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EGFP reporter protein: its immunogenicity in *Leishmania*-infected BALB/c mice

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Abstract Optical reporter genes such as green fluorescent protein (GFP) and luciferase are efficiently and widely used in monitoring and studying the protective/therapeutic potential of candidate agents in leishmaniasis. But several observations and controversial reports have generated a main concern, whether enhanced GFP (EGFP) affects immune response. To address this issue, we studied the immunogenicity of EGFP in vivo by two lines of stably transfected parasites (*Leishmania major*^{EGFP} or *L. major*$^{EGFP\text{-}LUC}$) in BALB/c model and/or as a recombinant protein (rEGFP) produced in vitro by bacteria in parallel. Disease progression was followed by footpad swelling measurements and parasite burden in draining lymph nodes using microtitration assay and real-time PCR, and immune responses were also evaluated in spleen. EGFP-expressing parasites generated larger swellings in comparison with wild-type (*L. major*) while mice immunized with rEGFP and challenged with wild-type parasite were quite comparable in footpad swelling with control group without significant difference. However, both conventional and molecular approaches revealed no significant difference in parasite load between different groups. More importantly, no significant inflammatory responses were detected in groups with higher swelling size measured by interferon-γ (IFN-γ), interleukin (IL)-10, IL-5, and nitric oxide against frozen and thawed lysate of parasite as stimulator. Altogether, these results clearly revealed that EGFP protein expressed in prokaryotic and eukaryotic hosts is not an immunological reactive molecule and acts as a neutral protein without any side effects in mice. So, EGFP expressing *Leishmania* could be a safe and reliable substitution for wild-types that simplifies in situ follow-up and eliminates the animal scarification wherever needed during the study.

Keywords Reporter gene · EGFP · Luciferase · *Leishmania major* · In vivo infectivity

Introduction

Leishmaniasis is one of the major zoonotic infectious diseases transmitted by female sand flies (phlebotomine). Clinical symptoms appear from mild cutaneous lesion to progressive lethal visceral disease (Desjeux 2004). It is prevalent in six continents, and many countries including Iran which is considered as an endemic area for cutaneous and visceral leishmaniasis. Many people are at the risk of leishmaniasis, and about 1.5 million new cases are reported yearly (www.who.int). Major clinical forms of leishmaniasis include cutaneous, visceral, and mucocutaneous caused by different *Leishmania* species. Murine models of leishmaniasis have been applied to investigate the pathogenesis of the disease and to test new therapeutic approach (Mehta et al. 2008).

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Conventional methods to evaluate host–*Leishmania* interactions and infection follow-up are based on Giemsa staining, in vitro culture-based assays, labor-intensive microscopic techniques, and caliper-based assessments (Courret et al. 2003; Lang et al. 2005; Sacks and Anderson 2004). Although many of these techniques are useful for in vitro studies and have helped to describe many important features of microbial pathogens, they are intrinsically bound to some limitations such as parasites progression to undetectable sites must be considered (Lang et al. 2005). Successful labeling of invasive microorganisms by fluorescence and bioluminescence reporter genes recently provided a precise tool for infectivity evaluation and disease detection at early stages (Calvo-Álvarez et al. 2012). Fluorescence signals are discovered with minimal handling taking advantage of fluorescence microscopy and flow cytometry (Bolhassani et al. 2011; Lang et al. 2005). Green fluorescent protein (GFP) recombinants have been reported in many species of *Leishmania* (Beattie et al. 2008; Fumarola et al. 2004; Ha et al. 1996) and other parasites such as *Plasmodium* species (Jongco et al. 2006; Sultan et al. 1999). *Trypanosoma* (DaRocha et al. 2004), and *Toxoplasma* (Striepen et al. 1998). Other useful reporter genes include firefly luciferase which converts luciferin as substrate to oxyluciferine, which emits light after returning back to its steady state (Baldwin 1996). Emitted light is easily detectable by luminometry. Both GFP and luciferase reporters provide in vivo imaging of live anesthetized animals.

Despite wide application of GFP, some reports have shown side effects of GFP on cells (Koike et al. 2013). There are very controversial reports on toxicity of GFP. Many reports have shown that GFP is toxic (Liu et al. 1999; Re et al. 2004) or even reduce potential pathogen infectivity (Bennett et al. 2001). In contrast, some of them have confirmed that GFP expressed in transgenic mice is not toxic and live as long as wild-type mice (Hoffman 2008; Okabe et al. 1997).

Our preliminary observation has shown larger footpad swelling size in BALB/c mice infected with enhanced GFP (EGFP) recombinants *Leishmania major* which raised some questions to be addressed: (1) if expression of exogenous *egfp* gene in parasite impacts infectivity potential or virulence and (2) if EGFP is natively an immunogenic molecule. Therefore, there was a demand to evaluate clinical and immunological response in host against EGFP protein.

In this study, we used two forms of EGFP protein to assess the biological and immunological effects of EGFP in vivo: recombinant protein expressing in *Escherichia coli* and recombinant live parasite stably expressing the protein. We compared the infectivity rate of transgenic *L. major* strains expressing EGFP and EGFP-LUC with *L. major* wild-type with different methods in BALB/c mice. Furthermore, we compared effect of EGFP in recombinant protein (produced in prokaryote host) form or expressed by a recombinant parasite (expressed in eukaryotic host).

Materials and methods

Mice

Six to eight weeks old female BALB/c mice were obtained from the animal breeding stock facility of Pasteur Institute of Iran and housed in plastic cages with free access to tap water and standard rodent pellets.

Parasite culture

Wild-type *L. major* (MRHO/IR/75/ER) (WT) and recombinant parasites (transfected with reporter genes, *L. major*^{EGFP} (Bolhassani et al. 2011) and *L. major*$^{EGFP\text{-}LUC}$ (Taheri et al. 2015) were maintained by continuous passage in BALB/c mice to keep the infectivity and virulence. The promastigote forms were cultured at 26 °C in M199 medium (Sigma), supplemented with 5 % heat-inactivated fetal calf serum (hi-FCS, Invitrogen), 0.1 mM adenosine, 1 mM L-glutamine, 40 mM HEPES, 0.5 μg/ml Hemin (Sigma), and 50 μg/ml gentamicin (PAA). The promastigotes in metacyclic stationary phase were separated from cultured parasites by Ficoll gradient type 400 (Sigma) as described previously (Späth and Beverley 2001). Briefly, live concentrated parasites were uploaded onto a Ficoll gradient including 20 % Ficoll in H_2O at the bottom of tube, covered by10 % Ficoll in M199 medium without FCS in the middle and the parasite layer at the top. After centrifugation (1300 rpm for 10 min at room temperature), white layer corresponding to metacyclic promastigote was collected from the middle layer of gradient and washed with phosphate-buffered saline (PBS) for two times and counted parasites were used to infect animals. To prepare freeze-thawed (F/T) parasite antigen, stationary-phase promastigotes in PBS were repeatedly frozen in liquid nitrogen and thawed in 37 °C water bath for ten times.

