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Phlebotomus papatasi exposure cross-protects mice against *Leishmania major* co-inoculated with *Phlebotomus duboscqi* salivary gland homogenate

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ABSTRACT

Leishmania parasites are inoculated into host skin together with sand fly saliva and multiple exposures to uninfected sand fly bites protect mice against *Leishmania* infection. However, sand fly vectors differ in composition of the saliva and therefore the protection elicited by their salivary proteins was shown to be species-specific. On the other hand, the optimal vaccine based on sand fly salivary proteins should be based on conserved salivary proteins conferring cross-reactivity. In the present study we therefore focused on cross-protective properties of saliva from *Phlebotomus papatasi* and *Phlebotomus duboscqi*, the two natural vectors of *Leishmania major*. Two groups of mice exposed to bites of *P. papatasi* and two control, non-immunized groups were infected with *L. major* promastigotes along with either *P. papatasi* or *P. duboscqi* salivary gland homogenate. All mice were followed for the development of *Leishmania* lesions, parasite burdens, specific antibodies, and for production of NO, urea, or cytokines by peritoneal macrophages. Protection against *Leishmania* infection was observed not only in exposed mice challenged with homologous saliva but also in the group challenged with *P. duboscqi* saliva. Comparing both exposed groups, no significant differences were observed in parasite load, macrophage activity, or in the levels of anti-*L. major* and anti-*P. papatasi*/*P. duboscqi* antibodies. This is the first study showing cross-protection caused by salivary antigens of two *Phlebotomus* species. The cross-protective effect suggests that the anti-*Leishmania* vaccine based on *P. papatasi* salivary proteins might be applicable also in areas where *L. major* is transmitted by *P. duboscqi*.

1. Introduction

The causative agents of leishmaniasis, protozoans from the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), are transmitted to the vertebrate hosts by the bites of female sand flies (Diptera: Phlebotominae). During the bloodfeeding process, sand fly saliva is obligatorily inoculated into the feeding lesion and if the females are infected, it plays a crucial role in the establishment of *Leishmania* infection in vertebrate host (reviewed in Gomes and Oliveira (2012)). In naive mice, it aggravates the development of the disease by suppression of the immune system (Titus and Ribeiro, 1988), conversely, in mice repeatedly exposed to sand fly saliva, it elicits strong Th1-derived immune milieu protective against leishmaniasis (Belkaid et al., 1998; Kamhawi et al., 2000; Oliveira et al., 2008; Rohoušová et al., 2011; Thiakaki et al., 2005; Xu et al., 2011).

Repeated exposures to sand fly bites induce saliva-specific immune response, both humoral and cell-mediated. While anti-saliva antibody response correlates with the intensity of exposure (Hostomska et al., 2008; Vlkova et al., 2011; Vlkova et al., 2012) and can be used as a reliable epidemiological tool (e.g. Clements et al., 2010; Gidwani et al., 2011), specific cellular immunity, particularly the delayed type hypersensitive reaction (DTH), is responsible for protection against leishmaniasis (Gomes et al., 2008; Kamhawi et al., 2000; Valenzuela et al., 2001). In the murine model, this immune response is characterized by powerful recruitment of lymphocytes and macrophages (MΦ) to the site of bite and it correlates with elevated production of IFN-γ and IL-12 (Kamhawi et al., 2000; Valenzuela et al., 2001).

Macrophages are the key cells responsible for healing or for progress of *Leishmania* infection (reviewed in Horta et al. (2012)). Their biological properties and function depend on the type of

Abbreviations: DUB, *Phlebotomus duboscqi*; EXP, exposed; *L.*, *Leishmania*; *Lu.*, *Lutzomyia*; NB, nitrocellulose membrane; nEXP, non-exposed; *P.*, *Phlebotomus*; p.i., post infection; PAP, *Phlebotomus papatasi*; PMΦ, peritoneal macrophages; SGH, salivary gland homogenate; SI, stimulation index.

