Functional evaluation of gene silencing on macrophages derived from U937 cells using interference RNA (shRNA) in a model of macrophages infected with *Leishmania* (*Viannia*) *braziliensis*

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SUMMARY

Leishmaniasis development is multifactorial; nonetheless, the establishment of the infection, which occurs by the survival and replication of the parasite inside its main host cell, the macrophage, is mandatory. Thus, the importance of studying the molecular mechanisms involved in the Leishmania–macrophage interaction is highlighted. The aim of this study was to characterize a cellular model of macrophages derived from U937 cells that would allow for the identification of infection phenotypes induced by genetic silencing with interference RNA in the context of macrophages infected with *Leishmania* (*Viannia*) *braziliensis*. The model was standardized by silencing an exogenous gene (*gfp*), an endogenous gene (*lmna*) and a differentially expressed gene between infected and non-infected macrophages (*gro-β*). The silencing process was successful for the three genes studied, obtaining reductions of 88·9% in the GFP levels, 87·5% in LMNA levels and 74·4% for Gro-β with respect to the corresponding control cell lines. The cell model revealed changes in the infection phenotype of the macrophages in terms of number of amastigotes per infected macrophage, number of amastigotes per sampled macrophage and percentage of infected macrophages as a result of gene silencing. Thus, this cell model constitutes a research platform for the study of parasite–host interactions and for the identification of potentially therapeutic targets.

Key words: *Leishmania*, monocyte-derived macrophages, gene silencing, RNA, small interfering, U937 cells.

INTRODUCTION

Leishmaniases are a group of diseases that affect people in 98 countries and are considered as neglected diseases by the World Health Organization (WHO) (Alvar *et al*. 2012). Depending on the infecting species and the host's immune response, different clinical forms with a wide range of signs and symptoms can be presented. For decades, the understanding of the disease's pathogenesis has been a challenge for researchers and clinicians interested in its cure and prevention. A large part of the knowledge available on the pathogenesis of and immune response to Leishmaniasis has been achieved based on studies with animal models, including dogs, hamsters, non-human primates, wild rodents and mouse lines (BALB/c and C57BL/6 mice are sensitive and resistant to the infection, respectively) (Loria-Cervera and Andrade-Narvaez, 2014). *In vitro* models such as macrophages derived from primary monocytes and RAW 264·7, J774, DH82, THP-1 and U937 cell lines have been used with lower frequencies (Maia *et al*. 2007; Hsiao *et al*. 2011).

In host–parasite interaction studies, functional genomics plays an important role, seeking to identify the biological functions of genes, and different strategies are used for this purpose. *Knock-out* mice have been the most widely used models for the study of Leishmaniasis, especially in the study of the immune response, with important results for the understanding of the disease (Rosas *et al*. 2003). However, this strategy is expensive and does not allow for the rapid evaluation of a phenotype, and it is not always possible to obtain *knock-out* mice for vital genes (Harborth *et al*. 2001). An alternative is *knock-down* by interference RNA in cell lines infected with the parasite.

The *in vitro* silencing of macrophage genes by interference RNA can produce different results depending on the silencing strategy used and the type of interference RNA (Rao *et al*. 2009). In each cellular model, it is important to evaluate the functionality of the silencing system and its effect on the phenotype of interest. These characteristics have not been evaluated in macrophages derived from U937 cells infected with *Leishmania* (*Viannia*) *braziliensis*. This study evaluated whether the cell model

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of macrophages derived from U937 cells with genetic silencing using interference RNA, would allow for the identification of infection phenotypes induced in macrophages infected with *L.* (*V.*) *braziliensis*. The results obtained in this work indicate that in this model, it is possible to detect changes in the susceptibility of the macrophages to the parasite infection as a consequence of genetic silencing.

MATERIALS AND METHODS

Cell cultures

The U937 cell line (American Type Culture Collection-CRL-1593·2, USA) was cultured in RPMI-1640 medium (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS); HEK-293FT cells (Invitrogen, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®,CA, USA) according to the manufacturer's instructions. The two cell lines were incubated at 37 °C in a 5% CO_2 atmosphere. The promastigotes of *L.* (*V.*) *braziliensis* (MHOM/BR/00/M2903, Centre National De Reference Des Leishmania, Montpellier, France) were cultured at an initial concentration of 1×10^6 parasites per mL in Schneider's medium (Sigma-Aldrich), supplemented with 10% FBS at 26 °C for 6 days to reach the stationary phase.

Selection of genes to be silenced

With the aim of evaluating the cellular model regarding the silencing process and its capacity for detecting different infection phenotypes, three genes were selected for silencing: *gfp*, as a reporter gene, the constitutively active gene *lmna* as an endogenous positive control and the *gro-β* gene, which, based on microarray (log2-fold expression change = 1·92) (GEO accession number GSE61211) and RT–qPCR assays (log2-fold expression change = 2·69), showed differential expression between the control macrophages *vs* macrophages infected with *L.* (*V.*) *braziliensis* (Ovalle-Bracho *et al*. 2015).

Design and generation of lentiviral constructs

Four DNA sequences coding for shRNAs targeting the human *gro-β* gene (GenBank accession NM_002089·3) and four negative control nonsense sequences with no counterparts in the human genome were designed. The DNA fragments, which were synthesized and pre-annealed, were ligated into the entry vector pENTR/H1/TO using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen) in accordance with the manufacturer's instructions. OneShot® TOP10 *Escherichia coli* (Invitrogen) bacteria were transformed with the ligation product, and the constructs were confirmed by digestion with the BamHI–HF restriction enzyme (New England BioLabs, UK) and sequencing (data not shown). Then, a Gateway recombination reaction was performed between the entry vector and the target vector pLenti4/BLOCK-iT-DEST (Invitrogen) to generate the corresponding lentiviral constructs. The recombination product was used to transform OneShot® Stbl3™ bacteria (Invitrogen). The constructs were confirmed by sequencing. The constructs pLenti-6·3-V5-GW-EmGFP and pLenti4-GW/H1/TO-lamin^{shRNA} were acquired from Invitrogen and utilized for the expression of the *gfp* gene and the short hairpin RNA (shRNA) targeted against the *lmna* gene, respectively. The shRNA sequences are shown in Table 1.