Cloning, expression and purification of recombinant EGFP

The entire open-reading frame of *egfp* gene was isolated from pLEXSY-*egfp* (Bolhassani et al. 2011) using *Bam*HI/*Kpn*I restriction enzymes and subcloned into the *Bgl*II/*Kpn*I restriction site of pQE30 plasmid (QIAGEN) and transformed in M15 (pREP4) strain as bacterial host by standard heat-shock transformation method (30 min/ice and 90 s/42 °C). One recombinant clone was propagated in LB culture media supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin. Protein production was induced with 1 mM IPTG (isopropyl-1-thio-β-D-galactoside, Sigma; OD 0.8 at 600 nm

wavelength) for 4 h with agitation at 37 °C. Bacterial pellet was harvested at 4000 rpm for 20 min and stored at −70 °C. To separate and purify recombinant EGFP (rEGFP) from inclusion bodies, the pellets were dissolved in 2× loading buffer (4.5 mM Tris–HCl, pH 6.8, 10 % (*v*/*v*) glycerol, 2 % (*w*/*v*) sodium dodecyl sulfate (SDS), 5 % (*v*/*v*) 2-mercaptoethanol, 0.05 % (*w*/*v*) bromophenol blue), boiled for 5 min, and electrophoresed on 12.5 % SDS-polyacrylamide gel electrophoresis (PAGE) gel. Proteins were purified following reverse staining by 0.2 M imidazole/0.1 % SDS/0.2 M $ZnSO_4$ and eluted using 50 mM ammonium carbonate/0.01 SDS solution (Ortiz et al. 1992). The purified rEGFP was concentrated by ultrafiltration (Amicon) with a 10-kDa cutoff of and was dialyzed in PBS.

The whole bacterial lysate (before and after induction) and purified recombinant protein were electrophoresed on 12.5 % SDS-PAGE minigel and transferred onto nitrocellulose membranes (Protean, Schleicher & Schuell, Germany) and blocked in 2.5 % BSA/0.1 % Tween20 in TBS solution (10 mM Tris–HCl (Sigma), pH 7.4, 150 mM NaCl) overnight at 4 °C. The membranes were then incubated for 2 h at room temperature with 1:6000 diluted monoclonal anti-GFP-HRP (Acris Antibodies GmbH) antibodies in blocking buffer. After washing, the membranes were incubated with substrate (DAB, Sigma) solution (0.05 %, *w*/*v*, DAB powder solubilized in 50 mM Tris–HCl, pH 7.4) along with 0.01 % (*v*/*v*) H_2O_2 (Sigma). The reaction was stopped by H_2O immediately after specific bands were developed. The concentration of protein was determined using BCA kit (Pierce). Wild-type (WT) parasite was used as a negative control.

Immunization and challenge program

The mice were divided into several groups (n=20 per group) and inoculated with different regimens. For immunization, 100 μl PBS containing 30 μg rEGFP alone (G1) or mixed with equal volume of incomplete Freund's adjuvant (IFA; Sigma; G2) were injected intradermally to each mouse. IFA alone (100 μl/mouse) was also injected as control (G3). Three weeks later, immunized groups received the same regimes as booster. Three weeks after booster, the immunized mice were challenged subcutaneously (s.c.) in the left hind footpad with WT metacyclic *L. major* at stationary phase (5×10^6 parasite/50 μl PBS/mouse). Group 4 (G4) was non-immunized but infected with *L. major*^{WT} as positive control. Two other groups of mice (n=20 mice per group) were inoculated by recombinant metacyclic *L. major*^{EGFP} (G5) or *L. major*$^{EGFP\text{-}LUC}$ (G6) at stationary phase (5×10^6 parasite/50 μl PBS/mouse). Group 7 (G7) remained non-immunized and unchallenged as negative control (naïve). All groups were weekly monitored for inflammation by footpad swelling measurement using a metric caliper up to 8 weeks.

Parasite burden by limiting dilution assay

At different time points, four mice from each group were randomly sacrificed to dissect and weigh the popliteal lymph nodes (LNs). The LNs were homogenized separately with plastic tissue grinder in Schneider's *Drosophila* medium (Sigma) containing 10 % hi-FCS and 50 μg/ml gentamycin. The cell suspensions were serially diluted fivefold (ranging from 1^1 to ~ 10^{-14}) and dispensed into 96-well microtitration flat bottom plates (Orange) in duplicate. Each well was examined with invert microscope at a magnification of ×40. Incubation and microscopic observation was continued for maximum 2 weeks. The last dilution for which the well contained at least one viable promastigote was the final titer selected for further calculations. The number of parasite was calculated as follows: $-\log_{10}$ (last parasite dilution/weight of LN) (Buffet et al. 1995). Remaining cells were kept frozen in −20 °C for genomic DNA extraction.

Parasite burden by real-time PCR

Quantification of *Leishmania* number was carried out with RV1 (forward, 5′-CTTTTCTGGTCCCGCGGGTAGG-3′) and RV2 (reverse, 5′-CCACCTGGCCTATTTTACACCA-3′) primers, amplifying a 120-bp long fragment from minicircle kinetoplast DNA (kDNA) of *L. major* (present in about 10,000 copies in each parasite cell). To draw a standard curve, genomic DNA of *L. major* (strain MRHO/IR75/ER) was extracted from 10^7 promastigote using genomic DNA extraction kit (AccuPrep® Genomic DNA Extraction Kit, Bioneer) according to the manufacturer's instructions. DNA was serially diluted ranging from 10^6 to 10^2 parasites. PCR reaction mixture included 1× SYBER GREEN reaction master mix (QIAGEN), 10 pmol of each forward and reverse primers and ~20 ng of sample (DNA extracted from individual LNs). In each experiment, a no template tube was used as negative control to ensure lack of contamination or nonspecific reaction. PCR cycling was fulfilled by Applied Biosystems 7500 Real-time PCR system as follows: denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 40 s and one cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Each sample was run in duplicate and data was analyzed by 7500 system SDS Software against a standard curve obtained from promastigotes' kDNA.

