Molecular Immunology 67 (2015) 501–511

Contents lists available at ScienceDirect

Molecular Immunology

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

Leishmania tarentolae secreting the sand fly salivary antigen PpSP15 confers protection against *Leishmania major* infection in a susceptible BALB/c mice model

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ARTICLE INFO

Article history:
Received 3 April 2015
Received in revised form 18 June 2015
Accepted 2 August 2015
Available online 19 August 2015

Keywords:
L. tarentolae-PpSP15
Sand fly
Saliva
L. major
BALB/c mice CpG
Vaccine

ABSTRACT

Cutaneous leishmaniasis is a zoonotic, vector-borne disease causing a major health problem in several countries. No vaccine is available and there are limitations associated with the current therapeutic regimens. Immune responses to sand fly saliva have been shown to protect against *Leishmania* infection. A cellular immune response to PpSP15, a protein from the sand fly *Phlebotomus papatasi,* was sufficient to control *Leishmania major* infection in mice. This work presents data supporting the vaccine potency of recombinant live non-pathogenic *Leishmania* (*L.*) *tarentolae* secreting PpSP15 in mice and its potential as a new vaccine strategy against *L. major*. We generated a recombinant *L. tarentolae*-PpSP15 strain delivered in the presence of CpG ODN and evaluated its immunogenicity and protective immunity against *L. major* infection in BALB/c mice. In parallel, different vaccination modalities using PpSP15 as the target antigen were compared. Humoral and cellular immune responses were evaluated before and at three and eight weeks after challenge. Footpad swelling and parasite load were assessed at eight and eleven weeks post-challenge. Our results show that vaccination with *L. tarentolae*-PpSP15 in combination with CpG as a prime-boost modality confers strong protection against *L. major* infection that was superior to other vaccination modalities used in this study. This approach represents a novel and promising vaccination strategy against Old World cutaneous leishmaniasis.

1. Introduction

Leishmaniasis, caused by the intracellular parasite *Leishmania*, is a global vector-borne disease with an incidence of two million new cases each year [1]. Cutaneous leishmaniasis (CL) is the most common manifestation of this disease [2] and its highest prevalence rates are reported from Afghanistan, Iran, Syria, Algeria, Brazil and Colombia [3]. The CL lesions are mostly self-healing but can sometimes leave severe scars which lead to social stigmatization [4,5]. The parasites are transmitted by the bite of an infected sand fly. During this process, the sand fly injects saliva into the host together with the parasite. Prior exposure of animals to saliva through bites of uninfected sand flies confers protection against *Leishmania major* infection [1,6]. Additionally, immunization with certain molecules present in sand fly saliva, such as PpSP15, confers the same level of protection against CL [7]. It has been shown that triggering the immune response against sand fly salivary components may indirectly enhance anti-leishmanial immunity [6]. Protective salivary proteins share a similar property and are characterized by the induction of a delayed type hypersensitivity response with a Th1 profile [8,9]. Vaccination against sand fly salivary proteins is an interesting approach in *Leishmania* vaccinology and could provide an additive effect when administered together with an anti-*Leishmania* vaccine. Recently, a study showed the value of combining a sand fly salivary vaccine, PpSP15, with live recombinant *Leishmania tarentolae* stably expressing the cysteine proteinases CPA and CPB. This study was the first to

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http://dx.doi.org/10.1016/j.molimm.2015.08.001
0161-5890/

demonstrate the enhanced protection from leishmaniasis resulting from the inclusion of a vector salivary component with the *Leishmania* vaccine [10]. Another recent study also showed that delivery of the salivary protein LJM11 by *Listeria* is a promising vaccination strategy that induces long-term protection against ulcer formation following a natural challenge with infected sandflies [11].

Non-pathogenic *L. tarentolae* has the ability to target dendritic cells and secondary lymphoid organs and to create Th1 responses and IFN-γ production [12,13]. *L. tarentolae* has never been associated with any form of human leishmaniasis [14], and although it is capable of infecting mammalian cells and transforming into amastigote-like forms, it is not able to persist long enough within macrophages [15]. Previously, we have shown that vaccination with a *L. tarentolae* recombinant strain expressing selected immunogenic components of *Leishmania infantum*, including the A2 and cysteine proteinases A and B genes as a tri-fusion protein, conferred protection against a *L. infantum* infection in both mice and dog models [16] (in press).

Here, we used recombinant live non-pathogenic *L. tarentolae* secreting PpSP15 as a new vaccine strategy against *L. major* infection. We evaluated the efficacy of PpSP15 in the presence of CpG ODN (oligodeoxynucleotides) as an adjuvant for activation of both innate and acquired immune responses. It has been shown that vaccination with *L. major*/CpG changes the immunological features of *Leishmania* infections in C57BL/6 mice by enhancing early inflammatory responses (IL-6, IL-12, TNF-α) and expanding both Th1 and Th17 cells; these two populations seem to be required for vaccine-mediated protection and early control of parasite growth [17]. Our results demonstrate that *L. tarentolae* expressing PpSP15 in its secretory form and CpG ODN can boost the immune response resulting in a high production of IFN-γ and IL-17 both pre- and post-challenge against *L. major* in susceptible BALB/c mice. This is the first report showing the effect and applicability of live non-pathogenic *Leishmania* secreting a sand fly salivary protein in the presence of CpG ODN. Utilization of *L. tarentolae* as a vaccination tool can have several applications in various infectious diseases [18,19].

2. Materials and methods

2.1. Ethics statement

All animal experimental procedures were reviewed and approved by the Institutional animal care and research advisory committee of the Pasteur Institute of Iran (Research deputy dated October 2013). All animal experiments were carried out in strict accordance with the recommendations in the guidelines based on the specific national ethics for biochemical research issued by the research and technology deputy of the ministry of health and medicinal education (MOHM) of Iran (issued in 2005).

2.2. Reagents

All solutions were prepared using MilliQ™ ultrapure water (Milli-Q System, Millipore, Molsheim, France). G418 and Sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich (Sigma, Deisenhofen, Germany). All materials for PCR, enzymatic digestion and agarose gel electrophoresis were acquired from Roche Applied Sciences (Mannheim, Germany). SYBR green for Real Time PCR was purchased from QuantiFast™ (SYBR® Green PCR Kit, QIAGEN, Germany). Plasmid purification was carried out using the Endofree plasmid Giga kit (QIAGEN). Cell culture reagents including M199 medium, HEPES, L-glutamine, adenosine, hemin, gentamicin, DMEM and Schneider were purchased from Sigma (Darmstadt, Germany) and Gibco (Gibco, Life Technologies GmbH, Karlsruhe, Germany), respectively. Fetal Calf Sera (FCS) was obtained from Gibco (Gibco, Life Technologies GmbH, Karlsruhe, Germany). Cytokine kits including IFN-γ, IL-5, IL-6, IL-17 and TNF-α were purchased from DuoSet R&D Kits (Minneapolis, MN, USA). The goat anti-mouse IgG1-HPR and goat anti-mouse IgG2-HPR were provided by Southern Biotech (Toronto, Canada).