Lentivirus production

The constructs pMD2.G (VSV-G) and pCMV-delta-8·7 were used. The constructs were distributed in equimolar fractions for a total of 30 μg of DNA prior to the co-transfection of $1{\cdot}8 \times 10^7$ HEK-293FT cells in a 75 cm^2 culture recipient using 60 μl of Lipofectamine 2000 (Invitrogen). The cells were incubated overnight under the culture conditions described.

The first lentivirus harvest was performed after 16 h, and 10 mM sodium butyrate (Sigma) in DMEM culture medium supplemented with 10% FBS was added to the culture. The remaining lentivirus harvests were performed after 24, 48, 72 and 96 h. After each harvest, DMEM medium supplemented with 10% FBS was added to the cells in culture. The lentiviral suspensions were filtered through 0·45 μm membranes and concentrated using a Millipore Centricon Plus-20® concentration column (Millipore, Germany) according to the manufacturer's instructions.

The titration of the lentiviral vectors bearing the construct for the expression of *gfp* was determined by transduction of HEK-293FT cells with serial dilutions of the lentiviral stock, and a titre of $3{\cdot}06 \times 10^7$ Transduction Units/ml (TU ml^{-1}) was obtained. The titration of the remaining lentiviral vectors was determined via an enzyme-linked immunosorbent assay to measure the p24 protein using the lenti-Xp24 Rapid Titer Kit (Clontech-Takara, Japan). The titres for the lentiviruses bearing the other constructs were as follows: $1{\cdot}9 \times 10^{10}$ lentiviral particles mL^{-1} (LP mL^{-1}) for the *lmna* shRNA construct, $2{\cdot}12 \times 10^{10}$ LP mL^{-1} for the *gro-β* construct and $2{\cdot}01 \times 10^{10}$ LPmL^{-1} for the negative control.

Lentivirus-mediated transduction for gfp expression

A total of 100,000 U937 cells were cultured in 1 mL of RPMI. The lentiviruses were added at a

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Table 1. shRNA sequences. shRNA sequences used to generate the cell lines used as negative silencing controls and to silence the *gfp*, *lmna* and *gro-β* genes. Each shRNA sequence is composed by four parts: link sequence (four nucleotides: CACC), sense sequence (19–21 nucleotides), loop (four nucleotides: CGAA) and antisense sequence (19–21 nucleotides).

Name	Number	Sequence
shRNAs–NR	1	5′-CACCGTCTGTCACCTAGTTCGTACGCGAACGTACGAACTAGGTGACAGAC-3′
	2	5′-CACCGGCGCACCCGACGACGTAGGTCGAAACCTACGTCGTCGGGTGCGCC-3′
	3	5′-CACCGGTCCGACGCATACTAATACGAATATTAGTATGCGTCGGACC-3′
	4	5′-CACCGCCTATAATTACGGGTCTCCTCGAAAGGAGACCCGTAATTATAGGC-3′
shRNA–*lmna*	1	5′-GCTGGACTTCCAGAAGAACACGAATGTTCTTCTGGAAGTCCAGTT-3′
	1	5′-GATCCCAACACTTGTCACTACTTTTTCAAGAGAAAAGTAGTGACAAGTGTTGTTTTTT-3′
shRNAs–*gfp*	2	5′-GATCCCATGCCCGAAGGTTATGTATTCAAGAGATACATAACCTTCGGGCATGTTTTTT-3′
	3	5′-GATCCGCATGGATGAACTATACAATTCAAGAGATTGTATAGTTCATCCATGCTTTTTT-3′
	1	5′-CACCGCATCGCCCATGGTTAAGACGAATCTTAACCATGGGCGATGC-3′
	2	5′-CACCGCATCCAAAGTGTGAAGGTGAAGTCGAAACTTCACCTTCACACTTTGGATG-3′
shRNAs–*gro-β*	3	5′-CACCAAGATGCTGAAAAATGGCAAATCCGAAGATTTGCCATTTTTTCAGCATC-3′
	4	5′-CACCGAAGGAAGGAGGAAGCTTACGAATAAGCTTCCTCCTTCCTTC-3′
	5	5′-CACCGCGCCCATGGTTAAGAAAATCGAAATTTTTCTTAACCATGGGCG-3′

multiplicity of infection (MOI) of 306 infective units (IFU)/cell in combination with Polybrene (9 μg mL^{-1}) and were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Forty-eight hours after transduction, the U937 cells were quantified, and the percentage of GFP expressing cells was determined via fluorescence microscopy.

Lentivirus-mediated transduction for the silencing of gfp, lmna and gro-β

For the silencing of the *gfp* gene, 10,000 U937 cells expressing *gfp* (U937-GFP) were inoculated in a 96-well plate in 80 μL of RPMI medium supplemented with 10% FBS. Lentiviruses (Santa Cruz Biotechnology, TX, USA) were added at an MOI of 10 IFU/cell in conjunction with Polybrene (9 μg mL^{-1}) and were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Forty-eight hours after transduction, the U937 cells were quantified, and the percentage of GFP expressing cells was determined through fluorescence microscopy. The transduction efficiency, defined as the percentage of cells not expressing GFP, was determined. The cells obtained were amplified and selected with 5 μgmL^{-1} puromycin for 8 days (Santa Cruz Biotechnology) yielding the U937-GFP/shRNA-GFP cell line.

For the silencing of the *lmna* and *gro-β* genes, 100,000 U937 cells were inoculated in 1 mL of RPMI medium, and lentiviruses were added at MOIs of 380 and 0·2 IFU cell^{-1}, respectively. The transduced cells were selected with 200 μg mL^{-1} Zeocin (Invitrogen), yielding the U937/shRNA-LMNA and U937/shRNA-Gro-β cell lines.

Generation of silencing negative controls

The negative controls were generated transducing U937 cells with a pool of four lentiviral vectors bearing constructs expressing shRNAs directed against sequences which had no close identity with other transcripts of the human genome. Three cell lines were obtained: 'U937/shRNA-NR' (NR, non-relevant), transduced at MOIs of 10, 400 and 0·2, as control lines for the silencing of *gfp*, *lmna* and *gro-β*, respectively.