Cytokine measurement

Before and 8 weeks after challenge, dissected spleens of four randomly selected mice from each group were separately homogenated. Red blood cells were lysed by 5 min ACK lyses solution treatment (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2). After washing the cells, single cell suspension of splenocytes were resuspended in phenol-red free

DMEM (Sigma) supplemented with 5 % hi-FCS and 100 U/ml gentamycin then were plated at 3×10^6 cells per well in 48-well plates. The cells were induced with 10 μg/ml of the rEGFP or F/T as antigen and 5 μg/ml concanavalin A (conA, Sigma) as positive control. Wells without any stimulation were used as negative control. Five days later, the supernatants were harvested and cytokines (including interferon-γ (IFN-γ), interleukin (IL)-10, and IL-5) were assayed by enzyme-linked immunosorbent assay (ELISA) using R&D kits according to manufacturer's instructions. All samples and standards were evaluated in duplicates and two independent experiments.

Nitric oxide assay

Before and 8 weeks after challenge, nitric oxide (NO) released in harvested supernatants of splenocytes after antigen stimulation were determined with an equal volume of Griess reagent (0.1 % *N*-(1-naphthyl)-ethyl-enediamine dihydrochloride and 1 % sulphanil amide in 5 % H_3PO_4) and incubated 10 min at room temperature. Absorbance was determined at 570 nm, and NO value of each sample was calculated using standard curve (sodium nitrite). All samples were run in duplicates.

Real-time imaging

In order to evaluate in vivo infection in living mice, four mice of each recombinant parasite-infected groups were randomly selected and were imaged while anesthetized using intraperitoneal ketamine (10 %)–xylazine (2 %). Monitoring was fulfilled in anesthesia time as described previously (Taheri et al. 2015). To get rid of the background fluorescence, body was epilated before imaging. The *L. major*$^{\text{EGFP-LUC}}$-infected mice (G6) were given D-luciferin potassium salt (Caliper Lifescience, dissolved in calcium and magnesium-free PBS at 15 mg/ml concentration) intraperitoneally 5 min before anesthetization. In vivo imaging was performed individually for each mouse using KODAK imaging system (In Vivo Imaging system F Pro). The images were captured using three different modes at different exposure times: luciferase (10 min), white (1 s), and GFP (30 s). To record LUC signaling, the black–white and rainbow color images were overlaid together. Parasite burden was quantified on ROI area selected; and number of pixel/ROI was counted to quantify emitted light using Molecular Imaging V.5.0.1.27 software.

Statistical analysis

For analyzing data, Graph-pad Prism 5.0 (Graph-pad Software Inc 2007, San Diego, California, USA) was applied and statistics were analyzed with one-way ANOVA (multiple-comparison Tukey post hoc test) when required comparison between two groups were performed with Student's *t* test (non-parametric test, Mann–Whitney). *p* values less than 0.05 ($p<0.05$) were considered significant.

Results

Expression and purification of recombinant EGFP

To prepare recombinant EGFP protein, the open-reading frame of *egfp* gene (~750 bp) was isolated from eukaryote pLEXSY-*egfp-neo* plasmid as source of *egfp* gene in this study and was subcloned in prokaryote expression plasmid pQE30. Recombinant EGFP (27 kDa) was induced with 1 mM IPTG and was purified by reverse staining on 12.5 % SDS-PAGE. The quality of purified protein was subsequently evaluated with Coomassie blue (Fig. 1a) and immunoblot (Fig. 1b) using a specific anti-GFP-HRP antibody.

Comparison of footpad swelling and parasite load in different groups of mice

In order to study the precise impact of EGFP expression by *Leishmania*-bearing *egfp* on infectivity, recombinant protein expressed by *E. coli* (rEGFP) adjuvanted by IFA was used in parallel with recombinant parasites. The mice were clustered in seven groups and injected with different regimens. According to Fig. 2a, groups 1 and 2 were immunized intradermally by rEGFP with or without IFA. The IFA alone was injected to group 3 as negative control. All groups were immunized two times at 3 weeks intervals with rEGFP alone or mixed with IFA. Group 4 received PBS two times as control. Three weeks after last immunization, all immunized groups were challenged with WT *L. major*. Two other groups of mice were challenged with *L. major*$^{\text{EGFP}}$ (G5) or *L. major*$^{\text{EGFP-LUC}}$ (G6) recombinant parasites, respectively (5×10^6 metacyclic stationary phase promastigotes in 50 μl PBS injected subcutaneously in the left hind footpad). Group 7 as a negative control was non-immunized and unchallenged (naïve) group. Figure 2b depicts the timeline of the study.

All groups (immunized and unimmunized) were weekly monitored for clinical and inflammation signs derived from leishmanial infection at infection site for 8 weeks. Footpad swelling began early after infection in all groups. There was no significant difference in footpad swelling between EGFP immunized groups and unimmunized control (G4). Expectedly, lesion size in groups receiving recombinant parasites (G5 and G6) developed with higher speed at fourth week in comparison with other group. The difference even increased and was significant in the following weeks (Fig. 3a, $p>0.05$). Eight to four weeks footpad ratio was 1.46, 1.57, and 1.64 times for *L. major*$^{\text{WT}}$ (G4), *L. major*$^{\text{EGFP}}$ (G5), and *L. major*$^{\text{EGFP-LUC}}$ (G6) groups, respectively.