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activation which determines the processing of L-arginine, a common substrate for two different enzymatic pathways. In the Th2 milieu (alternative way of MΦ activation), the metabolism of L-arginine is diverted towards the production of L-ornithine, the precursor of polyamines, which are utilized by *Leishmania* parasites for their intracellular growth (Kropf et al., 2005). On the contrary, in the Th1 environment, macrophages are activated by IFN-γ (classical way of MΦ activation) and L-arginine is transformed into L-citrulline and nitric oxide (NO); the latter, together with other toxic intermediates, successfully eliminates *Leishmania* parasites (Murray and Cartelli, 1983). Therefore individual salivary proteins inducing strong Th1 DTH reaction were suggested as promising candidates for anti-*Leishmania* vaccine and thus are the subject of intensive scientific research (da Silva et al., 2011; Gomes et al., 2008; Morris et al., 2001; Oliveira et al., 2006; Tavares et al., 2011; Valenzuela et al., 2001; Xu et al., 2011).

However, several facts complicate the utilization of salivary compounds in the control of *Leishmania* infection; such vaccines might have limited use due to different saliva composition between various sand fly species (Ribeiro et al., 2010; Rohoušová et al., 2012; Volf and Rohousová, 2001; Volf et al., 2000). Indeed, the immunity elicited by sand fly salivary proteins (Drahota et al., 2009; Rohousova et al., 2005; Volf and Rohousová, 2001) as well as the protection (Thiakaki et al., 2005) was shown to be species-specific. On the other hand, the vaccine could be cross-protective between phylogenetically related vector species with more conserved salivary proteins (Ribeiro et al., 2010; Rohoušová et al., 2012; Volf et al., 2000) and as such could be applicable in more endemic foci.

In this study, we focused on *Phlebotomus papatasi* and *Phlebotomus duboscqi*, two sand fly species which serve as the natural vectors of *Leishmania major* (Killick-Kendrick, 1999; Ready, 2013). Using a BALB/c model, *P. papatasi* pre-exposed mice were challenged with *L. major* along with *P. papatasi* or *P. duboscqi* salivary gland homogenate and examined for the lesion size development, parasite load, macrophage activity as well as for antibody response. As far as we are aware, this is the first study describing the cross-protection between *Phlebotomus* sand fly species.

2. Methods

2.1. Ethical statement

All animals used in this study were maintained and handled strictly in accordance with institutional guidelines and legislation for the care and use of animals for research purpose Czech Act No. 359/2012 coll on Protection Animals against Cruelty in present statutes at large that complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24,773/2008-10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic. All efforts were made to minimize the number and the suffering of experimental animals during the study.

2.2. Sand flies and salivary gland dissection

Colonies of *P. papatasi* (originating from Turkey, colonized in 2005) and *P. duboscqi* (originating from Senegal, 1994) were reared under standard conditions as described in Volf and Volfova (2011). Salivary glands were dissected from 3 to 5 day-old female sand flies, placed into Tris-buffered saline (TBS) (20 mM Tris, 150 mM NaCl, pH 7.8) and stored at −20 °C until needed. Before use, salivary glands were disrupted by three cycles of freezing-thawing to prepare salivary gland homogenate (SGH). In SGH of both species the concentration of salivary proteins was measured using Qubit™ Fluorometer (Invitrogen). The protein concentrations were as follows: 0.296 μg/gland in *P. papatasi* and 0.347 μg/gland in *P. duboscqi*.

2.3. Leishmania parasites

L. major (strain MHOM/IL/67/LRC-L137 Jericho II) promastigotes were maintained at 23 °C in RPMI 1640 medium with HEPES (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 0.1% amikin (Bristol-Myers Squibb), 1% BME vitamins (Sigma), and 0.5% sterile urine. Before use, parasites were washed in 0.9% saline solution by centrifugation for 8 min at 2900 × *g*, resuspended in saline and the concentration of parasites was determined using Burker chamber.

2.4. Mice exposure and infection

Twenty-eight BALB/c mice in total (8-week-old females) from Anlab (Czech Republic) were used within three independent experiments. This mouse strain is highly susceptible to *Leishmania* infection and widely used to study the protective effect against leishmaniasis (e.g. Belkaid et al., 1998; Kamhawi et al., 2000; Rohoušová et al., 2011; Thiakaki et al., 2005). Mice were divided into four groups of seven mice each. Two groups of mice were intraperitoneally anesthetized by a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg) and were exposed individually to 35 females of *P. papatasi*, twice in 1 week interval (EXP groups) (Fig. 1). The snout part and eyes were covered with damp cotton wool to avoid drying and sand fly feeding on these parts. During each exposure, an average of 29 females fed on each mouse (standard error = ±0.7). The other two groups remained without any exposure to sand flies (nEXP groups). Blood samples were collected from the tail vein of all mice before exposure and 3 days after the last exposure. The obtained sera were kept at −20 °C until needed.