2.3. Generation of recombinant L. tarentolae-PpSP15-EGFP

The *L. tarentolae* Tar II (ATCC 30.267) strain was grown at pH 7.2 and 26 °C in M199 medium (Sigma) supplemented with 5% heat-inactivated fetal calf serum (FCS, Gibco), 40 mM HEPES, 0.1 mM adenosine, 5 μg/ml hemin and 50 μg/ml gentamicin. The pLEXSY-neo vector (EGE-233, Jena Bioscience, Germany) was chosen as an integrative vector for transfecting the PpSP15 fused to EGFP into *L. tarentolae* in a secretory form. First, the PpSP15 gene fragment was cloned in-frame into the pEGFP-N3 and then subcloned into *Not*I and *Xba*I sites of the pLEXSY-Neo vector. For transfection, 4×10^7 log-phase parasites were washed and re-suspended in 300 μl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5), mixed with 50 μl H_2O containing 15 μg of linearized pLEXSY-PpSP15-EGFP, stored on ice for 10 min and electroporated (Bio-Rad Gene Pulser Ecell, Germany) at 450 V and 500 μF as described previously [16]. The electroporated promastigotes were incubated for 24 h in a drug-free M199 medium containing 10% FCS at 26 °C and then plated on solid media (composed of M199 media supplemented with 10% FCS, 50 μg/ml G418, and 1% Noble agar (wt/vol). After 15–20 days some colonies were picked and propagated in liquid M199 medium with 10% FBS and the drug concentration was gradually increased to 240 μg/ml. Integration of the expression cassette into the *ssu* locus was confirmed by PCR using genomic DNA samples of the cultured colonies as a template and *ssu* forward primer F3001 (5′-GATCTGGTTGATTCTGCCAGTAG-3′) and reverse primer A1715 (5′TATTCGTTGTCAGATGGCGCAC-3′) hybridizing within the 5′UTR of the target gene. Integration of the expression cassette into the ssu locus yielded a 1-kb fragment not obtained in control reactions where the template is the expression plasmid or genomic DNA from the wild-type host strain.

For further evaluation, the *L. tarentolae*-PpSP15 promastigotes were examined for GFP expression by epi-fluorescence microscopy (Nikon, E 200, ACT-1 software, Digital sight Camera, Japan). Promastigotes were centrifuged at 3000 rpm for 15 min, washed once with and resuspended in PBS and mounted on microscope slides. EGFP-expressing *L. tarentolae* promastigote were used as positive control. Similarly, EGFP expression was evaluated in *L. tarentolae*-PpSP15 by flow cytometry, using a FACS caliber flow cytometer equipped with a 488-nm laser, in parallel with wild type *L. tarentolae* and EGFP-expressing *L. tarentolae* promastigotes (*L. tarentolae*-EGFP) as negative and positive controls, respectively. From each sample, 50,000 events were recorded.

For Western blot analysis, the stationary-phase promastigotes of *L. tarentolae*-PpSP15 were centrifuged at 3000 rpm for 10 min. The supernatant was concentrated (∼1:5) using a Freeze Dryer (Christ L-1, Alpha 2–4, Germany) and then mixed with 6X SDS-PAGE sample buffer (4.5 mM Tris–HCl, pH 6.8, 10%, v/v glycerol, 2%, w/v SDS, 5%, v/v 2-mercaptoethanol, 0.05%, w/v bromophenol blue) on ice, then boiled for 5 min and separated by SDS-PAGE in a 15% (w/v) polyacrylamide gel (SDS gel apparatus; Bio-Rad). The protein bands were transferred onto a nitrocellulose membrane (Schleicher and Schuell Bioscience, Dassel, Germany) using a Bio-Rad wet blotting system and incubated with a blocking solution (TBS with 0.1% Tween 20 and 2.5% BSA) overnight, followed by probing with an anti-GFP antibody (1:5000 v/v; polyclonal antibody to GFP-HRP; Acris antibodies GmbH) for 2 h at 37 °C. Diaminobenzidine tetrahy-

drochloride (DAB, Sigma, Germany) was used as the substrate to detect the immunoreactive protein bands.

2.4. DNA Vaccine construct

As previously reported, the plasmid VR1020-PpSP15 was purified using the Endo Free Plasmid Giga kit according to the manufacturer's instructions (Endofree plasmid Giga kit, QIAGEN, Germany) [10].

2.5. Antigens and Salivary gland preparation

Frozen and thawed (F/T) *L. tarentolae* PpSP15 and *L. major* (MRHO/IR/75/ER) antigens were prepared from stationary phase promastigotes. The parasites were washed with PBS and exposed to liquid nitrogen and water bath (37 °C) 10 times. For the salivary gland homogenate (SGH), salivary glands of *Phlebotomus papatasi* females, Israel strain, were prepared and used as described previously [10], then reconstituted just before use in the listed experiments.

2.6. Vaccination regimens and infectious challenge

Female BALB/c (H-2^d) mice, 6–8 weeks old (weighting 20 ± 5 g), were obtained from the Pasteur Institute of Iran's animal breeding facilities. All animals were housed in plastic cages with appropriate water and standard rodent pellets in an air-conditioned room under a constant 12:12 h light–dark cycle at room temperature and 50–60% relative humidity. BALB/c mice were divided into seven groups ($n = 20$ per group) and they were vaccinated using different prime/boost modalities in the left hind footpad at a three-week interval (Table 1). Group 1 was vaccinated with stationary phase *L. tarentolae*-PpSP15 plus CpG adjuvant (5′-TCCATGACGTTCCTGACGTT-3′) as a prime and boost (Live/Live) vaccine; Group 2 was vaccinated with *L. tarentolae*-PpSP15 plus n-CpG (5′-TCCAGGACTTCTCTCAGGTT-3′ as a non-CpG) as a prime and boost (Live/Live) vaccine; Group 3 was vaccinated with VR1020-SP15 as a prime and boost (DNA/DNA) vaccine; Group 4 was vaccinated with VR1020-SP15 and with *L. tarentolae*-PpSP15 as a prime and boost (DNA/Live) vaccine; Group 5 was vaccinated with the empty vector VR1020 (control group); Group 6 was vaccinated with *L. tarentolae* plus *n*-CpG (control group); Group 7 received only PBS (control group). In a separate experiment, an additional Group 8 ($n = 8$) was vaccinated with *L. tarentolae* plus CpG (control group).

L. major $EGFP^+$ (MRHO/IR/75/ER) parasites were used for the infectious challenge. The parasites were kept in a virulent state by continuous passage in BALB/c mice. A homogenized lymph node (LN) from an infected BALB/c mouse was isolated and the promastigotes were cultured in RPMI-1640 media supplemented with 10% FCS and 100 μg gentamycin/ml at 26 °C. Stationary-phase promastigotes were harvested, washed in PBS and resuspended at a final concentration of 2×10^5 cells/50 μl and injected in the right hind footpad of each mouse three weeks after the booster immunization. For G1–G4 and G7, a total of 0.5 pair of sand fly SGH was mixed with *L. major* $EGFP^+$ parasites and injected.

2.7. Footpad and parasite burden measurements using Real Time PCR

Footpad (FP) swelling was monitored weekly by measuring the increase in thickness and wideness of the inoculated and the uninfected footpad with a metric caliper. Furthermore, the parasite burdens were determined at 8 and 11 weeks after challenge (except G8, which was assessed only at 8 weeks post challenge). At each time point, four mice from each group were sacrificed and the lymph nodes (LNs) were excised, weighed and then homogenized. Genomic DNA was extracted using DNeasy Blood & Tissue kit (QIAGEN). Two sets of primers targeting a 120-bp region of kinetoplastid minicircle DNA of *L. major* named RV1 (5′-CTTTTCTGGTCCCGCGGGTAGG-3′) and RV2 (5′ CCACCTGGCCTATTTTACACCA-3′) were used. The absolute copy number of the target sequence was quantified using the Applied Biosystems 7500 Real Time PCR system. *L. major* genomic DNA was used in ten-fold dilutions corresponding to 2×10^5 parasites and used in Real Time PCR to draw the standard curve. For quantification of parasites in each LN, 30 ng of DNA was subjected to the reaction containing 5 pmol of each forward and reverse primers, 12.5 ul Qiagen QuantiFast SYBR Green Master Mix in a total volume of 25 μl. All reactions were performed in duplicate. Conditions for PCR amplification were as follows: 95 °C for 5 min; 40 cycles consisting of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 40 s.

