PBS. After suspension in 1 mL of PBS, centrifugation at 11,200 *g* for 1 min at room temperature (RT) was performed. Pellet was suspended in *Laemmli* buffer with 2.5 % of β-mercaptoethanol, and protein extracts were sonicated for 20 cycles of 20 s at 4 °C (Bioruptor Plus®, Diagenode®). Protein extracts samples were heated for 5 min at 95 °C, centrifuged at 15,000 *g* for 5 min and electrophoresed in a 10 % SDS-polyacrylamide gel. Protein gels were transferred to a PVDF membrane (Amersham®, GE Healthcare Life Sciences®) for 1 h at 80 V for western blot analysis. Blocking was carried out for 1 h at RT with 5% non-fat milk in PBS containing 0.05 % Tween 20 (PBST). Incubation with purified rabbit α-mCherry antibody (1:5000 dilution) was performed overnight (O.N.) at 4 °C. After washing in PBST, membranes were incubated with anti-rabbit IgG secondary antibody (GαR-PO, dilution 1:10000) for 1 h at RT. Detection was done by chemiluminescence (ECL Clarity®, Biorad®) and the Amersham® Imager 600 (GE Healthcare®).

2.7. RNA electrophoresis and membrane transfer (Northern blot)

RNA from *L. infantum* WT after axenic differentiation or infection of J774 macrophages line was obtained by TriReagent® (Sigma-Aldrich). RNA samples were separated on 1% agarose 6% formaldehyde gels in MOPS buffer (20 mM Morpholineopropanesulfonic Acid, 5 mM Sodium Acetate, 2 mM EDTA, pH7.0) at 20 V O.N. For the transference of RNA to

the positively charged nylon membrane (Roche®), the classical method of Southern in 1975 (Southern, 1975), was performed. As size marker, RNA from Thp1 human macrophages was included to the membrane. After transfer, RNA was fixed to the membrane by exposure to UV light (150 mJ - UV Stratalinker 1800/Stratagene®) for 2 min. After cross-linking, the membrane was incubated for 5 min in a 0.5 % acetic acid solution, and then stained with 0.04 % methylene blue for 5 min. The membrane was washed with distilled H_2O to remove the excess of dye and incubated in the prehybridization solution (DIG Easy Hyb Granules - Roche®) at 42 °C for 1 h under constant shaking. After incubation, the denatured DIG-labeled probe was added (Amastin - FW: 5'TAGGATG-GAGGTAGTAGGAG'3, RV: 5'CGGAAGAAGAGGGAGGAG3') and hybridization was carried out at 42 °C O.N. under constant shaking. The membrane was washed with stringent solutions: twice for 5 min with 2xSSC 0.1 % SDS (SSC 20x – 3 M NaCl, 0.3 M Trisodium citrate, pH 7.0) at RT under constant agitation; twice with 0.5XSSC 0.1 %SDS preheated to 68 °C for 15 min; once with 0.1X SSC 0.1 %SDS preheated to 68 °C for 15 min. After this step, the membrane was washed with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3 % Tween 20) for 5 min at RT. Afterwards, the membrane was incubated in blocking buffer (Blocking Reagent – Roche®) for 1 h with constant shaking at RT. After blocking, the buffer was removed and the membrane was incubated with *anti-Digoxigenin*-AP Roche® (at 1/10,000 dilution) for 1 h. After three cycles of washing, detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) was applied for 5 min. Around 20 drops of CSPD were added on the membrane and incubated for 5 min at RT and for 10 min at 37 °C. Detection was developed by chemiluminiscence and image capture was performed an Armersham 600® scanner.

2.8. Macrophage cell culture and in vitro infection

Dog DH82 macrophages and mouse J774 macrophages were purchased from the Rio de Janeiro Cell Bank (BCRJ), Rio de Janeiro, Brazil. The DH82macrophages were grown in DME medium, and J774macrophages were grown in RMPI medium, both supplemented with 10 % FBS and 50 µg/mL of gentamicin at 37 °C and humidified atmosphere containing 5 % CO_2.