One week after the last exposure, mice of all four groups were intradermally challenged in the right ear with 10^4 stationary phase promastigotes of *L. major* in the presence of either (1) the equivalent of 0.5 salivary gland of *P. papatasi* (groups: EXP + P.pap and

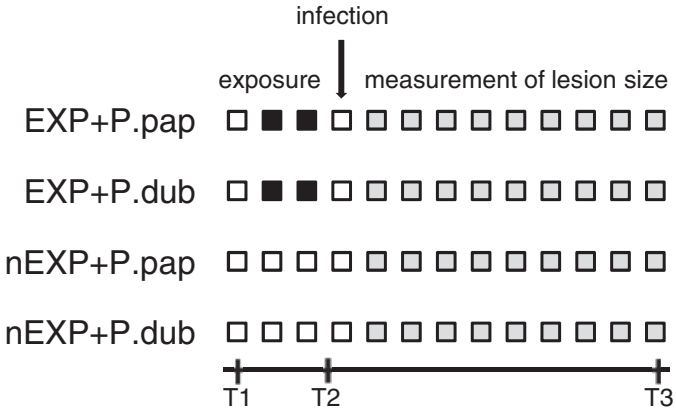

Fig. 1. Timeline of the experiment. Mice were divided into four groups. Two groups of mice were exposed to *Phlebotomus papatasi* bites (black squares, EXP), two groups remained unexposed (white squares, nEXP). One week after the second exposure, all mice were intradermally challenged with 10^4 *L. major* promastigotes in the presence of either (1) the equivalent of 0.5 salivary gland of *P. papatasi* (+P.pap) or (2) the equivalent of 0.5 salivary gland of *P. duboscqi* (+P.dub). The development of *Leishmania* lesion size was measured weekly for 9 consecutive weeks (gray squares). Blood samples were collected from the tail vein of all mice before exposure (T1), 3 days after the last exposure (T2) and 9 weeks post infection (T3). Each square represents 1 week.

nEXP + P.pap) or (2) the equivalent of 0.5 salivary gland of *P. duboscqi* (groups: EXP + P.dub and nEXP + P.dub). The infectious dose by needle inoculation was chosen to approximate the number of parasites naturally transmitted by sand fly bites (e.g. Kimblin et al., 2008; Maia et al., 2011; Warburg and Schlein, 1986). Lesion size was measured weekly for 9 consecutive weeks using a digital caliper. Mice were sacrificed 9 weeks after infection and sampled for blood, both ears, draining lymph nodes, and peritoneal macrophages (PMΦ).

2.5. Detection and quantification of Leishmania parasites in mice

Parasite numbers were determined by quantitative PCR as previously described (Myskova et al., 2008) with some modifications. Briefly, total DNA was isolated from homogenized samples using DNA tissue isolation kit (High Pure PCR Template Preparation Kit; Roche) according to the manufacturer's instruction. *Leishmania* parasites were quantified using SYBR Green detection method (iQSYBR Green Supermix, Bio-Rad). Kinetoplast DNA was targeted using primers described by Mary et al. (2004). One microliter of eluted DNA was used for reaction which was performed in duplicate. Thermal cycling scheme was 3 min at 95 °C followed by 45 repetitive cycles: 10 s at 95 °C, 10 s at 56 °C, and 10 s at 72 °C (Myskova et al., 2008). Calibration was performed in the range of 10^1–10^6 *Leishmania* promastigotes blended with homogenized liver from non-infected mouse. The liver without *Leishmania* parasites served as a negative control.