2.8. Cytokine assays

Four mice from each group were sacrificed before challenge and also at three and eight weeks post challenge. Their spleens were isolated and homogenized with a tissue grinder. Then red blood cells were lysed with ACK lyses buffer (NH_4Cl 0.15 M, $KHCO_3$ 1 mM and Na_2EDTA 0.1 mM) for 5 min. Splenic cells were then washed and resuspended in complete DMEM (containing 5% FCS, 0.1% L-glutamine, 1% HEPES, 0.1% 2ME, and 0.1% gentamicin). Splenic cells were seeded at 3.5×10^6 cells/ml density in the presense of SGH (2 pairs/ml), *L. tarentolae*-PpSP15-EGFP F/T (20 μg/ml), *L. tarentolae*-EGFP F/T (20 μg/ml), and *L. major* F/T (20 μg/ml). A medium-only and Concanavalin A (5 mg/ml) stimulated cells were also used in all experiments as a negative and a positive control, respectively. Plates were incubated at 37 °C in 5% CO_2 humidified atmosphere. Cell culture supernatants were collected after 24 h for IL-6 and TNF-α assays, 72 h later for IL-17 and IL-5 assays, and 96 h later for IFN-γ assay. Cytokine production was measured at different time points in two independent experimental repeats using sandwich ELISA DuoSet R&D kits according to the manufacturer's instructions. The minimum detection limit was 2 pg/ml for mouse IFN-γ, 7 pg/ml for IL-5, 5 pg/ml for TNF-α, 1.8 pg/ml for IL-6, and 5 pg/ml for IL-17. All measurements were done in duplicates.

2.9. Antigen-specific antibody response

Blood samples were collected from the orbital plexus of each mouse before and six weeks after challenge. Serum samples were analyzed using ELISA for specific IgG1, IgG2a and ratio of IgG2a/IgG1 isotype responses against SGH (2 pair/ml), *L. tarentolae*-PpSPp15 F/T (10 μg/ml), *L.tarentolae* F/T (10 μg/ml), and *L. major* F/T (10 μg/ml). In brief, 96-well maxisorb plates (Greiner) were coated with antigens overnight at 4 °C. Plates were blocked with 200 μl of BSA 1% in PBS at 37 °C for 2 h to prevent nonspecific binding. 100 μl of sera was added (1:100) and incubated at 37 °C for 2 h. After three washes, goat anti-mouse IgG1-HPR (1:10,000) or goat anti-mouse IgG2a-HPR (1:10,000) were added to each plate and incubated at 37 °C for 2 h. After three washes, ABTS Peroxidase Substrate System (KPL) was used and plates were incubated at 37 °C for 30 min. The reactions were stopped with SDS 1% and the absorbance was measured at 405 nm using an ELISA reader (TECAN, USA).

2.10. Statistical analysis

Statistical analysis was performed using Graph-Pad Prism 5.0 for Windows (Graphpad Software Inc. 2007, San Diego, California, USA). Depending on the data passing normality tests, either an ANOVA or a Mann–Whitney *U* test was performed and *P* values less

Table 1
Vaccination regimens in different mice groups.

Groups	Prime	Boost	Challenge	Modality
G 1	*L. tarentolae* (PpSP15-EGFP) + CpG 2×10^7	*L. tarentolae* (PpSP15-EGFP) + CpG 2×10^7	*L. major* + SGH Footpad S.C. 2×10^5	rLive/rLive vaccine
G 2	*L. tarentolae* (SP15-EGFP) + nCpG 2×10^7	*L. tarentolae* (SP15-EGFP) + nCpG 2×10^7	*L. major* +SGH Footpad S.C. 2×10^5	rLive/rLive vaccine
G 3	VR1020-SP15 100 μg/mice	VR1020-SP15 100 μg/mice	*L. major* + SGH Footpad S.C. 2×10^5	DNA/DNA Vaccine
G 4	VR1020-SP15 100 μg/mice	*L. tarentolae* (SP15-EGFP) 2×10^7	*L. major* + SGH Footpad S.C. 2×10^5	DNA/rLive Vaccine
G 5	VR1020 100 μg/mice	VR1020 100 μg/mice	*L. major* Footpad S.C. 2×10^5	Control
G 6	*L. tarentolae* + nCpG 2×10^7	*L. tarentolae* + nCpG 2×10^7	*L. major* Footpad S.C. 2×10^5	Live/Live vaccine Control
G 7	PBS	PBS	*L. major* + SGH Footpad S.C. 2×10^5	Control
G 8	*L. tarentolae* + CpG 2×10^7	*L. tarentolae* + CpG 2×10^7	*L. major* Footpad S.C. 2×10^5	Live/Live vaccine Control

than 0.05 were considered significant. The specific test employed is indicated in each figure legend.

3. Results

3.1. Generation of a recombinant L. tarentolae secreting PpSP15-EGFP

Recombinant *L. tarentolae* stably expressing the secretory PpSP15 sand fly salivary antigen with the EGFP gene was generated by introducing the linearized pLEXSY-PpSP15-EGFP vector into the 18S rRNA *ssu* locus of *L. tarentolae* as described in Materials and Methods in Section 2. The presence of PpSP15-EGFP gene in transgenic *L. tarentolae* was confirmed first at the level of DNA (Fig. 1A). Amplification of a 750-bp sequence for EGFP (Fig. 1A, lane 2) and a 397-bp sequence for presence of PpSP15 (Fig. 1A, lane 4) was confirmed. The expression and secretion of PpSP15-EGFP was assessed by Western blot in the concentrated supernatant of stationary phase *L. tarentolae*-PpSP15-EGFP. An immunoreactive band of 42 kDa was detected in *L. tarentolae*-PpSP15-EGFP using an anti-GFP antibody (Fig. 1C, lane 1). *L. tarentolae*-EGFP and wild type *L. tarentolae* were included as positive and negative controls, respectively (Fig. 1C, lanes 2 and 3). Expression of EGFP in *L. tarentolae*-PpSP15-EGFP and *L. tarentolae*-EGFP parasites was confirmed using fluorescence microscopy (Fig. 1D). Flow cytometry analysis also confirmed the GFP expression in *L. tarentolae*-PpSP15-EGFP parasites, although with a lower signal intensity; this is most likely due to the fused PpSP15 peptide and also the secretion of the PpSP15-EGFP upon its biosynthesis (Fig. 1E).