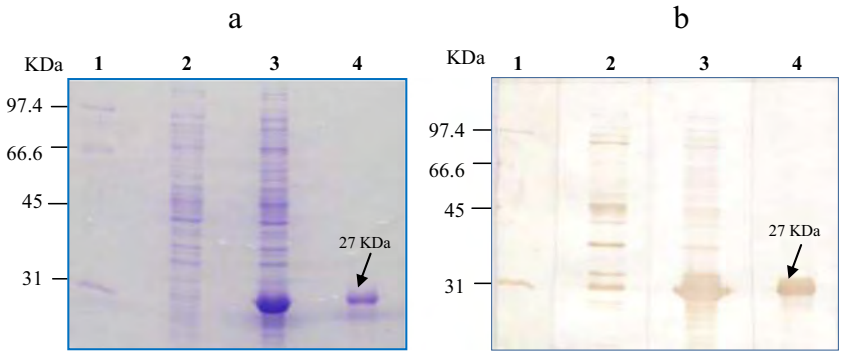

Fig. 1 Purification of rEGFP protein. The entire *egfp* gene from pLEXSY-*egfp* (as source of EGFP in this study) was subcloned in pQE30 plasmid, induced with 1 mM IPTG for 4 h and purified from SDS-PAGE gel using reverse staining. The induced bacteria, purified rEGFP, and a before-induction sample were confirmed on 12.5 % SDS-PAGE following Coomassie blue staining (**a**) and purified rEGFP protein was recognized using anti-GFP on immunoblot (**b**). *Line 1*, molecular weight marker; *line 2*, uninduced bacterial lysate; *line 3*, 4 h after induction; and *line 4*, the purified recombinant EGFP protein in *line 4*

To estimate and compare parasite load among all groups, conventional (serial dilution) and molecular methods (real-time PCR), at two different time points (4 and 8 weeks post-infection) were used. Conventional approach estimates number of parasite in homogenated LNs after amastigotes to promastigotes differentiation. Only at week 4 was a significant difference shown between *L. major*^{WT} (G4) and *L. major*^{EGFP} (G5) groups ($p>0.01$). No significant difference was detected among other groups at both time points (Fig. 3b).

Parasite burden was also estimated in LNs using extracted genomic DNA and specific primers for a conserved sequence of kDNA minicircle that allows comparing parasite amount between different groups more precisely. This method also confirmed non significant level of parasite load in LNs of all groups of mice (Fig. 3c). Together, both methods showed similar parasite load at both time points of analysis. This indicated that footpad size was not quite pertinent to number of parasite. Most importantly, footpad size and parasite load (evaluated by both limiting dilution and qPCR methods) was comparable between two recombinant parasites with no significant difference.

As shown in Fig. 3b, c, parasite burden increased during experiment in all groups (without any significant different). These results validated that pre-exposure (immunized) to EGFP protein did not affect disease progression.

Cytokine assessment in different groups with rEGFP or F/T antigens

In cutaneous leishmaniasis, exacerbation or recovery of diseases is in direct relation with activity of two types of

a

Group No.	Regimen	First immunization	Second immunization	Type of parasite
G1)	rEGFP	+	+	*L. major* wild-type
G2)	rEGFP+IFA	+	+	*L. major* wild-type
G3)	IFA	+	+	*L. major* wild-type
G4)	-	PBS	PBS	*L. major* wild-type
G5)	-	PBS	PBS	*L. major*^{EGFP}
G6)	-	PBS	PBS	*L. major*$^{EGFP-LUC}$
G7)	-	-	-	-

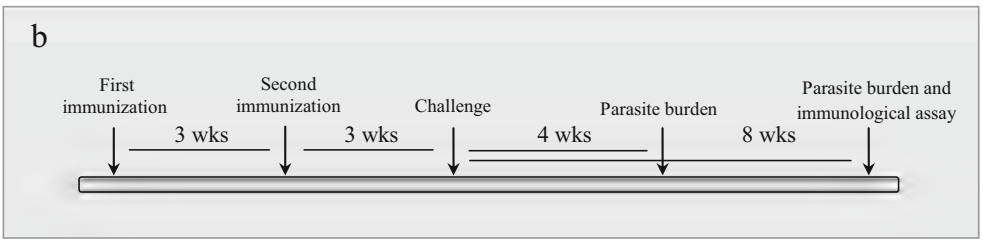

Fig. 2 Schematic diagram of immunization schedule and challenge. **a** Introduction of groups of BALB/c mice with different regimens of immunization and parasite for injection. Groups 1–3 were immunized with rEGFP alone, combined with IFA or IFA as a control, respectively, and then infected with wild-type *L. major*. Groups 4–6 were just challenged simultaneously with immunized groups using *L. major*^{WT} or recombinant *L. major*^{EGFP} and *L. major*$^{EGFP-LUC}$ (that intrinsically express EGFP). Group 7 as a negative control was non-immunized and unchallenged (naïve) group. **b** Schematic figure showing the experimental design for two immunizations with 3 weeks interval and challenge 3 weeks after last protein inoculation