For *in vitro* infection, 2.5 × 10^6 DH82 cells were plated in 6-wells

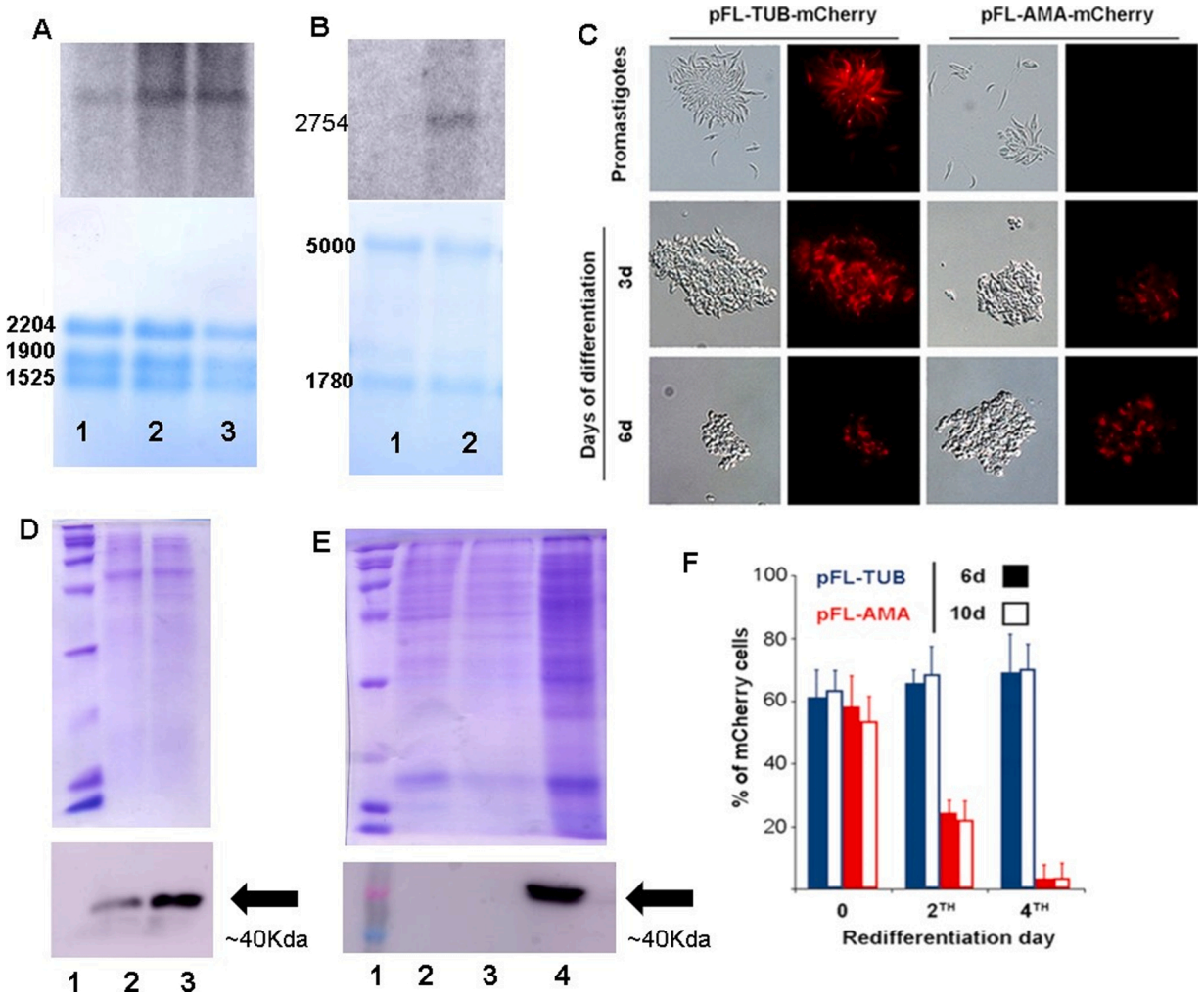

Fig. 2. Analysis of life-stage specific gene expression conferred by pFL-AMA plasmid during differentiation process. (A) Northern Blot (above) and methylene-stained transferred membrane (below) – The promastigotes *L. infantum* WT, time 0 h (Line 1), differentiation time 4 h (line 2) and 24 h (line 3). (B) Northern Blot (above) and methylene-stained membrane (below) - J774 macrophages (line 1) and *L. infantum WT* infected J774 macrophages after 48 h (line 2). (C) Representative fluorescence microscopy images from transfected parasites submitted to axenic differentiation during indicated times (400X). (D) SDS-PAGE electrophoresis in 10 % polyacrylamide gel (above) and Western Blot (below) - Molecular weight marker (line 1); Transfected *L. infantum* pFL-AMA-mCherry time 0 h (line 2), differentiation time 24 h (line 3). (E) SDS-PAGE electrophoresis in 10 % polyacrylamide gel (above) and Western Blot (below) - Molecular weight marker (line 1); J774 macrophages (line 2); *L. infantum* WT infected J774 macrophages after 48 h (line 3) *L. infantum* pFL-AMA-mCherry infected J774 macrophages after 48 h (line 4). (F) Percentage of positive mCherry cells during the re-differentiation process to promastigote, after 6 days (solid bars) or 10 days (open bars) over differentiation condition.

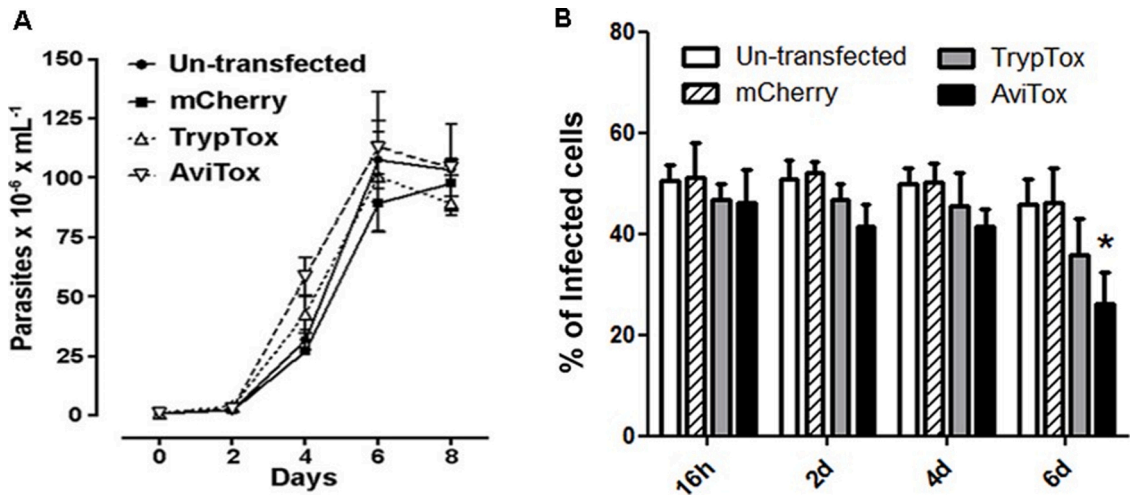

Fig. 3. Presence of pFL-AMA-Toxic plasmids are not affecting growth and parasite internalization process. (A) *L. infantum* parasites un-transfected or transfected with indicated plasmid were cultivated in promastigote-growth condition. (B) DH82 macrophages were infected, with indicated parasite lines, maintained for indicated times and fixed for Giemsa stain. Significance level was *$p < 0.05$.

plates and maintained for 16 h. After washing with PBS twice, parasites were added at ratio 1:5 cell/parasites in cell growth medium. Co-cultures were maintained 16 h at 37 °C and 5% CO_2 followed by two washes with PBS and trypsinization, and plated in 24-wells plates with coverglass to fixed Giemsa® (Sigma-Aldrich). Parasites were counted after two, four and six days of incubation. For Giemsa staining, samples were fixed with methanol (Synth® 99,8%) for 10 min and stained by Giemsa® (Sigma-Aldrich) in buffer (1/20 ratio of 0.07 M potassium phosphate monobasic KH_2PO_4 and 0.07 M bibasic sodium phosphate Na_2HPO_4 - pH 7.0) for 6 min, after drying for 24 h, preparations were observed by optical microscopy.

2.9. Parasitic burden analysis

Parasite burdens in spleen, liver and bone marrow of BALB/c mice were determined 10 weeks after infection or immunization. Spleen and liver were weighed and homogenized in 1 mL and 5 mL of parasite medium, respectively. Bone marrow was washed with 1 mL of parasite medium, both were diluted in 1: 4 series in 96-wells plates, which were maintained at 26 °C for 10 days. The parasitic burden was determined by the number of parasites per mL using inverted microscope, according to Buffet et al. (1995).