3.2. Evaluation of the immunogenicity of live recombinant L. tarentolae secreting PpSP15 plus CpG compared to three different vaccination regimens in BALB/c mice

It was previously shown that PpSP15 induces specific DTH responses and production of IFN-γ and IL-12 at the bite site in C57BL/6 mice [1,20]. In addition, C57BL/6 mice immunized with PpSP15 became resistant to infection with *L. major* and showed fewer skin lesions accompanied by clearance of infection after eight weeks [20]. Here, by generating a recombinant *L. tarentolae* expressing PpSP15 in a secretory form, we evaluated the protective potential of using live nonpathogenic *Leishmania* as a tool for vaccine development in susceptible BALB/c (H-2^d) mice in the presence of CpG ODN (Table 1). All eight groups of vaccinated and control BALB/c mice were challenged with 2×10^5 late-stationary phase *L. major* GFP$^+$ promastigotes in their right footpads in the presence (G1–G4, G7) or absence (G5, G6 and G8) of SGH. *L. tarentolae*-PpSP15 + CpG (G1) showed the smallest lesions of all groups 7 weeks post-challenge (Fig. 2). There was a significant ($p < 0.05$) difference between G1 and the other groups at 8 and 10 weeks post challenge (Fig. 2, $p < 0.05$). Although the level of footpad swelling is smaller in G2–G4 groups in comparison with the PBS group (G7), the difference was not significant ($p > 0.05$). The footpad of the mice in G1 showed minor tissue damage (no open lesions, data not shown) in comparison to the other groups. As an extra control group (G8), the effect of CpG ODN in combination with *L. tarentolae* was also assessed. As it is shown in Fig. S.1.A, there was a significant difference ($p < 0.05$) between G1 and G8 at 8 weeks post infection. Furthermore, there was no significant difference between G2, G6, G7 and G8 ($p > 0.05$).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2015.08.001

Only evaluating footpad swelling between groups is not an accurate assessment of disease severity and is often more representative of an inflammatory response. For this reason, the degree of protection against infection was determined by assessing the parasite load using real time PCR. The level of parasite propagation in the lymph node (LN) of each mouse within each group was quantified at 8 and 11 weeks post-challenge. Immunization in groups G1–G4 significantly reduced the infection levels in comparison to control-vaccinated groups G5–G7 (Fig. 3). Importantly, *L. tarentolae*-PpSP15 plus CpG (G1) was the most effective group in reducing parasite numbers ($p < 0.05$) in comparison to the other vaccine groups G2–G4 (Fig. 3). Interestingly, this decrease in parasite burden was more pronounced in the case of *L. tarentolae*-SP15 plus CpG-immunized mice (G1) in comparison to mice vaccinated with *L. tarentolae*-PpSP15 (G2) at 8 and 11 weeks post-challenge, confirming that a higher level of protection was observed in group G1 ($p < 0.001$). This may be due to the adjuvant effect of CpG ODN in shaping the immune response towards Th1. Eight weeks after challenge with *L. major* GFP$^+$, G1 and G2 showed a significantly lower parasite load than their control groups (G6 and G7) ($p < 0.0004$, Fig. 3.A). There is no significant difference between G2–G4 at 8 weeks after challenge. Furthermore, groups G2–G4 shows a lower lymph node parasite load than G5–G7, respectively (control groups). At week 11, the parasite load of G1–G4 remained significantly lower ($p < 0.05$) compared to their control groups (G5–G7) (Fig. 3 panel B). For further analysis, the parasite load was compared between G1, G2, G6–G8 at 8 weeks post challenge as shown in Fig. S.1.B. At this time point, G8 has a significantly lower level of parasite load as compared to G6 ($p < 0.05$) but not to G2. However, as it is shown in this figure, there is a significant