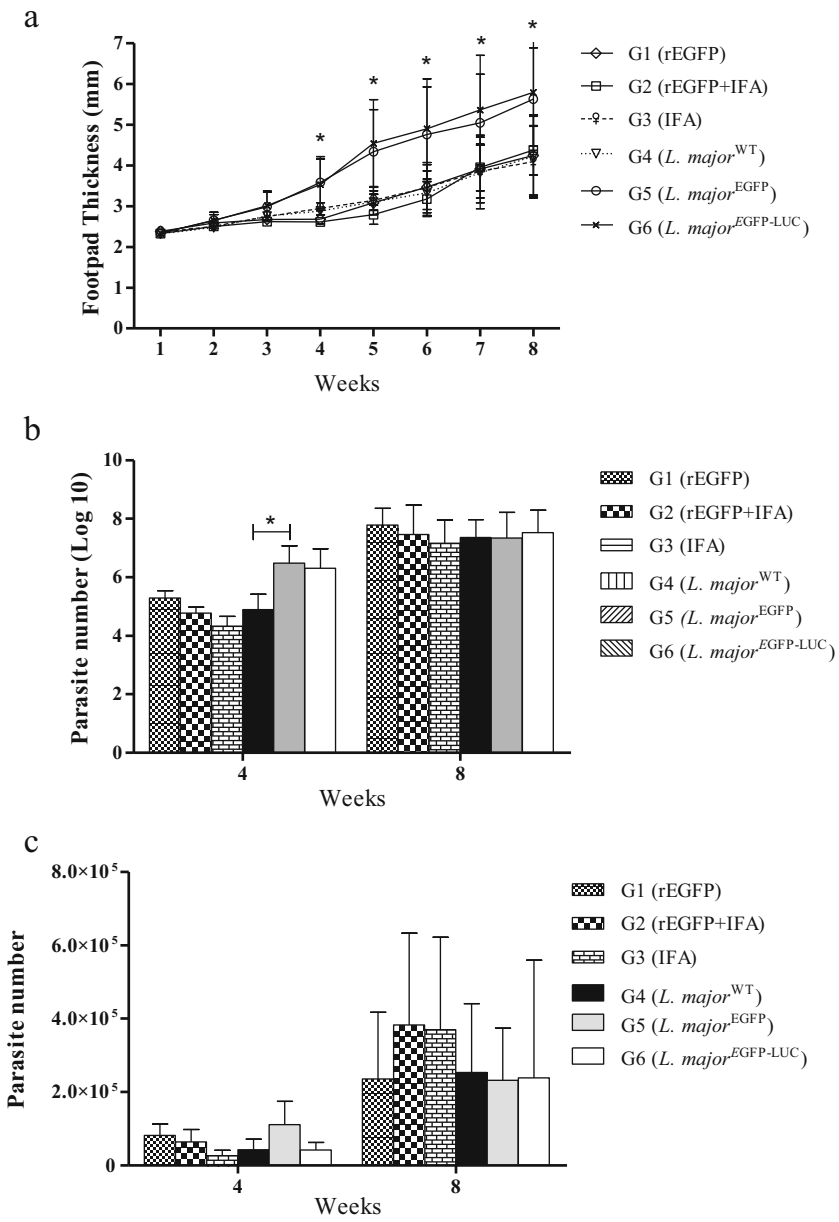

Fig. 3 Comparison of footpad swelling size and parasite burden in WT and recombinant parasites infected mice. **a** Footpad thickness was weekly recorded by metric caliper. Two recombinant parasites caused larger swelling size in footpad in comparison with WT and immunized groups (*stars* indicate significant difference, $p<0.05$). Quantification of parasite load in LNs of different groups of mice was performed by serial dilution (**b**) and real-time PCR (**c**) approaches at 4 and 8 weeks after challenge. LNs of four mice of each group (randomly selected) were individually isolated and homogenized in Schnieder's insect media. For serial dilution, homogenates were diluted (1:5 dilution factor) in 96-well microplate and observed by inverted microscope during 2 weeks. For real-time PCR, genomic DNA was extracted from LN's homogenates and used for quantification of parasite load. All tests were done in duplicate, and the results depict the number of parasites per LN as mean±SD. No significant difference was shown between immunized and non-immunized groups using both parasite count methods

T helper cells, Th1 or Th2. Here in this experiment, the infected mice with recombinant parasites showed more inflammation at infection site without any significant difference in parasite load in comparison with other mice groups. To further evaluate the impact of EGFP expression on Th2 response intensity, immune correlates of inflammation were assessed. To determine cytokine profile induced by rEGFP immunization as Th1 or Th2 response, IFN-γ, IL-10, and IL-5 were measured and compared between different groups. Single cell suspension of individual splenocytes of each group was separately restimulated in vitro with rEGFP or F/T antigens before and 8 weeks after challenge. Five days after stimulation, supernatants were collected and different cytokines as well as NO were quantified using relevant ELISA kits and Griess method, respectively.

Quantification of IFN-γ before challenge against rEGFP showed an increasing non significant difference among groups. rEGFP alone (G1) could induce more IFN-γ (148.50±86.32 pg/ml) and IL-10 (227.98±94.31 pg/ml) compared with immunized group with rEGFP+IFA (G2) (58.2±3.6 pg/ml and 111.41±29.57 for IFN-γ and IL-10, respectively) or IFA group (G3; 111.75±69.94 and 196.19 pg/ml for IFN-γ and IL-10, respectively) and non-immunized (G4) control group (83.95±25.74 and 199.18±40.37 pg/ml for IFN-γ and IL-10, respectively) (Fig. 4a, b). But this difference was not significant. No significant difference was detected in