2.6. Macrophage activation studies

PMΦ were obtained by peritoneal lavage of BALB/c mice using 10 ml of RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μg/ml gentamicin, 100 U/ml penicilin (in the text referred to as complete medium). After centrifugation (8 min, 4 °C, 260 × *g*), the cell pellet was resuspended in complete medium, mixed with Trypan Blue and counted by the Countess™ Automated Cell Counter (Invitrogen) according to the manufacturer's guidelines and following criteria: cell size = 8–20, roundness = 80, sensitivity = 5. Murine PMΦ were cultured in 96-well plates at a concentration of 2×10^5 living cells/ml at 37 °C, 5% CO_2. After 2 h, non-adherent cells were removed by washing with warm complete medium. Cells were then incubated alone in complete RPMI 1640, with a combination of IFN-γ (25 U/ml, AbD SEROTEC) and LPS (0.5 μg/ml, Sigma), or with *L. major* promastigotes (2×10^5 cells per well). After 72 h of incubation, the supernatant and cell lysate were used for nitrite/cytokine and urea analysis, respectively.

2.6.1. Nitrite analysis to measure NO production

The accumulation of NO_2^- produced by cultured macrophages over a 72 h period was determined in a microplate assay using Griess reagent. A total of 100 μl of culture supernatant was mixed with 50 μl of 60 mM sulfanilamide in 2.5% phosphoric acid and incubated at room temperature in the dark for 5 min. Thereafter, 50 μl of 12 mM *N*-1-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid was added and incubated in the dark for additional 5 min. The absorbance was read at 550 nm using the microplate reader (Tecan Infinite M200). The NO_2^- concentration was determined using sodium nitrite as a standard in the range of 12.5–100 μM.

2.6.2. Urea analysis to measure arginase activity

Arginase activity was analyzed in macrophages lysate by measuring the conversion of L-arginine to urea as previously described (Kropf et al., 2007) with slight modifications. Cells were lysed with a solution of Tris–HCl in combination with protease inhibitors (Complete Mini Roche, one tablet per 10 ml of solution), Triton X and $MnCl_2$. The enzyme was then activated by heating and arginine hydrolysis was carried out by incubation of the activated enzyme with arginine (Sigma–Aldrich) at 37 °C with 5% CO_2 for 120 min. The reaction was stopped with 400 μl of solution containing H_2SO_4, H_3PO_4 and distilled water. Color reaction was developed in the presence of 20 μl 550 mM α-isonitrosopropiophenone (dissolved in 100% ethanol) after incubation at 100 °C for 45 min. The absorbance was read at 540 nm. Urea concentration was determined using urea as a standard in the range of 0.004–0.6 mg/ml.

2.6.3. Detection of cytokine production

The production of IL-10 and TNF-α was determined by sandwich enzyme-linked immunosorbent assays (ELISA) (Fig. 1). Microtiter plate wells were coated with primary antibody (Purified Anti-mouse IL-10 or TNF-α; eBioscience) in concentration of 2 μg/ml in phosphate-buffered saline (PBS) (150 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4 \times 12H_2O$, 1 mMKH_2PO_4, pH 7.2) at 4 °C overnight. To block free binding sites, washed wells were incubated with 6% low fat dry milk diluted in PBS with 0.05% Tween 20 (PBS–Tween) for 2 h. Plates with 100 μl of undiluted macrophage supernatant per well were incubated at 4 °C overnight. Secondary antibodies conjugated with biotin (Biotin Conjugated Anti-mouse IL-10 or TNF-α; eBioscience) were diluted in PBS–Tween to a concentration of 2 μg/ml and incubated for 1 h at room temperature. To visualize the antigen–antibody complex avidine-peroxidase was applied at concentration of 500 μg/ml for 30 min at room temperature. Orthophenylendiamine and H_2O_2 in phosphate–citrate buffer (0.11 M $Na_2HPO_4 \times 12H_2O$, 0.5 M citric acid; pH 5.5) were used as a substrate solution. Absorbance was measured at 492 nm using a microplate reader (Tecan Infinite M200). Data are stated in the form of stimulation index (SI); each cytokine absorbance value was divided by the relevant negative control.