2.10. Protective capacity study against reinfection with L. infantum

Immunized BALB/c mice with transfected *L. infantum* with pFL-AMA-mCherry, -AviTox or-TrypTox and infected mice with *L. infantum* WT were challenged after 10 weeks with 1×10^6 *L. infantum* WT promastigotes by i.p. route. In parallel, control animals (without immunization) were also challenged. After 10 weeks of the challenge, animals were euthanized by cervical dislocation for parasitic burden analysis in spleen, liver and bone marrow.

2.11. Protective capacity study against infection with L. amazon

Immunized BALB/c mice with transfected *L. infantum* with pFL-AMA-mCherry, -AviTox or-TrypTox and infected mice with *L. infantum* WT were challenged after 10 weeks with 1×10^6 *L. amazonensis* promastigotes by s.c. in the plantar region of the left footpad (Araújo et al., 2012; Araujo and Giorgio, 2015). Control animals (without immunization) were also challenged at the same time. After 40, 60 and 80 days of challenge, the skin lesions were evaluated and, measured with a pachymeter. After 90 days of the challenge, the animals were euthanized by cervical dislocation for parasitic burden analysis. The footpad was homogenized in PBS to release the amastigotes for parasite counting (diluted 1/100) in a Neubauer chamber by conventional optical microscopy.

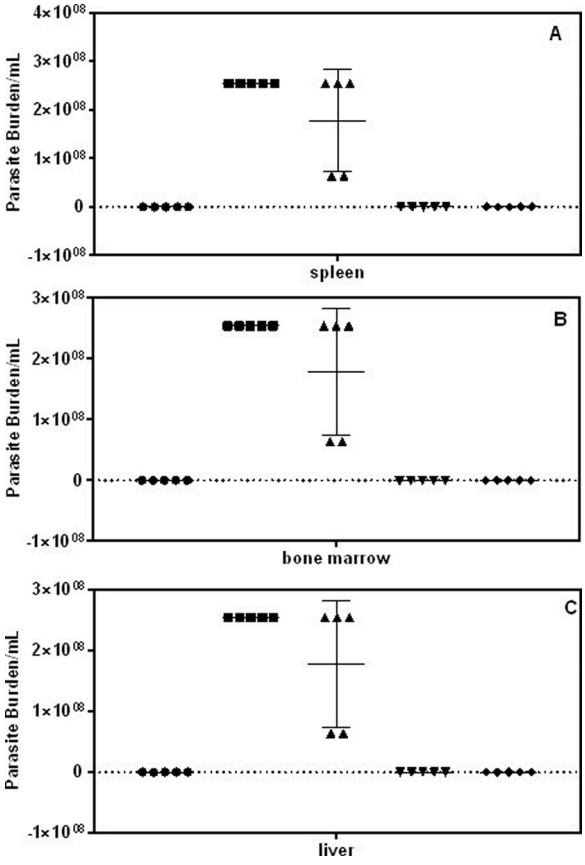

Fig. 4. Decreased parasitic burden in visceral organs of mice infected with *L. infantum* transfected with toxic plasmids. BALB/c mice were inoculated intraperitoneally with 2×10^6 promastigotes carrying or not plasmids. After 10 weeks, the spleen (A), liver (B) and bone marrow (C) the parasitic burden was calculated by limiting dilution.

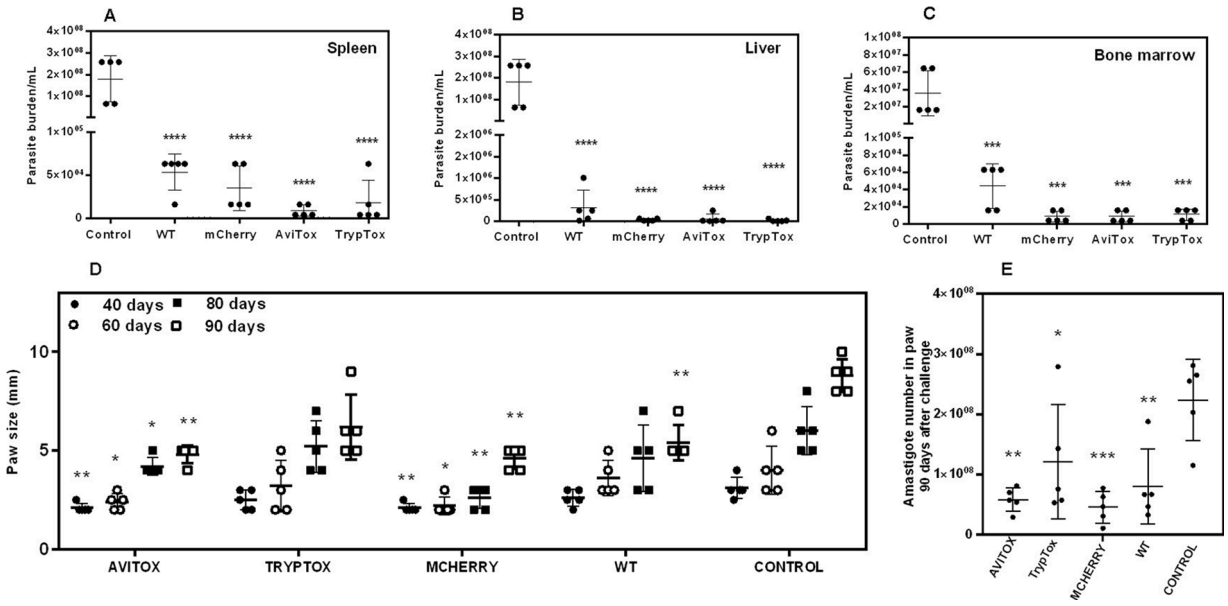

Fig. 5. Challenge with *L. infantum* WT (A, B and C) and *L. amazonensis* WT (D and E) in mice immunized with parasites transfected with the toxic gene. BALB/c mice were inoculated or not (control) *via* intraperitoneally with 2×10^6 promastigotes carrying or not plasmids. After 10 weeks animals were challenged with 1×10^6 promastigotes *L. infantum* WT (intraperitoneally.) or 1×10^6 promastigotes *L. amazonensis* WT (subcutaneously in paw). After 10 weeks they had been challenged, the parasitic burden was analyzed in spleen (A), liver (B) and bone marrow (C). At 40, 60, 80 and 90 days the paw was measured in mice challenged with *L. amazonensis* (D), and parasitic burden quantified in paw by amastigote count after 90 days after challenge (E). Significance level was *$p < 0.05$; **$p < 0,01$; ***$p <$ 0,001 and ****$p < 0,0001$ with the control group.