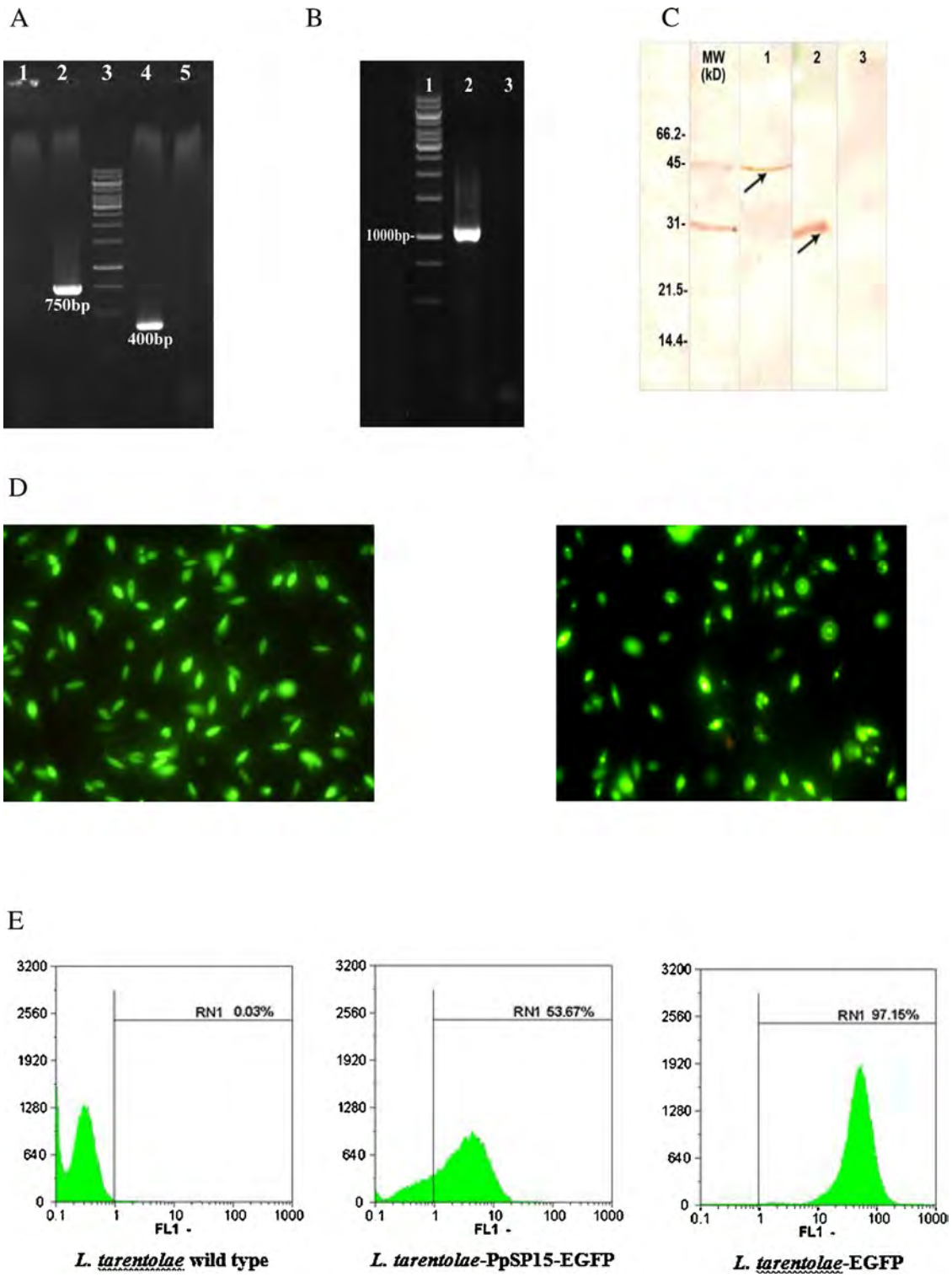

Fig. 1. Generation of recombinant *L. tarentolae*-PpSP15.
(A) Amplification of the 750-bp (lane 2) and 397-bp bands (lane 4), indicating the presence of *EGFP* and *PpSP15* genes in the recombinant *L.tarentolae*-PpSP15-EGFPstrain. Wild type *L. tarentolae* was used as negative control (Lanes 1 and 5). (B) Amplification of the 1 kb band (PpSP15-EGFP) confirms the integration into the genome of *L. tarentolae*-PpSP15-EGFP. Wild type *L. tarentolae* was used as negative control (Lane 3) (C) Western blot analysis for evaluating the production of PpSP15-EGFP in recombinant *L. tarentolae*-PpSP15-EGFP. A 42-kDa band corresponding to the PpSP15-EGFP protein was detected in the concentrated cell supernatant of recombinant parasites using anti-GFP antibody (lane 1), a 27-kDa band in lane 2 indicates the EGFP protein in *L. tarentolae*-EGFP strain, and lane 3 is the wild type *L. tarentolae* as negative control. (D) Detection of EGFP fluorescence in recombinant *L.tarentolae*-PpSP15-EGFP promastigotes (right) and *L. tarentolae*-EGFP promastigotes (left) using fluorescence microscopy. (E) Flow cytometry analysis of the promastigote form of recombinant *L. tarentolae*-PpSP15-EGFP (middle), *L. tarentolae*-EGFP (right) and *L. tarentolae* wild type (left) using FITC channel.

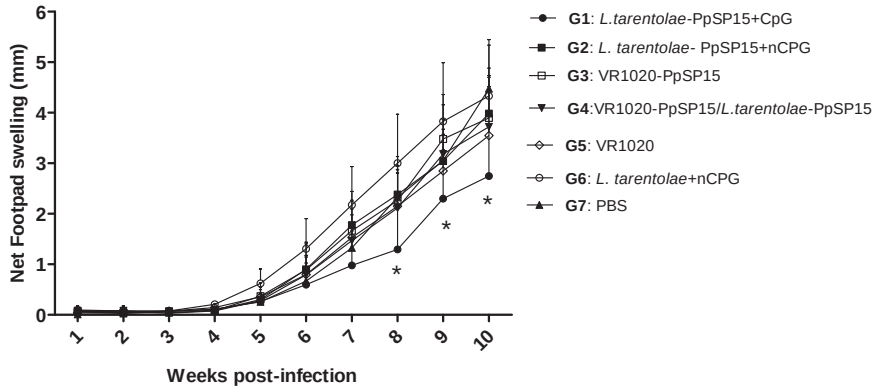

Fig. 2. The course of infection with *L. major* GFP⁺ in vaccinated and control groups of BALB/c mice.
Assessment of footpad swelling in vaccinated and control groups. Schematic presentation of the mean + SD footpad swelling measurements via caliper-based method in mm. BALB/c mice were immunized subcutaneously with *L. tarentolae*-PpSP15+ CpG (G1, rLive/rLive); *L. tarentolae* PpSP15 (G2, rLive/rLive); DNA PpSP15 (G3, DNA-PpSP15/DNA-PpSP15); primed with DNA PpSP15 and boosted with *L. tarentolae* PpSP15 (G4, DNA/rLive); DNA (G5, DNA/DNA control); *L. tarentolae* (G6, control); mice injected with PBS (G7, control). Three weeks after the booster immunization all animals were challenged with stationary phase *L. major* GFP⁺ (2×10^5/mice) plus SGH (0.5 pair) in the right footpad except for G5 and G6, which received only *L. major* GFP⁺. From the 7th week, a statistically significant difference ($p < 0.05$) was observed between G1 and the other groups. The differences are also shown at 10 weeks post challenge (the asterisk indicates the significant difference between values at the indicated time points) as determined by One-way ANOVA and multiple comparison Tukey post-test. This experiment was repeated twice and values are pooled in the figure (See in Fig. S.1.A)

additive effect when mice were vaccinated with a combination of *L. trentolae*-PpSp15 and CpG (G1) compared with *L. trentolae*-PpSP15 (G2) and *L. tarentolae* + CpG (G8). Altoghter, G1 had significantly the lowest parasite load at both 8 and 11 weeks post infection ($p < 0.001$).

It is worth mentioning that the quantity of parasites in the lymph nodes of G5 (DNA only) and G6 (*L. tarentolae* + nCpG) are significantly different from G7 (PBS only) at both 8 and 11 weeks post challenge (Fig. 3 panel A and B, $p < 0.05$). These differences may be due to CpG dinucleotide motifs within the plasmid DNA (in the case of G5) or *L. tarentolae,* which has a moderate ability to control parasite propagation.

3.3. *Immunization with live recombinant* L. tarentolae-*PpSP15 plus CpG induces IFN-γ and IL-17 production at different time points before and after infectious challenge*

The above experiments demonstrate that immunization with recombinant *L. tarentolae*-PpSP15 plus CpG confers a significant protection against *L. major* infection in BALB/c mice. In the next step we examined the production of different effector cytokines including IFN-γ, IL-5, TNF-α, IL-6 and IL-17, which can orchestrate the protection against *L. major* infection at different times pre- and post- challenge, in the supernatant of splenocytes isolated from immunized and control mice in response to frozen-thawed

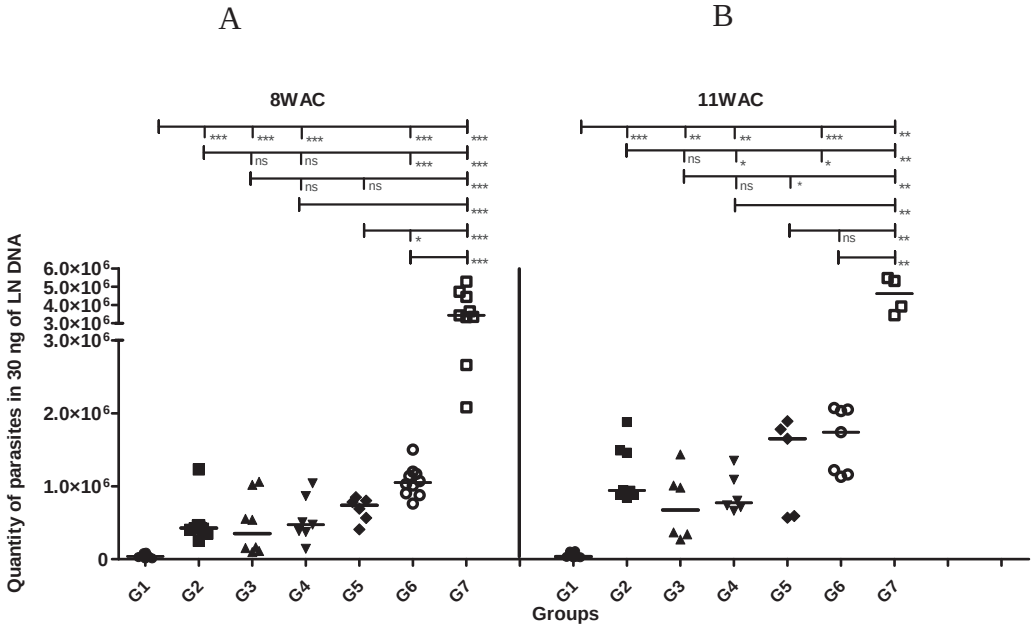

Fig. 3. Quantification of parasite DNA in the lymph nodes of vaccinated and control groups at 8 and 11 weeks post-infection.
Mice were sacrificed from each group at 8 (panel A) and 11 (panel B) weeks after challenge. Genomic DNA was prepared from each lymph node and the amount of *Leishmania* DNA was quantified by Real Time PCR as described in M&M. All reactions were performed in duplicate and the experiment was repeated twice and values are pooled in the figure. The asterisk indicates the significant difference between values at the indicated time points as determined by a Mann–Whitney *U* test ($p < 0.01$ denoted as **, $p < 0.0001$ denoted as ***). See also Fig. S.1.B.