2.7. Detection of anti-P. papatasi and anti-P. duboscqi saliva antibodies

Anti-*P. papatasi* and anti-*P. duboscqi* IgG antibodies were measured in sera of BALB/c mice obtained at three intervals: before exposure to *P. papatasi* bites (T1), 3 days after the last exposure to *P. papatasi* (T2) and 9 weeks post infection (T3). Microtiter plate wells were coated with *P. papatasi* or *P. duboscqi* SGH (equivalent of 1/5 salivary gland per well) in 20 mM carbonate–bicarbonate buffer (20 mM Na_2CO_3–$NaHCO_3$, pH 9.0–9.5) at 4 °C overnight. To block free binding sites, washed wells were incubated with 6% low fat dry milk diluted in PBS–Tween. Mice sera were diluted 1:200 in 2% low fat dry milk and incubated for 90 min at 37 °C. Secondary antibodies (goat anti-mouse IgG conjugated with peroxidase, Serotec) were diluted 1:1000 in PBS–Tween and incubated for 45 min at 37 °C. Reaction was developed and measured as described above. Similar protocol was used to determine anti-saliva IgG subclasses with the following modifications: sera were incubated overnight at 4 °C; secondary antibodies (goat anti-mouse IgG1 and IgG2a conjugated with peroxidase, Serotec) were diluted 1:9000 for IgG1 and 1:200 for IgG2a in PBS–Tween and incubated for 45 min and 2 h, respectively. Sera of two non-exposed and non-infected BALB/c mice were used as a negative control.

2.8. Detection of anti-L. major antibodies

Anti-*L. major* IgG antibodies were measured in sera of BALB/c mice obtained 9 weeks post infection. Stationary promastigotes of *L. major* were used as an antigen. Parasites were washed two-times in saline solution, centrifuged for 8 min at 2900 × *g*, counted in Burker chamber and frozen until used. Microtiter plate wells were coated with crude *L. major* promastigotes at a concentration of 10^7 cells/ml

at 37 °C for 2 h. To block free binding sites, washed wells were incubated with 6% low fat dry milk diluted in PBS–Tween. Mice sera were diluted 1:400 in 2% low fat dry milk and incubated for 60 min at 37 °C. Secondary antibodies (goat anti-mouse IgG conjugated with peroxidase, Serotec) were diluted 1:1000 in PBS–Tween and incubated for 45 min at 37 °C. Reaction was developed and measured as described above. Similar protocol was used to determine anti-*L. major* IgG subclasses with the following modifications: sera were incubated overnight at 4 °C; secondary antibodies (goat anti-mouse IgG1 and IgG2a conjugated with peroxidase, Serotec) were diluted 1:9000 for IgG1 and 1:200 for IgG2a in PBS–Tween and incubated for 45 min and 2 h, respectively. Sera of the two non-exposed and non-infected BALB/c mice were used as a negative control.

2.9. Western blot analysis

P. papatasi and *P. duboscqi* SGH was separated on 12% SDS–PAGE gel under non-reducing conditions using the Mini-Protean III apparatus (BioRad). For the salivary profile, separated proteins of both colonies (10 and 8.5 salivary glands for *P. papatasi* a *P. duboscqi* per well, respectively) were stained by silver. For the Western blot analysis, salivary proteins (10 gland pairs per well) were blotted onto a nitrocellulose membrane (NB) by Semi-Phor equipment (Hoefer Scientific Instruments) and NB was cut into strips. The strips were then blocked with 5% low fat dry milk in TBS with 0.05% Tween 20 (TBS-Tw) and subsequently incubated with sera diluted 1:100 for 1 h. Sera from BALB/c mice immunized 10 times by *P. papatasi* or *P. duboscqi* bites or never exposed to sand flies were used (none of those mice were infected by *L. major*). Then the strips were incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (Serotec) diluted 1:1000 in TBS-Tw. The chromogenic reaction was developed using a substrate solution containing diaminobenzidine and H_2O_2.

2.10. Statistical analysis

The data were analyzed using NCSS 6.0.21 software. Lesion size development was analyzed by general linear models (GLM) ANOVA and Scheffe's Multiple Comparison Test after data normalization using $\ln(x+1)$ transformation formula. The differences between and within groups (parasite load, macrophage activity and antibody response) were determined by the non-parametric Wilcoxon Rank Sum Test for Differences in Medians and Wilcoxon Signed-Rank Test for Difference in Medians, respectively. The non-parametric Spearman Rank Correlation Matrix was used to test correlations. GraphPad Prism 5.00 software was used for creating the correlation graphs.