2.12. Analysis of splenic lymphocyte populations

Splenic cells were obtained from mice 10 weeks after immunization with transfected *L. infantum* with pFL-AMA-mCherry, -AviTox or -TrypTox and infected with WT parasites. The organs were processed in a sterile environment and splenocytes disaggregated in RPMI medium. The mononucleated spleen cells were separated by Ficoll-PaqueTM® according to the manufacturer's instructions (GE Healthcare®). The splenocytes were subjected to cell surface labeling, using the anti-mouse monoclonal antibodies: anti-CD3PerCP, anti-CD4 PEC, anti-CD8a APC, anti-CD19 PE/Cy7 and anti-CD69 PE (all antibodies were purchased to Biolegend®). After incubation with specific antibodies for 30 min, the cells were fixed with 1% paraformaldehyde for 10 min, washed with PBS and submitted to flow cytometry (FACS-calibur cytometer - BD Biosciences®). CompBeads® anti-Mouse and anti-Hamster (BD Biosciences®) were used for compensation. Data were collected by the BD FACSDIVA® (BD Biosciences®) software and analyzed by the FlowjoX® software. In order to analyze the cellular activation, the Mean Fluorescence Intensity (MFI) of the CD69 expression in the T helper lymphocyte ($TCD4^+$), cytotoxic T lymphocyte ($TCD8^+$) and B lymphocyte ($CD3^-CD19^+$) were calculated. In supplement 2, we observed the gating strategy that was used from the cell populations of a control mouse (Zorgi et al., 2016).

2.13. Humoral immune response analysis

The IgG antibodies in immunized BALB/c mice with transfected *L. infantum* with pFL-AMA-mCherry, -AviTox or -TrypTox and infected with WT *L. infantum* were detected by ELISA. 96-well NEST® polystyrene plates were coated with 100 µL/well of SLA (Soluble Leishmania Antigen) antigen (Carrillo et al., 2015) in 0.1 M sodium carbonate buffer (Na_2CO_3 and $NaHCO_3$) pH 9.5 O.N. at 4 °C. The plates were then washed twice with PBS 0.05 % Tween-20 (PBST) and blocked with 200 µL/well PBST solution containing 1% BSA (Serum Bovine Albumin; Sigma-Aldrich®) for 1 h at 37 °C. After washing with PBST, diluted blood plasma (1/200 in PBST) was added 100 µl/well and incubated for 1 h at 37 °C. The plates were washed six times with PBST and 100 µl/well of 1/20, 000-diluted peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich®) was added for 1 h at 37 °C. After this time the plates were washed (six times with PBST) and the development of the reaction was performed by the addition of 100 µL/well of TMB (3,3', 5,5' - Tetramethylbenzidine Dihydrochloride - Sigma-Aldrich®), with 2 µL of H_2O_2 30 % in 0.05 M phosphate-citrate buffer, pH 5.0 for 30 min and stopped by the addition of 50 µL of 2 M H_2SO_4. The absorbance was measured at 450 nm (Synergy HT- Bio-TEK®).

For detection of IgG high avidity antibodies, 100 µl/well of chaotropic solution, Urea 6 M (Diezma-Díaz et al., 2017) was added for 15 min at 37 °C after incubation of the diluted blood plasma, and so the same protocol described above was followed.

2.14. Nitrite and cytokine production in co-culture of the macrophages and splenocytes

Peritoneal macrophages from mice 10 weeks after immunization or infection were obtained by washing the peritoneum with cold PBS, and the cells were isolated in a sterile environment. Cells were cultured in RPMI medium supplemented with 10 % SFB, after 3 h of adherence, 1×10^6 splenic cells were added (obtaining the cells was described above) with nonspecific stimuli (*Escherichia coli* lipopolysaccharide, LPS), specific (SLA) or *L. infantum* WT promastigotes 1/10 ratio, cell/parasite (PRO). After 48 h of stimulation, supernatants were collected and nitrite concentration quantified by the Griess® method (Sigma-Aldrich) (Linares et al., 2008). The profile of inflammatory cytokines was determined by Kit Cytometric Bead Array® (BD Biosciences®), following the manufacturer's standards.

2.15. Statistical analysis

Comparisons between multiple groups were done using one-way ANOVA with a Dunnett's (parasitic burden after challenge *L. amazonensis*), Tukey's (parasitic burden after challenge with *L. infantum* and activation cells) or Bonferroni's (cytokines production) multiple comparison test, and using two-way ANOVA with Tukey's multiple comparison test (antibodies production and nitrite production)

for comparisons between multiple groups verifying the homogeneity of variances. The paw size after challenge with *L. amazonensis* was analyzed by non-parametric test using Mann Whitney test, as these data do not follow a normal distribution. Comparisons whose probability of equality is less than 5 % ($p < 0.05$) were considered significant. Statistic analysis was performed using GraphPad Prism 7.0 software.