Silencing evaluation – Immunodetection of the GFP, LMNA and Gro-β proteins

Total protein extracts were obtained by lysing macrophages derived from the different cell lines using a radio-immuno-precipitation buffer (Mookerjee Basu *et al*. 2008) containing 0·125 M NaCl in 0·025M Tris–HCl (pH 8·0), 1% Triton X-100, 0·5% sodium deoxycholate, 0·1% SDS and 0·004% sodium azide in the presence of protease inhibitors (Roche, Switzerland). The extracts obtained were resolved by SDS–PAGE for 1·5 h at 100 V, transferred to a

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polyvinylidene fluoride (PVDF) membrane for 1 h and 15 min at 200 mA, and then blocked with a solution of 20 mM Tris–HCl, 150 mM NaCl (pH 7·5) and 5% skim milk. After the blocking step, immunodetection was performed using antibodies against the proteins GFP and LMNA (Santa Cruz Biotechnology) and Gro-β (Thermo Scientific, MA, USA). The immunodetection signal was revealed using the *immune-Star HRP substrate* kit (BIORAD, CA, USA). As a loading control, an SDS–PAGE gel was run in parallel with the same amount and type of sample and was stained with a 0·006% Coomassie Brilliant Blue solution. The PVDF membranes and loading control gels were visualized using ChemiDoc BIORAD equipment. Densitometry data from the images acquired were obtained using ImageJ software. The data were normalized with the corresponding loading control. The relative density of each sample was calculated by dividing each normalized value by the normalized value of the reference line (non-silenced line: U937 or U937/GFP). Western Blot assays were performed at least three independent times.

Evaluation of the infection phenotype

The infection phenotype was evaluated in terms of percentage of infected macrophages, number of amastigotes per infected macrophage and number of amastigotes per sampled macrophage. The macrophages derived from each of the cell lines were infected with *L.* (*V.*) *braziliensis* promastigotes previously opsonized with inactivated AB^+ serum at a proportion of 15:1. The macrophages were incubated for 2 h in the presence of the parasites, then washed with 1X PBS (pH 7·4) and incubated at 34 °C in a 5% CO_2 atmosphere for 72 h. After incubation the RPMI medium was removed, and the cells were washed with PBS pre-warmed at 37 °C. The infected macrophages were fixed with 2% paraformaldehyde for 25 min at 37 °C. The paraformaldehyde traces were inactivated with 50 mM ammonium chloride for 5 min at room temperature, and the cells were then permeabilized with 0·2% saponin for 5 min at room temperature. The cells were incubated for 1 h in a moist chamber at 37 °C with 10 mM *Vybrant® DiD cell-labelling solution* lipid stain (Invitrogen) to label the cell membranes and then with 1·25 μg mL^{-1} Hoechst DNA stain (Invitrogen) for 5 min at room temperature to stain the nuclei. Between each of the described steps, three washes with 1× PBS (pH 7·4) were performed. The macrophages were observed using a *Leica* DMI3000B fluorescence microscope with N2·1 filter for *DiD* (excitation at 644 nm and emission at 665 nm) and A1 filter for Hoechst (excitation at 352 nm and emission at 461 nm) at a magnification of 1000×. Images from 180 microscope fields per sample were acquired using each of the filters according to the emission spectrum of each fluorochrome using *LAS core* software.

The images acquired were analysed using *CellProfiler®* software (Carpenter *et al*. 2006), which was configured to identify and count macrophages and amastigotes and to determine the number of amastigotes per macrophage. The pipeline designed in *CellProfiler®* is illustrated in (Supplementary Figure 1), and representative image-processing steps are shown in Fig. 1A–F. Parameters such as object of interest (macrophage nuclei, parasite nuclei/kinetoplast, macrophage perimeter) and size in pixels were first manually determined for subsequent input into the pipeline. The minimum and maximum values of these parameters were calculated from images of 125 macrophages and 125 amastigotes using *CellProfiler®*. These values were then taken as a baseline to standardize the counting methodology on *CellProfiler®*. All images were re-examined with the standardized counting protocol to test its reproducibility. Using this methodology, it was possible to differentiate macrophages from amastigotes by its size and its signal intensity and to determine: (1) the percentage of infection (calculated based on the number of infected macrophages per each 100 macrophages sampled of the tested populations), (2) the number of parasites per sampled macrophage and (3) the number of parasites per infected macrophage. The infection assays and the determination of infection phenotypes for each cell line were performed in triplicate on at least three independent occasions.

Statistical analysis

- Protein expression levels obtained by *Western blot*: The geometric means of the relative optical densities normalized with the loading controls for the three replicates of the cell lines evaluated were calculated. To quantify the differences between signals obtained in the Western blot assays, the geometric means of the relative densities were expressed as percentages, and the difference between the percentage of the reference line and the percentage of each line evaluated was interpreted as the expression difference (Biosciences, 2013).
- Infection phenotype: To determine if there were statistically significant differences in the infection percentages between generated cell lines with respect to the parent line, the χ^2 test was used. To establish if there were differences between number of amastigotes per infected macrophage or number of amastigotes per sampled macrophage of the cell lines evaluated, the Kruskal–Wallis test was used, followed by Dunn's multiple comparison test.

The statistical analyses were performed using the Stata 13 and GraphPad Prism 5·0 software with a significance level of $P < 0·05$.