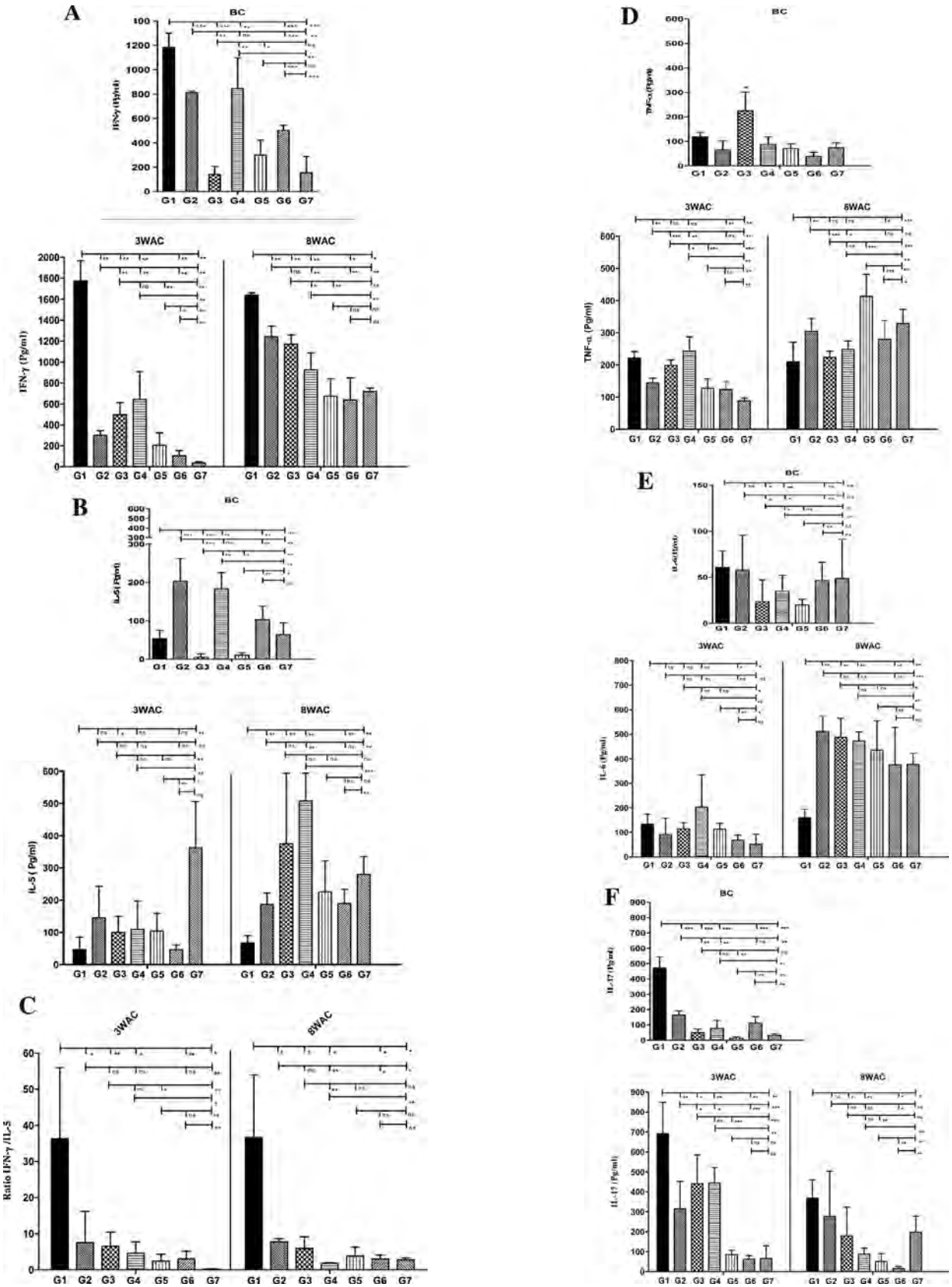

Fig. 4. Cellular immune responses of vaccinated and control BALB/c mice pre- and post-challenge.

Cytokine production from the splenocytes of 4 mice in different groups before (BC), 3 weeks (3WAC) and 8 weeks (8WAC) after last immunization was assayed by ELISA. Each bar represents the Mean ± SD in pg/ml (for all cytokines). IFN-γ (panel A), IL-5 (panel B), IFNγ/IL-5 ratio (panel C), TNF-α (panel D), IL-6 (panel E) and IL-17 (panel F) production after stimulation with *L. tarentolae*-PpSP15 F/T and *L. major* F/T antigens.

Fig. 4. (*Continued*)

In each panel, the upper graphs show the level of specific cytokine production to *L. tarentolae*-PpSP15 F/T at before challenge (BC) and the lower graph illustrate *L. major* F/T antigens as stimulators. The number of independent repeats was two and all tests were done in duplicate. The values are pooled in the figure. Mann–Whitney *U* test was used for statistical analysis ($p < 0.05$ denoted as *, $p < 0.001$ denoted as **, $p < 0.0001$ denoted as *** and non-significant denoted as n.s.). See also Fig S 2A–C

(F/T) antigens of *L. tarentolae*-PpSP15 (before challenge, BC) and *L. major* (at 3 and 8 weeks after challenge, 3WAC and 8WAC). IFN-γ levels remained high in pre- and 3 weeks post- challenge after F/T antigens stimulation with both strains in G1 compared to the other vaccinated groups (G2–G4) and the control groups G6 and G7 ($p < 0.05$, Fig. 4A). Pre challenge, there are significant differences in the levels of IFN-γ production against *L. tarentolae*-PpSP15 F/T in G2 and G4 compared to G5–G7 (control groups, Fig. 4A). At 8 weeks post challenge, all vaccinated groups (G1–G4) produced significantly higher IFN-γ levels against *L. major* F/T in comparison to all control groups ($p < 0.05$, Fig. 4A). It is worth mentioning that the level of IFN-γ is increased in G8, another control group, but the difference is not significant when compared with G6 and G7 (Fig. S2A). We further investigated whether splenocytes from vaccinated groups secreted the Th2-associated cytokine IL-5. As shown in Fig. 4.B, before challenge, G1 compared to G2, G4 and G6 has a significantly lower level of IL-5 production in response to F/T antigens of *L. tarentolae* PpSP15 ($p < 0.05$). In addition, there is no IL-5 production in groups G3 and G5 in response to SGH as recall antigen. Furthermore, at 3 and 8 weeks post challenge, G1 has the lowest level of IL-5 production upon antigen stimulation with *L. major* F/T antigens ($p < 0.05$). At 3 weeks post challenge there is no significant differences in IL-5 production between G2–G5 ($p > 0.05$). At 8 weeks post challenge, there is a sharp increase of IL-5 in G3 and G4 in comparison to other vaccinated groups ($p < 0.05$, Fig. 4B). As shown in Figure S2B, there are no significant differences between G2, G6 and G8 at 8weeks post-challenge.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2015.08.001

The post-challenge IFN-γ/ IL-5 ratio in response to stimulation with *L. major* indicates a bias towards a Th1 response and improved vaccine efficacy. This ratio was significantly higher in G1 compared to the other vaccinated groups, including G2–G4 and the control groups G5–G7 at 3 and 8 weeks after challenge in response to stimulation with *L. major* F/T antigens ($p < 0.05$, Fig. 4C). We also observed a high ratio in G2 compared to G6 and G7 but not in G3 compared to G5 at the same time and against the same antigen. As it is shown in Fig. S.2.C, this ratio is not significantly different between G2 and G8 at 8 weeks post challenge.