3. Results

3.1. pFL-AMA plasmid confers amastigote stage specific expression in L. infantum

Plasmid pFL-AMA was constructed using the 3'-UTR regulatory region of the *L. infantum* amastin gene (LINF_340024300 and LINF_340024200). As expected, the amastin transcripts increased during differentiation from promastigote to amastigote in *L. infantum* WT. The increment in amastin gene expression was also detected in J774 macrophages after infection by *L. infantum* WT (Fig. 2A and B). mCherry expression was demonstrated by immunofluorescence both promastigotes and amastigotes transfected with pFL-TUB-mCherry (controls), and only in amastigotes transfected with pFL-AMA-mCherry after 3 and 6 days of differentiation (Fig. 2C).Similarly, the expression of mCherry, as determined by western blot (Fig. 2D and E), increased in pFL-AMA-mCherry-transfected parasites after 24 h of differentiation (Fig. 2D). Additionally, infection on J774 macrophages with pFL-AMA-mCherry-transfected promastigotes led to a detectable expression of mCherry after 48 h of infection (Fig. 2E), indicating that the plasmid directed the expression of the protein *in vivo*. Finally, we analyzed whether, after reversing the axenic differentiation from promastigote to amastigote, there is a shutdown in the expression of plasmid pFL-AMA.For this purpose, transfected promastigotes with either pFL-TUB-mCherry or pFL-AMA-mCherry were submitted to conditions of axenic amastigote differentiation for 6 or 10 days and then, the amastigote-like forms were differentiated in promastigote growth medium at 26 °C. The expression of the fluorescence reporter gene was evaluated every 2 days by flow cytometry (Fig. 2F). A significant decrease in the number of mCherry expression cells was observed in transfected parasites with pFL-AMA-mCherry plasmids, whereas the expression of the fluorescent protein remained at similar levels in the transfected parasites with pFL-TUB-mCherry. In summary, the data supported that plasmid pFL-AMA was able to direct a stage-specific expression of a gene linked to the 3' regulatory regions of an amastin gene.

3.2. pF L-AMA plasmid-based constructs expressing potential toxic proteins

Once demonstrated the stage-specific gene expression of pFL-AMA plasmid, genes coding for putative toxic proteins were included in the plasmid, replacing mCherry ORF. For this purpose, the activate form of *bovine trypsin* (GenBank: CAA38513.1) and *egg avidin* (GenBank: CAC34569.1) were selected.

Promastigotes transfected with pFL-AMA-toxic genes showed a similar growth rate than un-transfected parasites or transfected with pFL-AMA-mCherry plasmid (Fig. 3A). Aiming to address *in vivo* studies, firstly we showed that the presence of toxic plasmids in parasites did not affect macrophage internalization or amastigote differentiation in the host cell. In order to achieve this, DH82 cell line macrophages were infected *in vitro* with promastigotes of *L. infantum* transfected with pFL-AMA-toxic genes or controls, and the percentage of infected cells were recounted over six days, using Giemsa staining (Fig. 3B). No significant differences were observed between the different parasites lines used during the first few days of the experiment. However, on day six, a significant decrease in the infection index was observed in transfected parasites with pFL- pFL-AMA-AviTox. Similar data were found in J774 macrophage line (data not shown).

After immunization with parasites transfected with toxic and non-toxic genes, we observed evident signs of hepatomegaly and splenomegaly in mice infected with parasites WT or transfected pFL-AMA-mCherry. In contrast, no apparent visceral leishmaniosis signs were observed in mice infected with parasites carrying pFL-AMA-Toxic plasmids (data not shown). In fact, mice inoculated with parasites transfected with the toxic plasmids, did not present parasites in the spleen. (Fig. 4A), liver (Fig. 4B) or bone marrow (Fig. 4C), whereas parasite burden was observed in mice infected with *L. infantum* WT or parasites transfected with pFL-AMA-mCherry after 10 weeks of inoculation. We conclude that amastigotes carrying pFL-AMA-Toxic plasmids did not survive into the mammalian host.

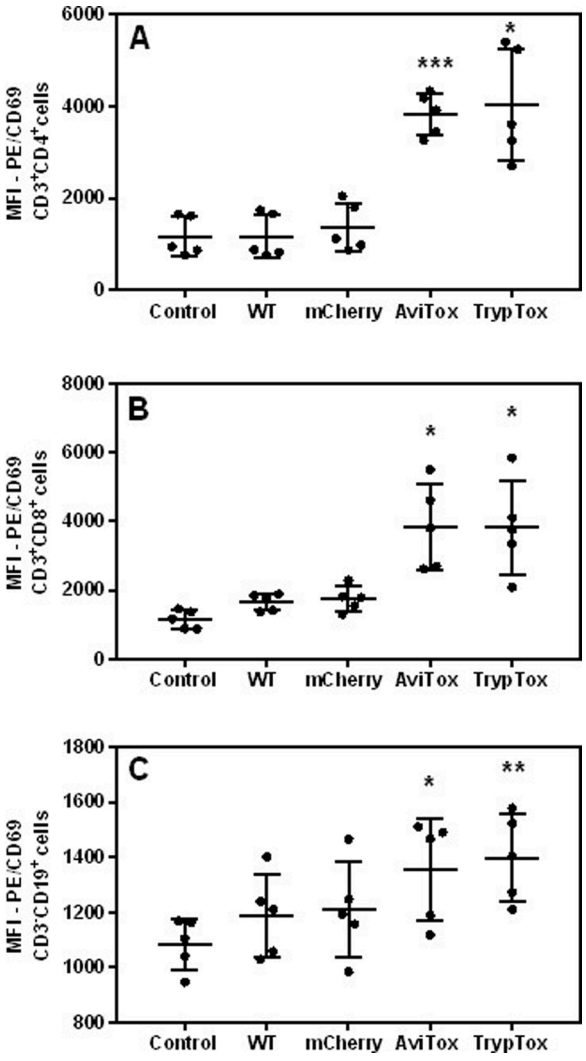

Fig. 6. Activation of TCD4 (A), TCD8 (B) and B (C) splenic cells after 10 weeks of immunization with the toxic genes transfected parasites. BALB/c mice were immunized with 2×10^6 promastigotes carrying or not plasmids by intraperitoneal route or not inoculated (control), and after 10 weeks the spleens were analyzed by flow cytometry. Significance level was *$p < 0.05$; **$p < 0,01$ and ***$p < 0,001$ with control group.