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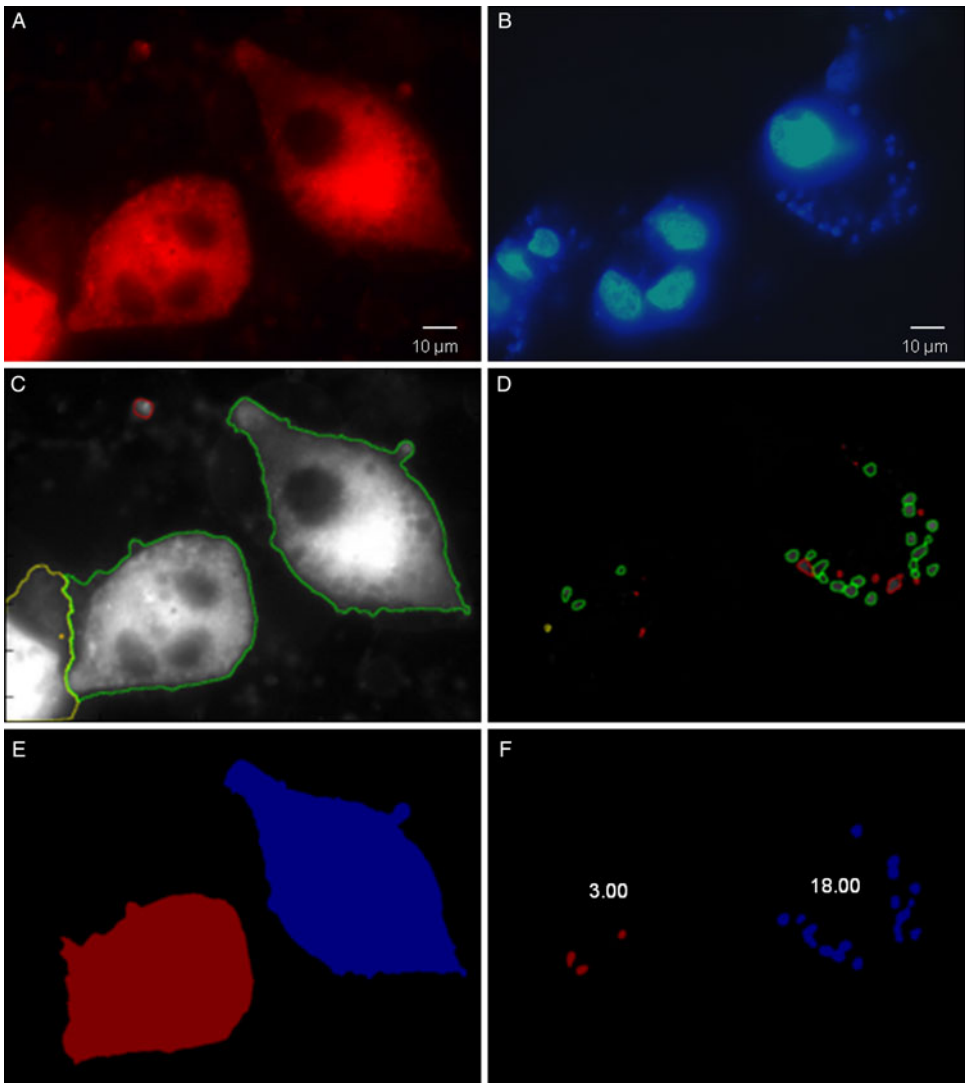

Fig. 1. Image analysis of macrophages infected with *L. (V.) braziliensis* using *CellProfiler*® software. (A) Image of macrophage membranes stained with 10 mM DiD. (B) Macrophage nuclei, kinetoplast-like structures and amastigote nuclei stained with 1·25 μg mL^{-1} Hoechst. Once the images were acquired, *CellProfiler*® software was used to identify the objects of interest in each of the pictures: macrophages in the image are stained with DiD (C), and amastigotes in the image are stained with Hoechst (D). After identification of the objects of interest, the amastigotes were associated with their respective host cell (E), assigning the same colour for both types of objects (F). With these data, the number of amastigotes per infected macrophage, the number of parasites per sampled macrophage and the infection percentage in each cell line were assessed.

RESULTS

Validation of gene silencing and phenotypic evaluation in U937-derived macrophages using gfp as a reporter

To confirm the feasibility of gene silencing in the context of U937-derived macrophages, we selected an easily identifiable reporter gene, such as *gfp*. First, we generated a U937-derived cell line constitutively expressing *gfp* (U937–GFP) via lentiviral transduction, with 100% efficiency as confirmed by fluorescence microscopy (Fig. 2A). After initial characterization of this cell line, we further generated two derived cell lines, one expressing a pool of three constructs encoding different shRNAs directed against *gfp* (U937–GFP/shRNA–GFP) and the other transduced with a pool of four NR shRNAs as a negative control (U937–GFP/shRNA-NR). GFP expression, or lack thereof, was confirmed by fluorescence microscopy and Western blotting (Fig. 2A and B). GFP protein levels were reduced by 88·9% in the U937–GFP/shRNA–GFP cell line as compared with its parental cell line U937–GFP, or the corresponding negative control U937–GFP/shRNA-NR, as demonstrated by densitometric analysis from Western blots (Fig. 2C).

After gene silencing confirmation, we proceed to evaluate three phenotypic infection parameters, i.e. percentage of infection, number of amastigotes per infected macrophage and number of parasites per sampled macrophage in the corresponding cell