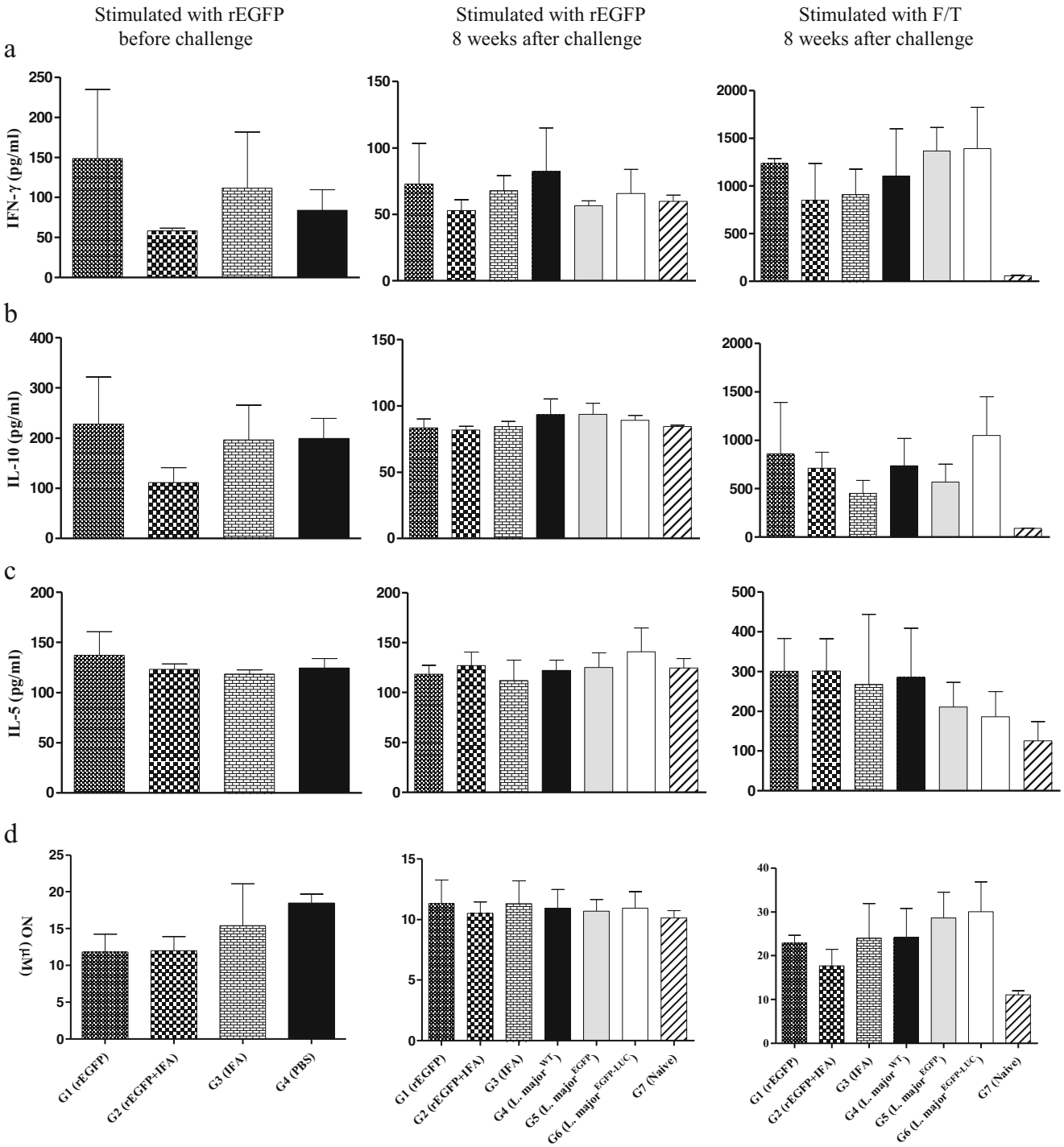

Fig. 4 Quantification of three cytokines and NO production by spleen cells between different groups of mice. Before and 8 weeks after challenge, splenocytes of four mice in each group were isolated and stimulated with rEGFP or F/T antigens prepared from *L. major*^{WT} strain. Five days later, supernatants were harvested and in vitro produced cytokines and NO were measured by ELISA and Griess assay, respectively. Data are shown as mean±SD of at least four mice per group and are representative of three experimental repeats. IFN-γ (**a**), IL-10 (**b**), IL-5 (**c**), and NO (**d**). Standard deviation in each group is presented by *bars*. *Stars* indicate significant ($p<0.05$); *ns* non-significant difference between groups

IL-5 level among immunized groups and control (G4) (Fig. 4c). The level of IFN-γ, IL-10, IL-5, and NO production in response to ConA as positive control ranges from 1122.57±669.57 to 1818.28±442.44 for IFN-γ, 807.5±329.8 to 1518±565.14 for IL-10, 222.27±55.8 to 365.79±191.7 for IL-5, and 15.91±2.3 to 35.1±

24.35 for NO. These results demonstrated that rEGFP alone or combined with IFA failed to produce more cytokines.

Eight weeks after challenge, cytokine profile against restimulation with rEGFP showed highest (82.46±32.59 pg/ml) but not significant levels of IFN-γ in WT group (G4) compared with other groups (ranging from 52.98±8.1 to 72.94±30.63) (Fig. 4a). Also, no statistical significance was observed between levels of measured cytokines (IL-10 and IL-5) in all groups of mice. Immunological factors regarding IFN-γ, IL-10, and IL-5 cytokines and nitric oxide level was comparable among immunized (G1, G2, and G3) and non-immunized groups (G4, G5, and G6) with no significant difference.

Eight weeks after challenge, lowest levels of IFN-γ were detected in mice immunized with rEGFP+IFA (G2; 850.74± 386.26 pg/ml) or IFA alone (G3; 911.56±265.69 pg/ml) against F/T stimulation. But, this difference was not significant among groups (Fig. 4a). Also, similar levels of IL-10 and IL-5 cytokines were detected in response to F/T stimulation in all groups (Fig. 4b, c).

Secreted NO by splenocytes was also measured against rEGFP or F/T stimulation before and 8 weeks after challenge. As shown in Fig. 4d, all groups of mice were compared in NO production level. Before challenge, two groups immunized with rEGFP (11.83±2.41 μM for rEGFP (G1) and 12± 1.91 μM for rEGFP+IFA (G2) groups) produced lowest NO level in comparison with controls (15.4±5.68 μM for IFA (G3) and 18.48±1.21 μM for WT (G4) groups).

Eight weeks after challenge, a similar amount of NO was detected in supernatant of all groups ranging from 10.51±0.9 to 11.31±1.9 μM, following induction with rEGFP. Also, in this time point, recombinant parasites produced higher levels of NO (28.6±5.88 and 30.05±6.85 μM for *L. major*^{EGFP} (G5) and *L. major*$^{EGFP\text{-}LUC}$ (G6), respectively) after induction with F/T antigens than WT (G4) control (24.23±6.59 μM) and immunized groups (ranging from 17.66±3.7 to 24.03±7.8 μM), which was not significant (Fig. 4d). The results demonstrated that EGFP expression by live *L. major* does not exacerbate Th2 response. In other words, stimulation using rEGFP protein could not produce any distinguished level of NO.

The results demonstrated that immunization with rEGFP protein had no effect on measured cytokines' and NO level. Although recombinant parasites induced higher level of IFN-γ, IL-10, and NO, the difference was not statistically significant. Taken together, these findings strongly support previous results from recombinant parasites that EGFP protein (in recombinant form or expressed in recombinant parasites) does not exacerbate Th2 immune response.

Monitoring of infectivity progression in vivo by dynamic measurement and imaging system

Besides classical methods, herein we monitored the disease level in two groups of infected animals taking advantages of reporter-expressing parasites and in vivo imaging technology that provides an excellent opportunity to observe and estimate extent of infection. Four and eight weeks after challenge, four mice were imaged; then the average of EGFP or LUC intensity emitted from footpad or LNs was estimated. As shown in Fig. 5a, in two out of four inoculated mice with *L. major*$^{EGFP\text{-}LUC}$ (G6), bioluminescent signal was observed not only in the site of inoculation (footpad) but also in the popliteal lymph nodes 4 weeks post-challenge. More spreading and migration of parasites was detectable in all mice at 8 weeks post-challenge. Figure 5b clearly indicates that the sum of intensity of LUC signals in EGFP-LUC-labeled transfectants (G6) 8 weeks after challenge is 2.1 times higher than LUC intensity at week 4. Also, four mice infected with *L. major*^{EGFP} (G5) were monitored by fluorescence signal that showed a direct correlation between EGFP and infectivity that was increased 1.59 times at week 8. No bioluminescence signals was seen in transfected mice with EGFP or mice infected with wild-type (G4) strain (data not shown). Totally, both reporter signals present that there was no any significant difference between two infected groups with recombinant parasites. This data is in direct concordance with footpad size swelling and parasite burden between these two groups.