3.3. Analysis of the capacity to eliciting immune protection of the parasites transfected with pFL-AMA-AviTox or –TrypTox plasmids against reinfection with L. infantum or L. amazonensis virulent parasites

Immunized mice with transfected and un-transfected parasites showed significant immunological protection after challenge with virulent *L. infantum* or *L. amazonensis* (Fig. 5). Immunized mice with transfected *L. infantum* pFL-AMA-AviTox or -TrypTox had a low parasitic

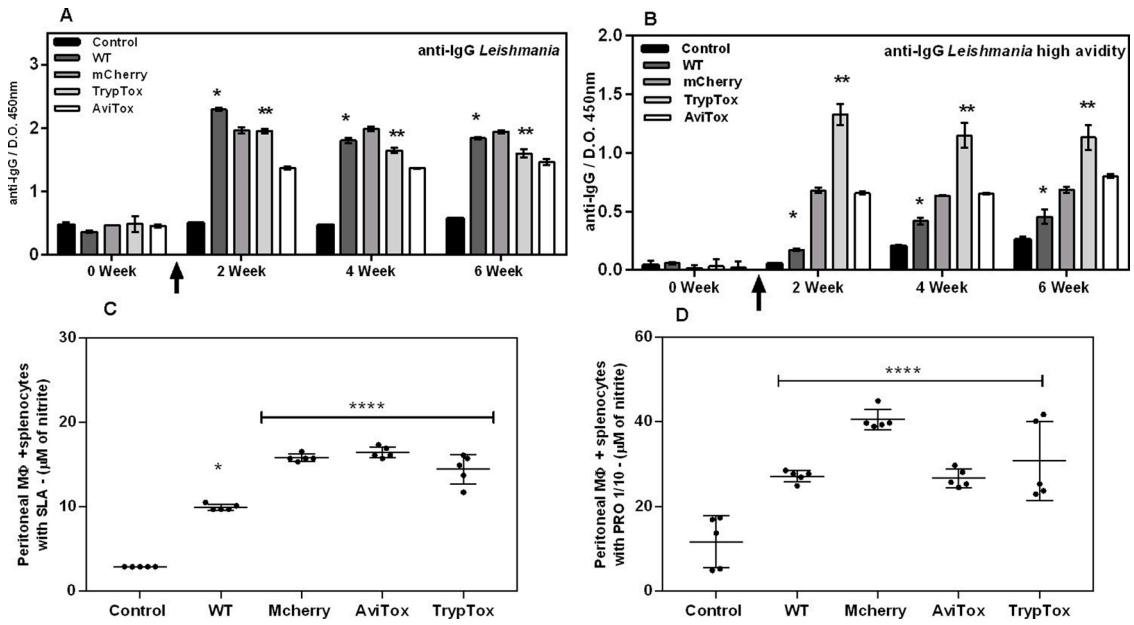

Fig. 7. Humoral immune response and nitric oxide production in mice after immunization with the transfected parasites with the toxic genes. BALB/c mice were immunized with 2×10^6 promastigotes carrying or not plasmids by intraperitoneal route or not inoculated (control). (A) Total *anti-Leishmania* IgG antibodies detected by ELISA. (B) High avidity *anti-Leishmania* IgG antibodies detected by ELISA. Significance level was *p < 0.05 between 2, 4 and 6 weeks of WT group and **p < 0.05 between 2, 4 and 6 weeks of TrypTox group. (C) Nitrite production in the co-cultures of peritoneal macrophages and splenocytes stimulated with SLA (Soluble *Leishmania* Antigens) and (D) 10:1 ratio of promastigotes of *L. infantum* (PRO), from mice after 10 weeks of the immunization. Significance level was *p < 0.05 and ****p < 0,0001 with control group.

burden on the spleen (Fig. 5A), liver (Fig. 5B) and bone marrow (Fig. 5C) when compared to control group animals. When animals were challenged with *L. amazonensis*, significantly decreased in the footpad lesion at 40, 60, 80 and 90 days was observed in the mice previously immunized with transfected parasites with toxic genes (AviTox) and non-toxic (mCherry), when compared with non-immunized. The mice infected with WT parasites showed significantly decreased in the footpad after 90 days of challenge with *L. amazonensis* (Fig. 5D). The low parasitic burden in the footpad in the immunized mice with transfected and un-transfected parasites were observed after 90 days of the challenge as compared to animals without immunization (Fig. 5E).

3.4. Induced immune response in mice after immunization with transfected L. infantum promastigotes with pFL-AMA-AviTox or –TrypTox

After 10 weeks immunization with parasites transfected with toxics genes, the mice showed an increased in the cell activation in $TCD4^+$ (Fig. 6A), $TCD8^+$ (Fig. 6B) and B cells, $CD3^-CD19^+$ (Fig. 6C), as evidenced by the high expression of CD69 of these cell populations in immunized mice when compared to control, non-immunized mice.

To elucidate humoral immune response, we analyzed the Total (Fig. 7A) and the high avidity (Fig. 7B) IgG production after immunization with transfected parasites with toxic genes. After 2 weeks high increased levels of Total and high avidity IgG *anti-Leishmania* were observed in mice and remains at the same levels until 6 weeks in immunized mice with transfected parasites with pFL-AMA-mCherry or -AviTox. But over time the high avidity and total IgG antibodies *anti-Leishmania* levels decrease significantly in immunized mice with transfected *L. infantum* with pFL-AMA-TrypTox and in infected mice with *L. infantum* WT (Fig. 7A and B).

Co-cultures of the splenic cells with peritoneal macrophages demonstrated an increase of nitrite production in cells from immunized mice with transfected parasites with toxic plasmids or un-transfected (WT) with both stimuli, SLA or infection *in vitro* (PRO) (Fig. 7C and D). Cells from control animals did not produce significative nitrite levels.

In relation of the cellular immune response, we also analyzed pro and anti-inflammatory cytokines production in co-culture of the splenic cells with peritoneal macrophages after 48 h, with SLA or PRO stimuli (Fig. 8). As observed in the Fig. 8A and B, the TNF-α (Tumor Necrosis Factor-alpha) levels are higher in the cell supernatants from mice immunized with transfected parasites with toxic genes (groups 1 and 2), non-toxic gene (group 3) and mice infected with WT parasites (group 4) compared to the control group (without infection, group 5) for both stimulus as SLA and PRO. But when we compared results from group 1 and 2 of mice with group 3, a significant increase or decrease of the production of TNF-α levels occurred with SLA or PRO stimuli, respectively and decrease of the production of TNF-α levels occurred with in PRO stimuli (Fig. 8A and B). Regarding IFN-γ, we observed that the groups immunized with transfected parasites with pFL-AMA-TrypTox (group 2) or mCherry (group 3) and infected with WT parasites (group 4) showed increased levels of this cytokine after stimuli with SLA or PRO (Fig. 8C and D) compared to the control group. In relation to group 3 (mCherry), groups 1 and 2 showed significant difference with an increase of production IFN-γ; in the group 2 (mice immunized with TrypTox-transfected parasites) and a decrease in the group 1 (AviTox) when stimuli SLA. Both groups immunized with toxic genes showed decrease levels IFN-γ (Fig. 8C and D) in stimulus PRO. MCP-1 (Monocyte Chemotactic Protein 1) production was increased in cells from immunized animals with transfected parasites with toxic or non-toxic genes in both stimuli (Fig.8E and F) when compared to control group. The immunized mice from groups 1 and 2 showed decrease of levels production of MCP-1 when compared with group 3 (Fig. 8C and D). The anti-inflammatory cytokine IL-10 production was increased in cells from infected and immunized mice, only with SLA stimuli (Fig. 8G and H) when compared with control group. In comparison to group 3, groups 1 and 2 showed increased levels of IL-10 production after stimuli SLA (Fig. 8G and H). The production of IL-6 by cells from groups 1, 2 and 3 showed an increase after stimuli by SLA or PRO (Fig. 8I and J) when compared to control (group 5). The group 2 (TrypTox-transfected parasites) showed an increase in the production of IL-6 after stimuli with SLA, compared to group 3 (non-toxic). All cytokines analyzed responded when cells were stimulated with LPS (data not shown), which were used