Before challenge, the highest level of TNF-α production belonged to G3 and there was no significant differences between other groups. In addition, after stimulating with *L. major*, the level of TNF-α in groups G1, G3 and G4 was the same at 3 and 8 weeks post challenge. In contrast, groups G2, G5–G7 had similar levels of TNF-α at 3 weeks post challenge and this level is significantly increased at 8 weeks post challenge ($p < 0.05$, Fig. 4D).

We also measured the secretion of IL-6 as a major pro-inflammatory cytokine pre- and post-challenge in all vaccinated and control groups. Before infection, the lowest level of IL-6 was observed in G3–G5. In addition, G1 and G2 had similar levels of IL-6 production as with G6 and G7, and there were no significant differences between these groups. At 3 weeks post challenge, there was no significant difference between G1, G2–G5 upon stimulation with *L. major* F/T. In contrast, at 8 weeks after challenge, the level of IL-6 production was almost similar between G2–G4 and significantly higher than G1 ($p < 0.05$, Fig. 4E).

It has been shown that activation of immune cells through CpG ODN as an adjuvant, in the presence of live *L. major*, results in the production of IL-17 from Th17 cells, which enhances protective cellular immunity against *L. major* infection [17]. We also investigated the IL-17 levels before and after challenge in all groups. As demonstrated, the level of IL-17 in G1 was significantly higher before challenge and 3 weeks post-challenge. Furthermore, other vaccinated groups including G2–G4 also produced significant levels of IL-17 compared to the control groups (G5–G7, Fig. 4F) at 3 weeks post-challenge. At 8 weeks after challenge, similar levels of IL-17 were observed between G1 and G2, and they were significantly higher than the rest of groups (G3–G6 and G7).

3.4. *Immunization with live recombinant* L. tarentolae-*PpSP15 plus CpG induces higher levels of antibody against* L. major *infection*

The levels of IgG1 and IgG2a antibodies against different antigens was determined in sera by ELISA before challenge and 6 weeks after challenge in all groups. Antibodies against *L. tarentolae*-PpSP15 F/T antigens in vaccinated group G1 showed significantly higher levels of IgG2a in comparison to other vaccinated groups G2, G4 and control groups G6 and G7 at BC (Fig. 5A). At this time point, considerable level of IgG1 production was also observed in G1, G2, G4 and G6 (Fig. 5B). Before challenge, there is low level of IgG2 and IgG1 production against SGH in G3 and G5 (Fig. 5A and B). At 6 weeks after challenge, in response to *L. major* F/T, groups G1, G2, G4–G6 had a similar level of IgG2a production and the lowest level belonged to G3 and G7 ($p < 0.05$, Fig. 5A). Furthermore, G1 showed significantly the lowest level of IgG1 compared to all other groups at this time point. There are no significant differences in IgG1 production between groups G2–G7 (Fig. 5B). Similarly, the ratio of IgG2a/IgG1 is shown in Fig. 5.C. In both time points, G1 has significantly the highest level of IgG2a to IgG1 compared with all other groups ($p < 0.05$).

4. Discussion

In this study, we used a live recombinant *L. tarentolae* as a vaccine delivery vector for PpSP15, the highly immunogenic and protective component of *P. papatasi* sand fly saliva. Beside the salivary component, by this strategy we take advantage of a plethora of parasite antigens, which can stimulate the immune system for better protective efficacy to combat against *L. major* infection. Additionally, the synthetic CpG ODN was used as a TLR9 agonist. CpG can bridge the innate and adaptive immune responses when used as a vaccine adjuvant [23]. There are several studies which clearly show the adjuvant activity of synthetic CpG ODN upon co-administration with vaccine antigens. It has already been shown that combining live pathogens with CpG ODN as a vaccine may modify the natural immune response to infection in an alternative manner to killed or subunit vaccines. Previous works with murine models indicates that activation of the innate immune system using CpG ODN reduced the severity and time course of infection and facilitated the clearance of virus [21,22] bacteria [23,24] and parasites [25]. In this study we showed for the first time that vaccination with *L. tarentolae*-PpSP15 plus CpG enhanced the early production of IFN-γ, TNF-α and IL-17, which in turn led to protection against *L. major*. It appears that CpG is an important component for the generation of Th17 cells and production of IL-17. Furthermore, generation of Th17 cells appears to be specifically associated with live parasite vaccination and cannot be raised in vaccines containing recombinant proteins or dead parasites [17]. It is worthy to mention that the role of Th17 cells in leishmaniasis is not clear, since there are some reports showing its disease-promoting effect [26] as well as its protective outcome both in mice and human [17,27,28]. In humans, studies have demonstrated a therapeutic role of Th17 cells where IL-17 has been correlated to protection against visceral leishmaniasis (ref). It appears that by inducing Th17 cells, other proinflammatory and inflammatory cytokines can facilitate the entry of other leukocytes to the site of vaccination and change the environment toward the Th1 response at early phase. In this study, four different vaccine modalities were administered and