Discussion

Recently, EGFP and LUC as nontoxic markers have been extensively utilized for living cells in different science of biology. Although there are some reporters about the toxic effect of EGFP expression in some cells (such as muscle (Wallace et al. 2013), Ku80-deficient hamster cells (Koike et al. 2013), retina (Rex et al. 2004), mouse fibroblast, hamster kidney cell and Huh-7 cells from hepatoma cancer (Liu et al. 1999), adult stem cells from rat hepatic (Taghizadeh and Sherley 2008), but recombinant parasites encoding reporters as EGFP have received great attention and are routinely used (Breton et al. 2005; Costa Sdos et al. 2011; Kamau et al. 2001; Mehta et al. 2008; Singh et al. 2009; Varela M et al. 2009) without adverse effects on parasite.

However, there are some controversial reports in several papers that generate a main question, whether antigenic properties of EGFP affect immune response in mice. Our previous experiences with *L. major*^{EGFP} parasite also potentiated this idea in our mind due to larger growing footpad size in BALB/c mice infected with recombinant *L. major*^{EGFP} compared with wild-type parasite (unpublished data). To address this question, we evaluated the infectivity level of both WT and recombinant parasites in vitro (Sadeghi et al. 2015) and found no significant difference by means of NO and MTT assay.

So, herein we focused more on parasite load and immunologic factors to address the question about infectivity potential of recombinant *L. major*^{EGFP} and *L. major*$^{EGFP\text{-}LUC}$ parasites,

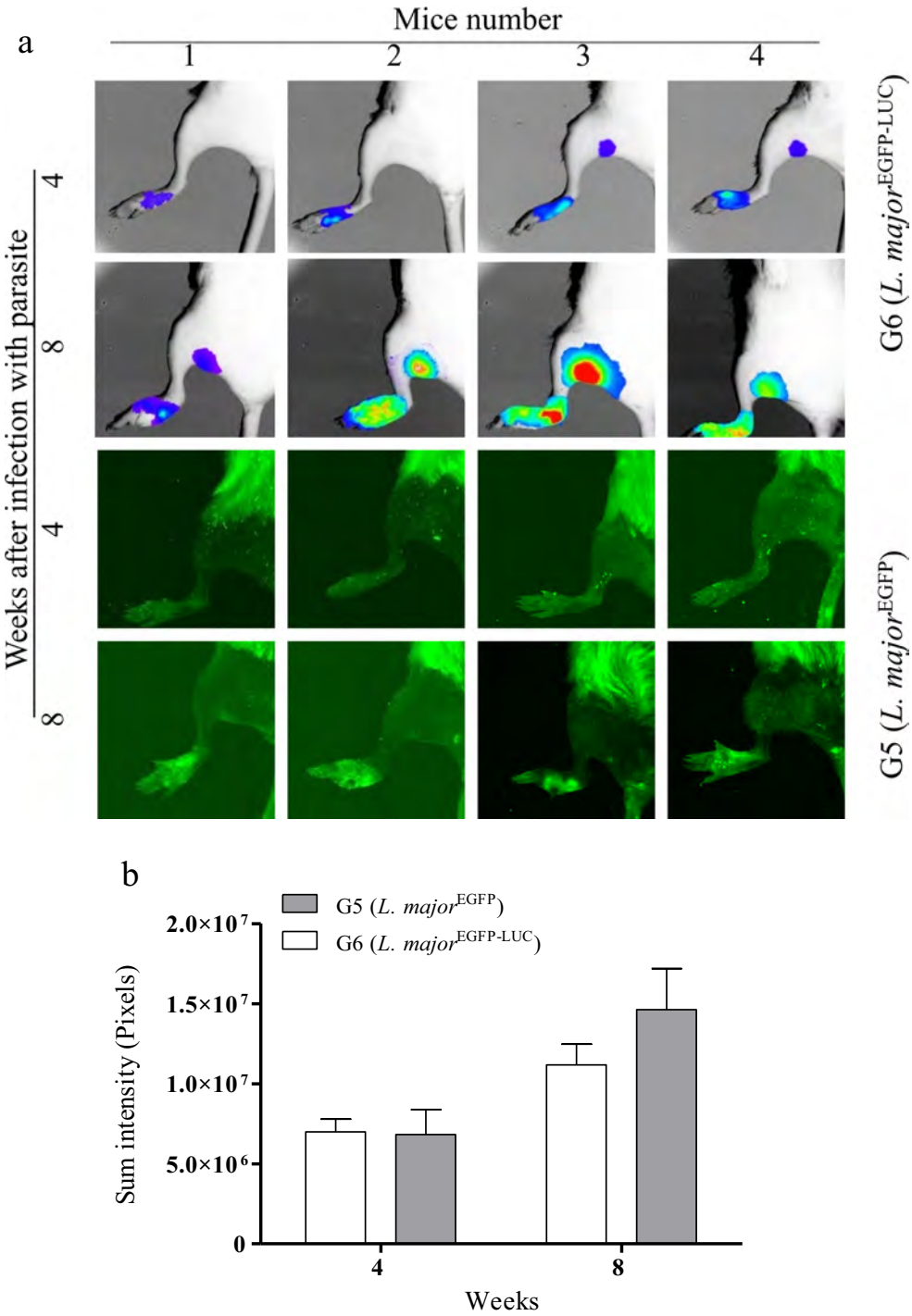

Fig. 5 Imaging and quantification of parasite burden using reporter genes. **a** Four anesthetized mice inoculated with *L. major*^{EGFP} (G5) or *L. major*$^{EGFP\text{-}LUC}$ (G6) were placed on imaging chamber. Bioluminescence and fluorescence lights were monitored in the footpad or popliteal lymph nodes 4 and 8 weeks after challenge. Images of luciferase activity in presence of luciferin as a substrate were recorded with LUC mode. For EGFP signal detection, settings were adjusted on GFP mode for 30 s. **a** *Rows 1 and 2* show bioluminescence images of *L. major*$^{EGFP\text{-}LUC}$-infected mice (G6), and *rows 3 and 4* depict biofluorescence images of *L. major*^{EGFP}-infected group (G5). **b** EGFP and LUC intensity was quantified using Molecular Imaging V.5.0.1.27 software

which are routinely used in our laboratory and other laboratories working on *Leishmania* parasite in mice. To this end, we evaluated impact of EGFP expression both in recombinant protein form and also expressed by parasite in in vivo condition.