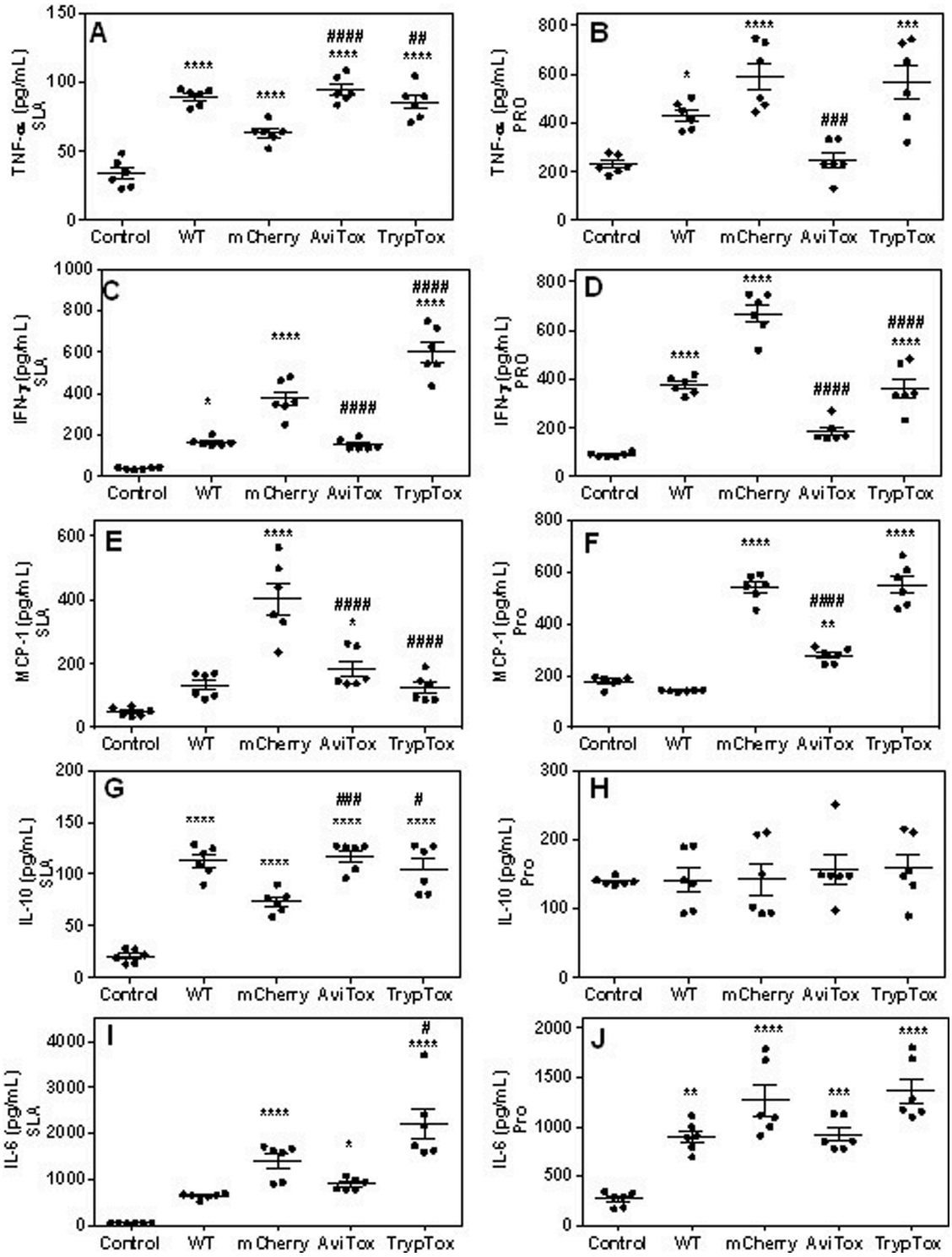

Fig. 8. Cellular immune response: Cytokine production in mice after immunization with the transfected parasites with the toxic genes. BALB/c mice were immunized with 2×10^6 promastigotes carrying or not plasmids by intraperitoneal route or not inoculated (control). After 10 weeks the cytokines production in the supernatant of co-culture of peritoneal macrophages and splenocytes stimulated with SLA (A, C, E, G and I) and PRO (B, D, F, H and J), were evaluated: TNF-α (A and B); IFN-γ (C and D); MCP-1 (E and F); IL-10 (G and H); IL-6 (I and J). Significance level was *p < 0.05, **p < 0,01, ***p < 0,001, ****p < 0,0001 with control group and # p < 0,05, ## p < 0,01, ### p < 0,001, #### p < 0,0001 with mCherry group.

as a positive control.