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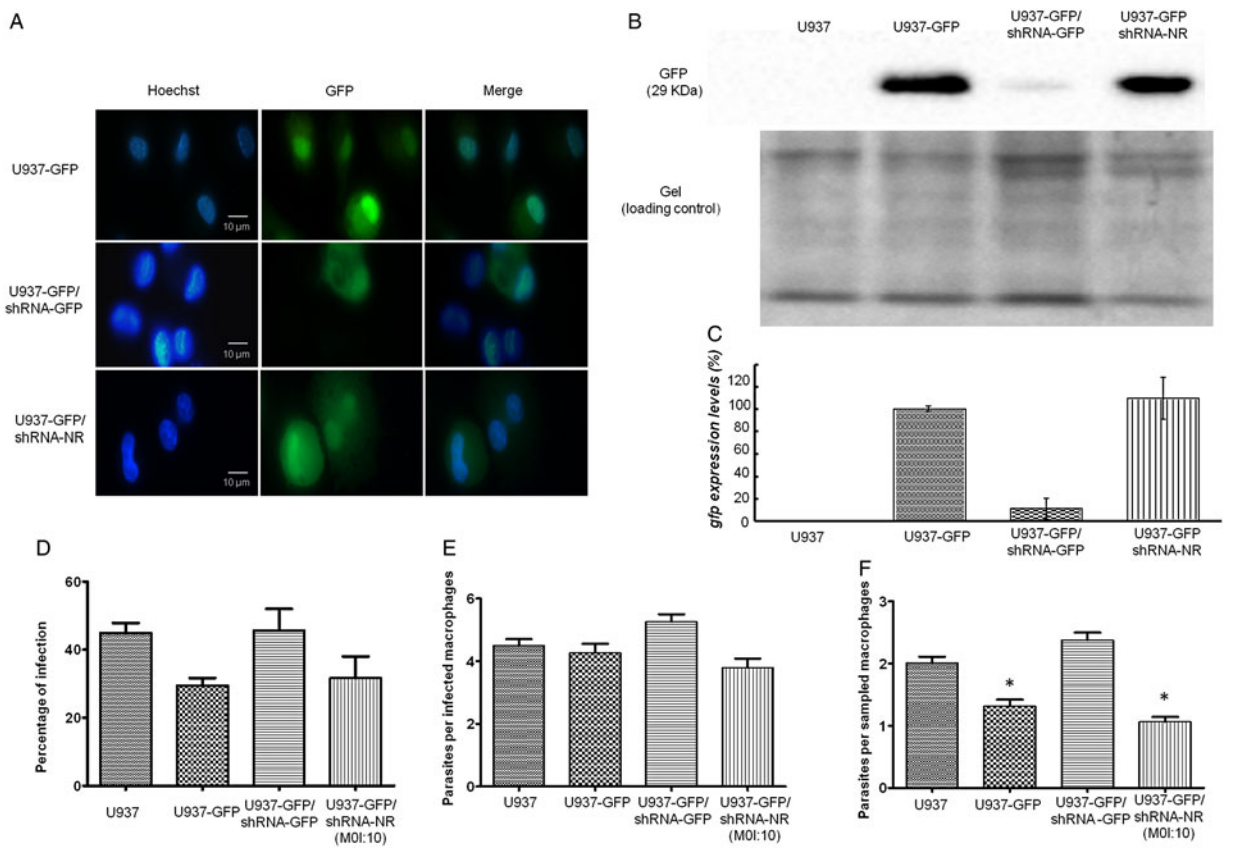

Fig. 2. Expression and silencing of the *gfp* gene in macrophages derived from U937 cells. (A) Representative images of the cell lines with expression and silencing of *gfp* and the negative control line, acquired by fluorescence microscopy taken at 1000× magnification. Nuclei stained with 1·25 μgmL^{-1} Hoechst (left), cells expressing GFP (centre) and the image resulting from the combination of the two fluorescence channels (right). (B) Representative Western blot of GFP protein levels expressed in percentages. The U937–GFP cell line was transduced with lentiviral vectors bearing a construct for the expression of shRNAs directed against *gfp* or shRNAs-NR. Cells form each cell line were differentiated to macrophages for subsequent protein extraction. For SDS–PAGE, 25 μg of total protein per lane was loaded into two 10% acrylamide/bisacrylamide gels. One of the gels was used for the Western blot, and the other was used as a loading control stained with Coomassie Brilliant Blue. A decrease in the GFP expression level in the U937–GFP/shRNA–GFP line was observed compared with the U937–GFP line and the negative control U937–GFP/shRNA-NR (MOI 10). (C) Average densitometric measurements of Western Blots for detection of GFP protein expression levels of the cell lines U937, U937–GFP/shRNA–GFP and U937–GFP/shRNA-NR normalized with respect to expression in the U937–GFP line (error bars represent standard deviation). Infection phenotype determination in the evaluated cell lines (D–F) was performed via fluorescence microscopy analysis of images in *CellProfiler*® software. The parameters of (D) infection percentage, (E) number of amastigotes per infected macrophage and (F) number of parasites per sampled macrophage were calculated. The means of three independent experiments are shown (error bars represent standard error of the mean). (*) indicates statistically significant differences calculated using Dunn's multiple comparison test ($P < 0{\cdot}05$) to identify if there were differences between number of amastigotes per infected macrophage or number of amastigotes per sampled macrophage of the cell lines evaluated.

lines. As shown in Fig. 2D–F, exogenous expression of *gfp* in U937–GFP was associated with reductions of approximately one-third in the percentage of infection and number of parasites per sampled macrophage when compared with the parental U937 cell line (Dunn's multiple comparison test; $P < 0{\cdot}05$). This infection phenotype was rescued by *gfp* gene silencing in the U937–GFP/shRNA–GFP cell line and had no effect on the corresponding negative control cell line U937–GFP/shRNA-NR-MOI: 10. Together, these results suggest that *gfp* expression is the cause of the lower percentage of infection observed in the U937–GFP cell line (χ^2; $P < 0{\cdot}05$). Interestingly, reduction was not observed with the number of amastigotes per infected macrophage, suggesting that once infected, U937–GFP macrophages seem to be equally suitable for parasite proliferation.

Validation of gene silencing and phenotypic evaluation in U937-derived macrophages using the endogenous gene lmna

The gene encoding the intermediate filament-type proteins Lamin A and C has become one of the standard positive controls used in silencing assays.