As shown, footpad swelling size monitored by weekly metric measurement was larger in infected mice with both recombinant parasites. But, the parasite load in draining LNs using two methods showed no significant difference between mice infected with recombinant parasites and control WT-infected group. The increasing of recorded reporter gene signals are related to multiplicity of infection at 8 weeks post-infection, but both signals showed a similar infection level at 4 weeks. Same proportional increase was confirmed by in vivo imaging for two recombinant parasites.

Limiting dilution method counts promastigotes after in vitro differentiation from amastigotes but many factors are

involved to limit precise estimation. qPCR instead accurately determines the presence and amount of intracellular living amastigote based on quantified DNA. However, both methods confirmed similar parasites load besides larger swelling. An estimation of disease progression was also made using an 8 to 4 footpad ratio that revealed proportional increase.

Immune response against recombinant parasites in vivo was further evaluated after challenge with all three parasite lines in mice. Characterization of immune response regarding cytokines and nitric oxide production following injection of different lines of parasite showed no significant difference between recombinant parasites and wild-type. Generally, susceptible strains of mice (BALB/c) develop predominantly Th2 response with high level of IL-4, IL-5, IL-10 and IL-13 secretion responsible for disease progression. Infected mice with recombinant parasites produced more IFN-γ and low IL-5 in comparison with wild-type-infected group. But no significant differences were shown in IL-5 and IL-10 levels. However, at 8 weeks post-infection, all cytokines were secreted in the same level without any detectable significant difference. Also, NO level as an important contributor in host defense system showed no significant difference between groups infected with three lines of parasites. So, the results of this study confirmed that the larger swelling was an inflammatory reaction in footpad due to presence of live recombinant parasites.

To determine immunogenicity of EGFP, rEGFP was produced and purified in *E. coli*. Mice were immunized with rEGFP in presence or absence of IFA as an adjuvant and then challenged with WT *L. major*. Disease progression and footpad size increase were the same in immunized and unimmunized mice. Furthermore, parasite load in LNs of all groups was identical. Evaluation of immune responses following the immunization with rEGFP plus IFA indicated this antigen does not exacerbate disease progression by intensified Th2 responses in BALB/c mice (A comparable level of IFN-γ and IL-5 was observed in this group in comparison with WT control group).

Several studies have shown that EGFP has immunogenic properties in BALB/c mice as well as rhesus macaque monkeys (Eixarch et al. 2009; Skelton et al. 2001). Although it has been shown that EGFP is less immunogenic in C57BL/6 mice in comparison with BALB/c mice, Han et al. were able to identify an immunodominant CTL epitope of EGFP that induced specific CTL responses in C75Bl/6 (Han et al. 2008). GFP-expressing DCs transduced with an adenovirus not only promotes dendritic cell maturation, but also elicits GFP-specific cellular immune responses (Re et al. 2004).

Steitz et al. was showed that in mice immunized with recombinant adenoviral-based vectors encoding EGFP (Ad-EGFP), high levels of antibody and cellular immune responses were measured (Steitz et al. 2010). Eixarch et al. showed a relation between EGFP expression level and anti-EGFP immune response. The C57Bl/6J mice receiving high doses of retrovirus expressing EGFP demonstrated more potential anti-EGFP cellular immune responses (IFN-γ) quite contrary to low-dose-receiving mice (Eixarch et al. 2009).

Nevertheless, there are very few studies on the effect of recombinant *Leishmania* parasites on immune response polarization in mice. In 2001, it was reported that EGFP expression by recombinant *Leishmania mexicana* delayed lesion development (Bennett et al. 2001; Millington et al. 2010). In contrast, recombinant *Leishmania amazonensis*^{GFP} kept the infectivity potential (Rocha et al. 2013) and recombinant *L. amazonensis*^{LUC} showed more infectivity but not significant in comparison with wild-type (Reimão et al. 2013). However, neither of these studies evaluated immune response factors correlating with infectivity of these recombinant parasites.

Immunogenicity of a protein is influenced by many factors, including type of host cell used as source of protein expression, type of expression and delivery system of the protein, conformation of expressed protein, type of parasite, antigen dose dependency in cell/tissue, persistent expression of protein, and host factors such as the genetic background (Han et al. 2008). In this study, we used two different sources for EGFP protein, a live parasite cell that constitutively expresses and enhances expression level during infection and a recombinant EGFP in a limited and stable level and without change during infection in mice. During disease progression, parasites proliferate and produce more EGFP that causes enhancement of inflammation. Taken together, this work supported that EGFP when used as a recombinant protein has lower effect on host immune response and parasite burden. In contrast, EGFP protein when produced by a live eukaryote organism like parasite is able to enhance inflammation in infected site.

Furthermore, the biological activity of expressed fusion EGFP-LUC protein by parasite in our study was very similar to EGFP alone, meaning that luciferase is albeit a non-toxic and neutral protein in BALB/c mice. Previous reports have confirmed that luciferase activity has no effect on tumor cells proliferation and viability in vitro and in vivo (Tiffen et al. 2010). Hence, EGFP or EGFP-LUC expressing *Leishmania* is a safe and reliable substitution for wild-types as robust non-invasive biological tools to track and quantification of parasite in live mice. These recombinant parasites simplify in situ follow-ups and eliminating animal scarification wherever needed during the study.

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Compliance with ethical standards All applicable institutional guidelines for the care and use of animals were followed. All mouse experiments including maintenance, feeding, handling program, and euthanasia were approved by the Institutional Animal Care and Research Advisory Committee of Pasteur Institute of Iran, based on the Specific National

Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran. All mice were kept in plastic cages with free access to tap water and standard rodent pellets in an air-conditioned room under a constant 12:12 h light–dark cycle at room temperature. This article does not contain any studies with human participants performed by any of the authors.

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