4. Discussion

To control gene expression in *Leishmania,* genetic tools frequently used in others models cannot be used, as unusual molecular mechanisms are involved. Meanwhile, in the last decades, significant progress has been made to adapt genetic systems from other organisms to this parasite, as bacterial tetracycline-operon (Yan et al., 2002; Kraeva et al., 2014), RNAi (Atayde et al., 2012), and different expression vectors were developed and studied (Nascimento and Leite, 2012). The expression vector, the pFL-AMA plasmid, represents a highly restricted genetic

system viable to direct gene expression in *Leishmania*, specifically to the amastigote stage of the parasite. The parasite's viability was affected by the replacement of the mCherry genetic sequence with sequences encoding toxic proteins (AviTox and TrypTox). Other studies with *L. mexicana* have shown that attenuated parasites for the BnSP-7 expression (basic phospholipase A2 from *Bothrops pauloensis* snake venom) showed reduced significance of the viability or rate of division after internalization in phagolysosomes, when compared to wild type strains. However, the expression of the BnSP-7 toxin caused high susceptibility to macrophages (Podešvová et al., 2020), requiring a better analysis for their use *in vivo*. According to our results, parasites transfected with toxic plasmids induce a possible death of the parasite when occurs the differentiation to amastigote form without affecting the viability of the host cell. In addition, mice inoculated with these transfected parasites showed no parasitic burden on visceral organs. However, some parasites can survive after losing the plasmid in the absence of the drug, and thus, viable parasites could explain the induced immune response and provided protection after challenges with virulent strains.

In both mice and humans, a primary *L. major* infection that cures represents the gold standard of acquired resistance. This resistance in cured mice is due to the rapid recruitment of TH1 $CD4^+$ cells to the skin site of the infected sandfly bite (Peters et al., 2009). However, immunity is most effectively achieved and maintained by the presence of persistent parasites, in order to maintain effective anti-parasitic T $CD4^+$ cells (Engwerda and Matlashewski, 2015). Therefore, host resistance and induced anti-leishmanial immunity effectivity is mediated by immune cell responses, both the innate and adaptive, and requires effective action of macrophages, dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T cells (Stanley and Engwerda, 2007). The immunization with transfected parasites with toxic genes enhances *anti-leishmanial* immunity, with reduced parasitic burden on visceral organs and visibly reduced paw lesions after challenge with virulent strains.

T $CD4^+$ lymphocytes are important for generating and maintaining both B cell and T $CD8^+$ cell responses (Igietseme et al., 2004). T $CD8^+$ cells are crucial to the host during infection because these cells may limit the spread of infectious agents, especially intracellular parasites such as *Toxoplasma gondii* (Grover et al., 2014) and *Leishmania sp* (Kaye et al., 2004). The B cells activation and the specific antibody production are essential elements of effector humoral immunity and host protection against a variety of pathogens (Pieper et al., 2013). However, for human leishmaniosis the humoral response is usually associated with disease progression, *anti-leishmania* antibodies that are produced at a low level in CL and at a very high level in VL play no role in protection (Mutiso et al., 2013). Immunized mice with parasites transfected with toxic genes showed high levels of *anti-Leishmania* IgG, but decreased with time of immunization. In addition, these mice also showed a humoral immune response maturation with high avidity (functional affinity) IgG antibody. The avidity of IgG antibodies is known to distinguish primary from secondary infections of many different microbial pathogens, such as toxoplasmosis, cytomegalovirus or rubella (Sirin et al., 2017). However, for leishmaniosis, avidity assays could be used to estimate the time and duration of infection, such as in *Leishmania donovani* infections (Redhu et al., 2006). The IgG avidity results could be used to monitor parasitological clearance in response to treatment or vaccination.

Immunity against leishmaniasis is very complex, however, it is well described that resistance to leishmaniasis is related to the development of T helper 1 (Th1) cells and pro-inflammatory cytokine production (eg IFN-γ, TNF-α, IL-12, IL-1) leading to macrophage activation and consequently parasite death (Schoenborn and Wilson, 2007). Macrophage activation is mainly mediated by nitric oxide production, a potent molecule involved in cytotoxic effects against microorganisms (Blanchette et al., 2009; Bogdan et al., 2000), playing an essential role in controlling *Leishmania* infection. Cytokines such as IFN-γ and TNF-α are important factors involved in leishmaniosis immunoprotection and immunopathology. IFN-γ is mainly secreted by Th1 $CD4^+$, $CD8^+$T lymphocytes and natural killer cells, and it stimulates the production of nitric oxide in activated macrophages inhibiting intracellular parasite growth. TNF-α is primarily produced by macrophages that plays a crucial role in *Leishmania* elimination through increased macrophage activity and nitric oxide synthesis (Schoenborn and Wilson, 2007). The IL-6 is involved in the acute phase response, B cell maturation and macrophage differentiation, and can act in the development of a Th1 and Th2 response (Diehl and Rincón, 2002; Titus et al., 2001). The MCP-1 has a potential role in *Leishmania* infection, which includes host defense functions, such as leukocyte recruitment, participation in cell-mediated immunity, cell activation and *anti-leishmanial* activity (Teixeira et al., 2006). The IL-10 is also produced after immunization, but at lower levels; it is a cytokine produced by active immune cells, mainly macrophages and subsets of T cells, the T regulatory (Treg). The IL-10 decreases the production of inflammatory mediators that can protect tissues from collateral damage caused by excessive inflammation (Sabat et al., 2010).

In our immunization data with transfected parasites with toxic genes, they showed a TH1 profile, with production of pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-6, however, they also showed levels of IL-10, an anti-inflammatory cytokine that can probably be related to the regulation of immune response avoiding tissue damage, especially in visceral organs, such as spleen and liver, where they are the focus of disease development in immunized and challenged animals. Our data clearly suggest that using live parasites transfected with toxic genes as immunogen is not only a genetic method that leads to parasite death specifically at the intracellular stage of *Leishmania*, but also induces a cell-mediated immune response that confers protection levels to both with *L. infantum* as *L. amazonensis*. Thus, upcoming system changes to prevent plasmid loss may be studied. For now, and considering the easy handling of the episomal plasmid in *Leishmania*, pFL-AMA represents a powerful tool to be used as an immunogen for leishmaniasis.

CRediT authorship contribution statement

Nahiara Esteves Zorgi: Investigation, Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing, Formal analysis. **Leonardo V. Arruda:** Investigation, Methodology. **Izadora Paladine:** Investigation, Methodology. **Guilherme A.S. Roque:** Investigation, Methodology. **Thalita F. Araújo:** Investigation, Methodology. **Marcelo Brocchi:** Conceptualization, Resources. **Manoel Barral:** Investigation, Conceptualization. **África Sanchiz:** Investigation, Methodology, Writing - review & editing. **José María Requena:** Investigation, Resources, Supervision, Writing - review & editing. **Daniel R. Abánades:** Investigation, Conceptualization, Methodology, Supervision. **Selma Giorgio:** Investigation, Resources, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2020.08.006.

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