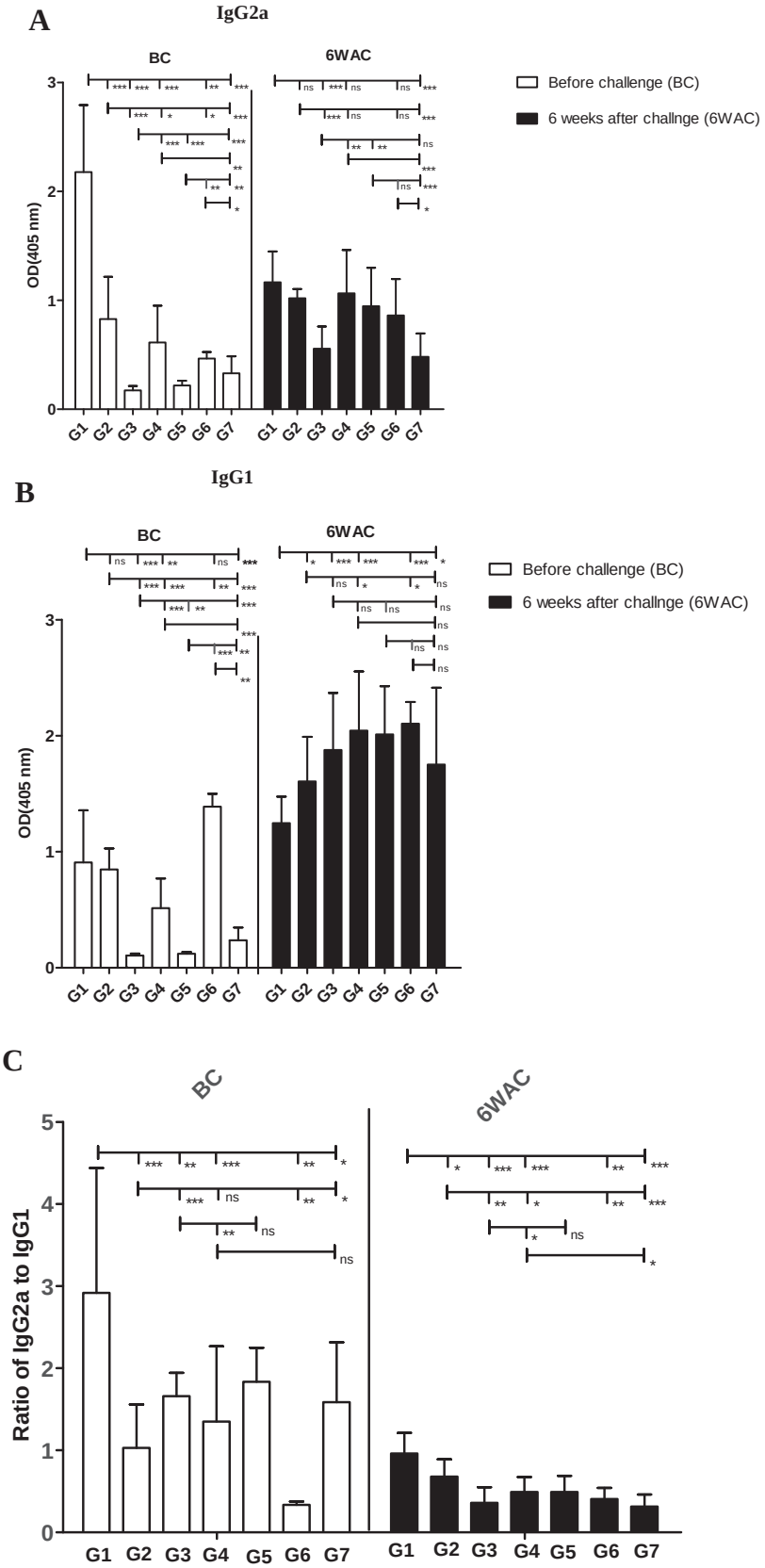

Fig. 5. Analysis of the specific humoral responses before and six weeks after challenge.
Sera were obtained from individual mice from each group ($n = 12–20$). Before-challenge sera were tested for anti *L. tarentolae*-PpSP15-EGFP F/T in G1, G2, G4, G6 and G7.SGH was used as an antigen in the case of G3 and G5. At 6WAC, *L. major* F/T was used for all groups. Panel A shows specific IgG2a, panel B shows IgG1 and panel C shows the ratio of IgG2a/IgG1. Results are representative of two independent experiments, each performed in duplicate. Mann–Whitney *U* test was used for statistical analysis ($p < 0.05$ denoted as *, $p < 0.001$ denoted as **, $p < 0.0001$ denoted as *** and non-significant denoted as n.s.).

compared. Among all, immunization of mice with recombinant *L. tarentolae*-PpSP15 (G1) in the presence of CpG elicited a significant protective immunity against *L. major* infection. All mice vaccinated with this modality showed smaller lesions and less tissue damage at 7 weeks after challenge in comparison to other vaccinated and control groups. As it is demonstrated, the *L. tarentoale* accompanied by CpG (G8) can also control the parasite propagation (Fig. S.1.A and B, Supplementary information). Therefore, CpG ODN can act as an important adjuvant for switching the immune response toward protection. This effect could be enhanced when it is combined with another antigen such as PpSP15. By comparing different strategies in this study, we confirmed a significantly lower parasite load in G1, G2, G3 and G4 compared to control groups at 11 weeks after challenge. Among all vaccinated groups, G1 has the lowest level of parasite quantity at both 8 and 11 weeks after challenge. Group G2, immunized with *L. tarentolae*-PpSP15 in the absence of CpG, did control the parasite propagation in the lymph nodes in comparison to G6 (*L. tarentolae* without CpG) at 8 and 11 weeks post challenge but to a lower extent in comparison to G1. In addition, the parasite load in groups G2 and G3 and G4 was similar and no significant differences were observed between different modalities. Furthermore, in group G1 we observed significantly higher levels of IFN-γ, TNF-α, IL-6 and IL-17 in the "silent" phase (week 3), and decreased level of IL-6 and TNF- α at 8 weeks after infectious challenge. The highest level of IFN-γ and lowest level of IL-5 observed in group G1 pre- and post- challenge induced a Th1 response. The IFN-γ/IL-5 ratio in splenocytes stimulated with *L. major* F/T from mice immunized with recombinant *L. tarentolae*-PpSP15 plus CpG (G1) was 4 to 12-fold higher at 3 and 8 weeks post challenge, respectively, than all other vaccinated and control groups. Furthermore, the highest level of IgG2a as well as Ig2Ga/IgG1 belonged to G1. Therefore, the combination of recombinant *L. tarentolae*-PpSP15 plus CpG (G1) clearly induces a stronger Th1 response against leishmanial infection. This data actually shows the effect of CpG in combination with a live vaccine, which can efficiently control parasite propagation in the lymph nodes of susceptible BALB/c mice.

As reported by [29], administration of CpG ODN to *L. major*-infected macaques reduced the severity of the lesions, but the effect highly depends on the time of administration of CpG ODN. Macaques that received CpG ODN prior to infection showed skin lesion acceleration. In contrast, those that received CpG ODN at the time of the challenge had delayed lesion development. Although we have not checked any other candidate antigens in this study, it could be feasible to combine salivary components from different sand fly vectors. This idea could be a wise approach especially for application in highly endemic areas where different vector sand flies are present.

Despite substantial efforts to control leishmaniasis, no vaccine is available to date and the disease is still a threat to human health [30]. Immunization with live attenuated strains in murine models has been reported to induce a protective immune response in the host [31]. Studies in mice have suggested that CpG ODN could be used in conjunction with leishmanization to improve the immune response and limit lesion development without sterilizing immunity. Instead of using pathogenic species, in this study we used a recombinant nonpathogenic *L. tarentolae* expressing PpSP15 with simultaneous administration of CpG ODN. This strategy resulted in a profound suppressive effect on parasite propagation and an adequate control of the disease.

Taken together, these finding suggest that CpG ODN with live recombinant *L. tarentolae* could offer an efficient approach for controlling leishmaniasis in a mouse model of infection. In future studies, it is necessary to consider the long term memory elicited by this strategy and whether an infected sand fly challenge can elicite a similar outcome.

5. Conclusions

This study demonstrated that immunization with recombinant *L. tarentolae*-PpSP15 plus CpG protects BALB/c mice against cutaneous leishmaniasis (*L. major*) when used as a prime-boost modality. Apart from the specific immunogenicity of PpSP15, CpG has an important role in inducing Th1 and Th17 cells in combination with a non-pathogenic live *L. tarentolae*. The major issue and limiting factor with a live vaccine is its safety, i.e., confirming that there can be no chance of it becoming virulent or reactivated in immunosuppressed individuals. *L. tarentolae* is non-pathogenic to humans and could be used even in immunocompromised individuals. Instead of using live pathogenic *Leishmania*, in a so-called Leishmanization method, we could replace it with a non-pathogenic recombinant *L. tarentolae*. This enables the combination of three important elements: the whole spectrum of *Leishmania* antigens, PpSP15 and CpG.

Acknowledgements

Special thanks to Amir Mizbani for English editing. The authors wish to thank A. Javadi (Pasteur Institute of Iran, Department of Immunology) and Mr. Sh. Alizadeh for their technical assistance. This work was supported by a grant from Pasteur Institute of Iran (ID 719).

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