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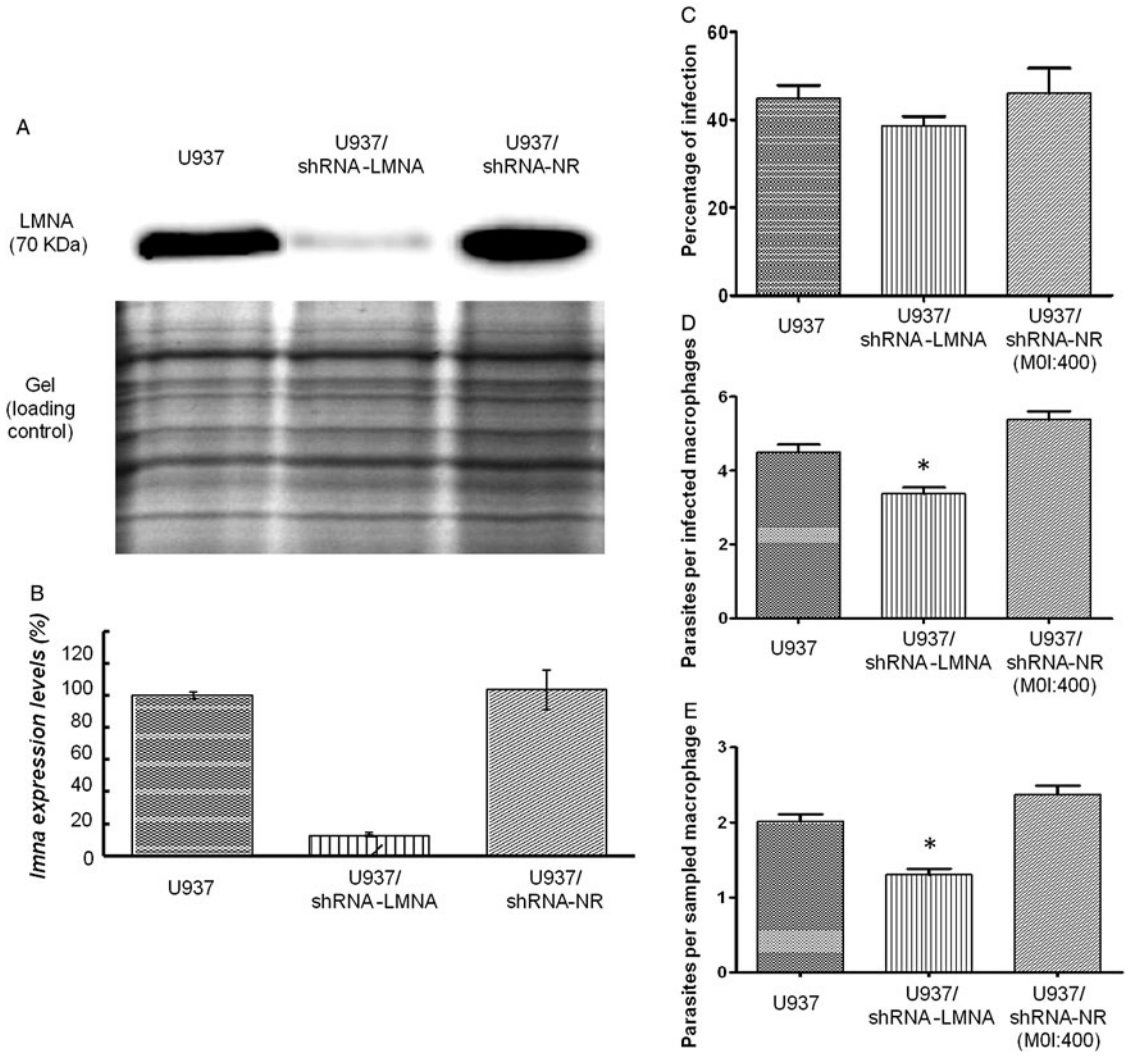

Fig. 3. Validation of gene silencing and phenotypic evaluation in U937-derived macrophages using the endogenous gene lmna. (A) Representative Western blot for detection of LMNA protein levels expressed in percentages. The U937 cell line was transduced with lentiviral vectors bearing constructs for the expression of shRNA directed against *lmna* or shRNAs-NR. Cells from each cell line were differentiated to macrophages for subsequent protein extraction. For SDS–PAGE, 50 μg of total protein per lane was loaded into two 8% acrylamide/bisacrylamide gels. One of the gels was used for the Western blot, and the other was used as a loading control stained with Coomassie Brilliant Blue. LMNA expression was decreased in the U937/shRNA–LMNA cell line compared with the U937 and negative control U937/shRNA-NR (MOI 400), cell lines. (B) Average densitometric measurements of Western blots for detection of LMNA protein expression levels in the cell lines were normalized with respect to expression in the U937 parental cell line (error bars represent standard deviation). Infection phenotype determination in the evaluated cell lines (C–E) was performed through analysis of fluorescence microscopy images using *CellProfiler*® software. The parameters of (C) infection percentage, (D) number of amastigotes per infected macrophage and (E) number of parasites per sampled macrophage were calculated. The means of three independent experiments are shown (error bars represent standard error of the mean). (*) indicates statistically significant differences calculated using Dunn's multiple comparison test ($P < 0{\cdot}05$) to identify if there were differences between number of amastigotes per infected macrophage or number of amastigotes per sampled macrophage of the cell lines evaluated.

For this reason, we generated a cell line with constitutive silencing of *lmna* (U937/shRNA–LMNA) and the corresponding negative control transduced with a pool of lentiviral vectors encoding NR shRNAs (U937/shRNA-NR-MOI: 400). Expression of the shRNA directed to *lmna* induced a reduction in LMNA protein levels of 87·5% when compared with the parental cell line or the corresponding negative control (Fig. 3A and B). *Lmna* gene silencing was associated with a slight reduction in the percentage of infection (χ^2; $P < 0{\cdot}05$) and more evident reductions in the parameters of number of parasites per sampled macrophage (Dunn's multiple comparison test; $P < 0{,}05$) and number of amastigotes per infected macrophage, as compared with the parental cell line and the negative control (Fig. 3C–E).

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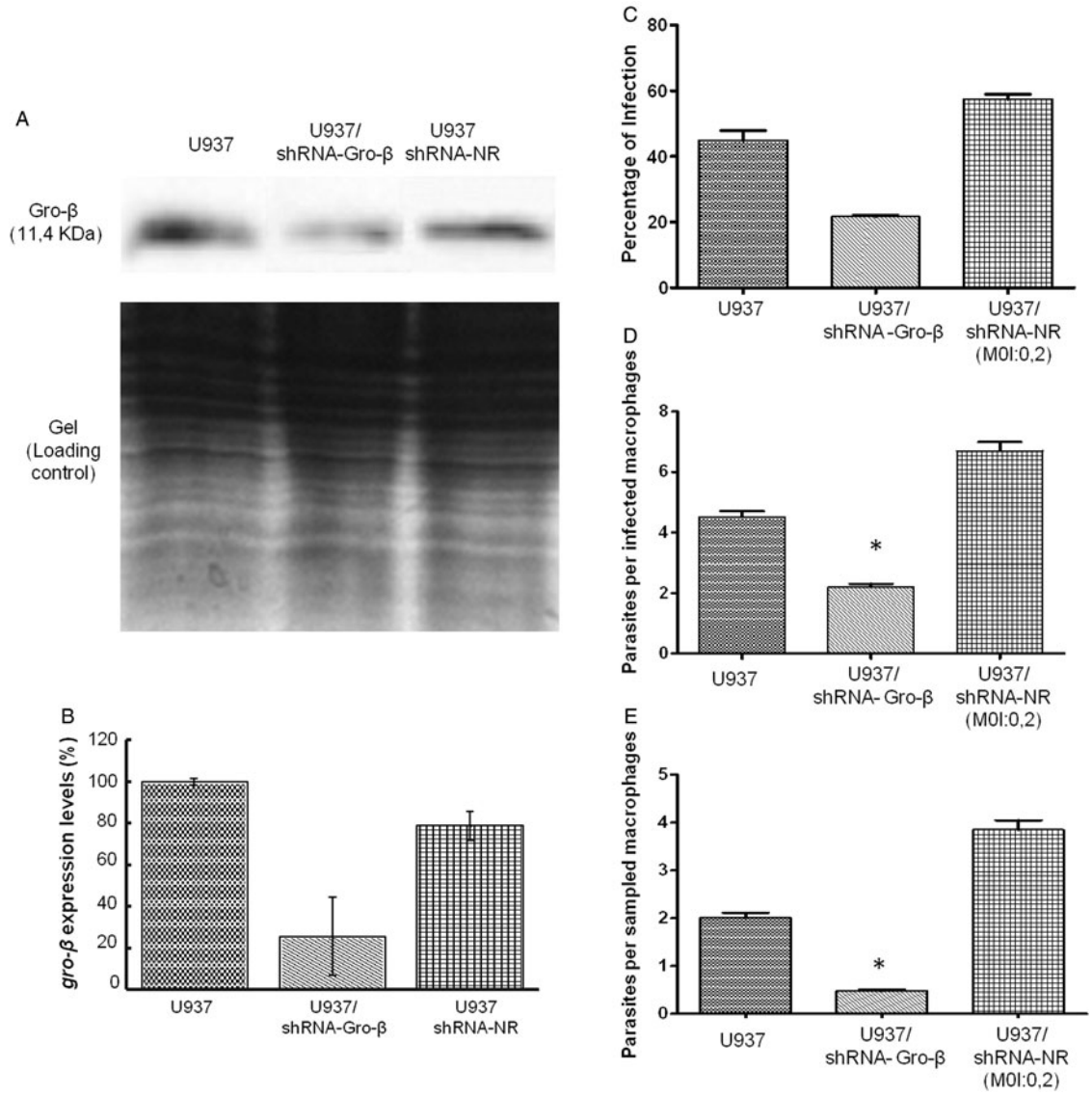

Fig. 4. Validation of gene silencing and phenotypic evaluation in U937-derived macrophages using the gene *gro-β*. (A) Representative Western blot for detection of Gro-β protein levels expressed in percentages. The U937 cell line was transduced with lentiviral vectors bearing a construct for the expression of shRNA directed against *gro-β* or shRNAs-NR. Cells from each cell line were differentiated to macrophages for subsequent protein extraction. For SDS–PAGE, 100 μg of total protein per lane was loaded into two 15% acrylamide/bisacrylamide gels. One of the gels was used for the Western blot, and the other was used as a loading control stained with Coomassie Brilliant Blue. Decreased Gro-β expression was observed in the U937/shRNA–Gro-β cell line compared with the U937 and negative control U937/shRNA-NR (MOI 0·2), cell lines. (B) Average densitometric measurements of Western blots for detection of Gro-β protein expression levels in the cell lines were normalized with respect to expression in the U937 parental cell line (error bars represent standard deviation). Infection phenotype determination in the evaluated cell lines (C–E) was performed through analysis of fluorescence microscopy images using *CellProfiler*® software. The parameters of (C) infection percentage, (D) number of amastigotes per infected macrophage and (E) number of parasites per sampled macrophage were calculated. The means of three independent experiments are shown (error bars represent standard error of the mean). (*) indicates statistically significant differences calculated using Dunn's multiple comparison test ($P < 0{\cdot}05$) to identify if there were differences between number of amastigotes per infected macrophage or number of amastigotes per sampled macrophage of the cell lines evaluated.

Validation of gene silencing and phenotypic evaluation in U937-derived macrophages using a differentially expressed gene in infected macrophages with L. (V.) braziliensis

After we demonstrated that U937 cell line gene modification can translate into changes in the Leishmania mediated-infection phenotype, we tested the silencing of a gene with precedents of overexpression associated with *L.* (*V.*) *braziliensis* infection for U937-derived macrophages. In a previous work, we performed a microarray-based gene expression analysis comparing non-infected and

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L. (*V.*) *braziliensis*-infected U937-derived macrophages (data not shown, paper in Press). That study identified a total of 218 macrophage genes with differential expression, and *gro-β* exhibited more than 2-fold upregulation by both microarray and real-time RT–PCR analyses. Therefore, we generated a cell line with silencing for *gro-β* (U937/shRNA–Gro-β) and the corresponding negative control (U937/shRNA-NR-MOI: 0·2). *Gro-β* gene silencing was associated with half-reductions of the parameters percentage of infection (χ^2; $P < 0{\cdot}05$) and number of amastigotes per infected macrophage, and an even larger reduction of number of parasites per sampled macrophage (Dunn's multiple comparison test; $P < 0{\cdot}05$) (Fig. 4C–E). During phenotypic determination, we obtained information that allowed us to generate the frequency distribution of the number of parasites per infected macrophage in the different sampled populations (Supplementary Figure 2). The U937/shRNA–Gro-β cell line had a median of two parasites per infected macrophage, whereas the parental cell line had a median of 4. Of note, in the U937/shRNA–Gro-β cell line, approximately 75% of the infected macrophages had one or two parasites, whereas such a subpopulation comprised approximately 49% of the parental cell line. These results reflect important reductions in not only the percentage of infected cells but also the number of parasites that manage to survive in those macrophages with *gro-β* gene silencing. Surprisingly, the cell line transduced with NR shRNAs was associated with increased values for the measured parameters of infection.

DISCUSSION

Deep knowledge regarding pathogen–host interactions is crucial for the understanding of an infectious disease. Current gene silencing technologies are the key for appropriate functional identification of each of the molecular nodes involved in the complex network of cellular components, typical of *Leishmania* spp. and its human host cell, the macrophage. In this work, we explored and obtained a proof of concept related to the use of the U937 cell line as a model for functional genetic screening in the context of *L.* (*V.*) *braziliensis*-mediated infection. First, we adapted a procedure to facilitate phenotypic identification of infected macrophages. The use of fluorescence microscopy allowed us to integrate a semi-automated phenotyping method with the image analysis software *CellProfiler*®, reducing inter-observer variability and providing enough information to obtain important quantitative data for characterizing the infection intensity. This procedure seems suitable for use in high-throughput and high-content screening analyses.

The U937 cell line has been extensively used as a pro-monocyte or human macrophage model in a variety of research topics, including *Leishmania* spp.-mediated infection. Despite being a reasonably good model for the study of Leishmania–macrophage interactions, the U937 cell line is challenging when genetic modification is desired. We employed lentiviral transduction and demonstrated that at high viral titres, it is possible to obtain 100% transduction efficiency.

Transduction with lentiviruses bearing the construct for GFP expression showed that all transduced cells expressed GFP, evidencing the transduction efficiency. These results are concordant with previous reports in which GFP expression levels in the U937 and SupT1 cell lines were higher than 96% after lentiviral transduction (Santhosh *et al*. 2008). The use of lentiviral vectors represents a great advantage when working with a cell line characterized as difficult to transfect, such as U937 (Martinet *et al*. 2003; Van De Parre *et al*. 2007; Tietz and Berghoff, 2012).

Next, we deployed lentiviral transduction to demonstrate the feasibility of gene silencing in U937-derived macrophages in the context of *L. braziliensis* infection. *lmna, gfp* and *gro-β* expression levels were silenced with shRNAs directed towards these genes.

Given the high expression levels of *lmna*, it is a difficult gene to silence (Elbashir *et al*. 2002; McManus and Sharp, 2002); therefore, achieving its silencing was considered a good functional indicator of the silencing machinery in the model used in this study. Our results indicate that LMNA protein expression was reduced by 87·5% in the silenced cells with respect to the parental cells. This result is concordant with studies performed in HeLa cells, where reductions in protein expression have been reported to be more than 90% with shRNA and 95% with small interfering RNA (siRNA) (Elbashir *et al*. 2001; Paul *et al*. 2002; Harborth *et al*. 2003). In a study performed with human endometrial cells, protein expression from the *lmna* gene was silenced by 88% using siRNA (Tulac *et al*. 2004).

Protein expression from the *gro-β* gene, which has been identified as differentially expressed between infected and non-infected macrophages, was reduced by 74·4% in comparison with the parent line. No similar studies in which this gene was silenced in macrophages were found in the literature.

With respect to infection phenotype, both *gfp* expression and its silencing were associated with different phenotypic changes in terms of percentage of infection, number of parasites per sampled macrophage and number of amastigotes per infected macrophage. Infection-related phenotypic changes were also detected when the endogenous genes *lmna* and *gro-β* were silenced. These results demonstrate that lentiviral-mediated genetic modification in U937-derived macrophages can translate to detectable phenotypic changes regarding *L.* (*V.*)

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braziliensis infection. Therefore, this study suggests that genetic modifications involving either exogenous gene expression (*gfp*) or endogenous gene silencing in the cellular model used here, could be used to identify macrophage genes that are functionally relevant to the *L.* (*V.*) *braziliensis* infection.

Although the results suggest that gene silencing is the cause of the changes in the infection phenotypes in the cell lines silenced, it is important to keep in mind that one of the difficulties inherent to this methodology are the off-target effects that can alter different cellular processes. The principal causes of the off-target effects are as follows: (1) homology of the shRNA sequence with other transcripts different from the gene of interest, (2) effects related to the erroneous processing of endogenous miRNAs or incorrect editing of the shRNA; and (3) cellular perturbation due to the abundant presence of vectors and exogenous double-stranded RNA (Fellmann and Lowe, 2014). This study evaluated the possibility of 'off target' effects caused by homology via alignments performed between the shRNA used and RefSeq, the NCBI human gene and transcripts database. These alignments confirmed that the sequence had no close identity with other transcripts and that there were no mismatches between the target sequence and the shRNA. Another cause of off-target effects is the cellular perturbation associated with the length of the interference RNA. According to Reynolds *et al.*, shRNA sizes larger than 23 base pairs can be recognized by Toll-like receptors, which trigger the immune response and thereby activate interferon-stimulated genes (Reynolds *et al*. 2006). This study attempted to control this effect using a 19-bp shRNAs. The possibilities of generating changes in the infection phenotype, in gene expression by only the transduction of lentiviral particles and in the expression of shRNA were evaluated with the negative control lines (U937/shRNA-NR); there were no differences between the parent line and the negative control line in the infection phenotype in two out of the three sets of assays performed. The negative control U937/shRNA-NR obtained after transduction with an MOI of 0·2 showed evident increase in the infection parameters of number of amastigotes per infected macrophage and number of parasites per sampled macrophage, when compared with the parental cell line. It seems possible that this behaviour reflects the effect of the selection required after transduction, given the fact that only a small fraction of the starting cell population would be effectively transduced with such a low titre (0·2 transducing units per cell).

Of note, we identified three types of infection phenotypes related to lentiviral-mediated genetic modification in U937-derived macrophages. The first type involved reductions in the percentage of infection and number of amastigotes per infected macrophage, such as those observed when GFP was exogenously expressed or *lmna* and *gro-β* genes were silenced. The second type was an increased susceptibility to infection observed in macrophages in which exogenous *gfp* was silenced compared with those cells expressing *gfp*. The final type involved a lack of change in the infection phenotype, such as that observed in cells transduced with the pool of NR shRNAs at high titres. Therefore, we consider that lentiviral-mediated genetic modification in U937-derived macrophages can be used for the functional identification of genes required for *L.* (*V.*) *braziliensis* infection.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/ 10.1017/S0031182015001304.

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CONFLICT OF INTEREST

None.

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