3. Results

3.1. Development of L. major infection

The development of *L. major* infection was monitored in four groups of mice; mice were followed for 9 weeks after the infection by measuring the ear lesion size, by quantification of *Leishmania* parasites in the ear dermis and in the draining lymph nodes, and by detection of IgG antibodies against *L. major* antigens (Fig. 2).

Importantly, since week 5, both immunized groups revealed significantly smaller *Leishmania* lesions when compared to the controls ($p < 0.05$) (Fig. 2A,B). Since the same week, no significant difference was detected between control groups. On the other hand, the differences were found between the immunized groups, for most of the time points with EXP + P. pap group having smaller lesion sizes than EXP + P. dub.

Nine weeks after infection, the inoculated ears and the corresponding draining lymph nodes of all mice were sampled and *Leishmania* burden was quantified using qPCR. In EXP + P.pap group, a significant reduction in the amount of the *Leishmania* parasites ($p < 0.05$) was detected, when compared to nEXP + P.pap group; fivefold in the inoculated ears and threefold in the draining lymph nodes (Fig. 2C,D). Similar results were achieved comparing the EXP + P.dub and nEXP + P.dub groups ($p < 0.05$); the parasite burden in the draining lymph nodes was 11-fold reduced in the immunized group and the same trend was observed also in the inoculated ears, although the difference was not significant ($p = 0.064$) (Fig. 2C,D). Moreover, the cumulative parasite load (the sum of parasites in the inoculated ear and in the draining lymph node) was also significantly reduced ($p < 0.05$) in both immunized groups, when compared to the either control group (data not shown). No significant difference in the amount of *Leishmania* parasites was found between the immunized groups, or the controls. Importantly, positive correlation was detected between the ear lesion size measured in the ninth week after infection and parasite burden in the inoculated ear ($\rho = 0.781$; $p < 0.05$) (Fig. 2F) as well as between the number of *Leishmania* parasites in the inoculated ear and in the draining lymph node ($\rho = 0.381$; $p < 0.05$) (Table S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.01.005.

The IgG antibodies against *L. major* were measured in the sera of mice in the ninth week after the infection as a marker of disease severity. No significant difference was found between the two immunized groups (EXP + P.pap and EXP + P.dub). However, significantly lower levels of specific antibodies ($p < 0.05$) were detected in the EXP + P.pap group, when compared to the nEXP + P.pap group (Fig. 2E). Although insignificantly, similar trend was observed between the group infected together with heterologous antigen (EXP + P.dub) and its control (nEXP + P.dub). Importantly, positive correlation was found between the levels of anti-*L. major* IgG and the *Leishmania* lesion size in the ears ($\rho = 0.583$; $p < 0.05$) (Fig. 2G) as well as between the levels of specific antibodies and the number of parasites in the inoculated ears ($\rho = 0.619$; $p < 0.05$) (Table S1).

Furthermore, we also determined levels of specific IgG2a and IgG1 antibodies. Anti-*L. major* IgG1 antibodies were the dominant IgG subclass, whereas IgG2a remained near the background levels of the negative control. No significant difference was detected between the groups for either IgG subclasses or the IgG1:IgG2a ratio (Fig. S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.01.005.

3.2. Macrophage activities

The activity of macrophages obtained by the peritoneal lavage was determined by measuring the production of nitric oxide (NO) (marker of classically activated macrophages), urea (marker of alternatively activated macrophages), and cytokine production in the ninth week after infection (Figs. 3 and 4).

No significant difference in the production of NO was detected between the groups (Fig. 3A). Differences in urea production were observed between the two groups challenged together with *P. duboscqi* saliva; EXP + P.dub produced approximately threefold more urea ($p < 0.05$) than nEXP + P.dub, both when the macrophages were stimulated with LPS and IFN-γ and in macrophages without any stimulation (Fig. 3B). A similar trend in urea production was observed in the exposed and non-exposed groups (EXP + P.pap compared to nEXP + P.pap and EXP + P.dub compared to nEXP + P.dub) incubated with *Leishmania* parasites.

When compared to nEXP + P.pap group, macrophages from the EXP + P.pap group produced significantly higher amount of TNF-α and lower amounts of IL-10 after stimulation with

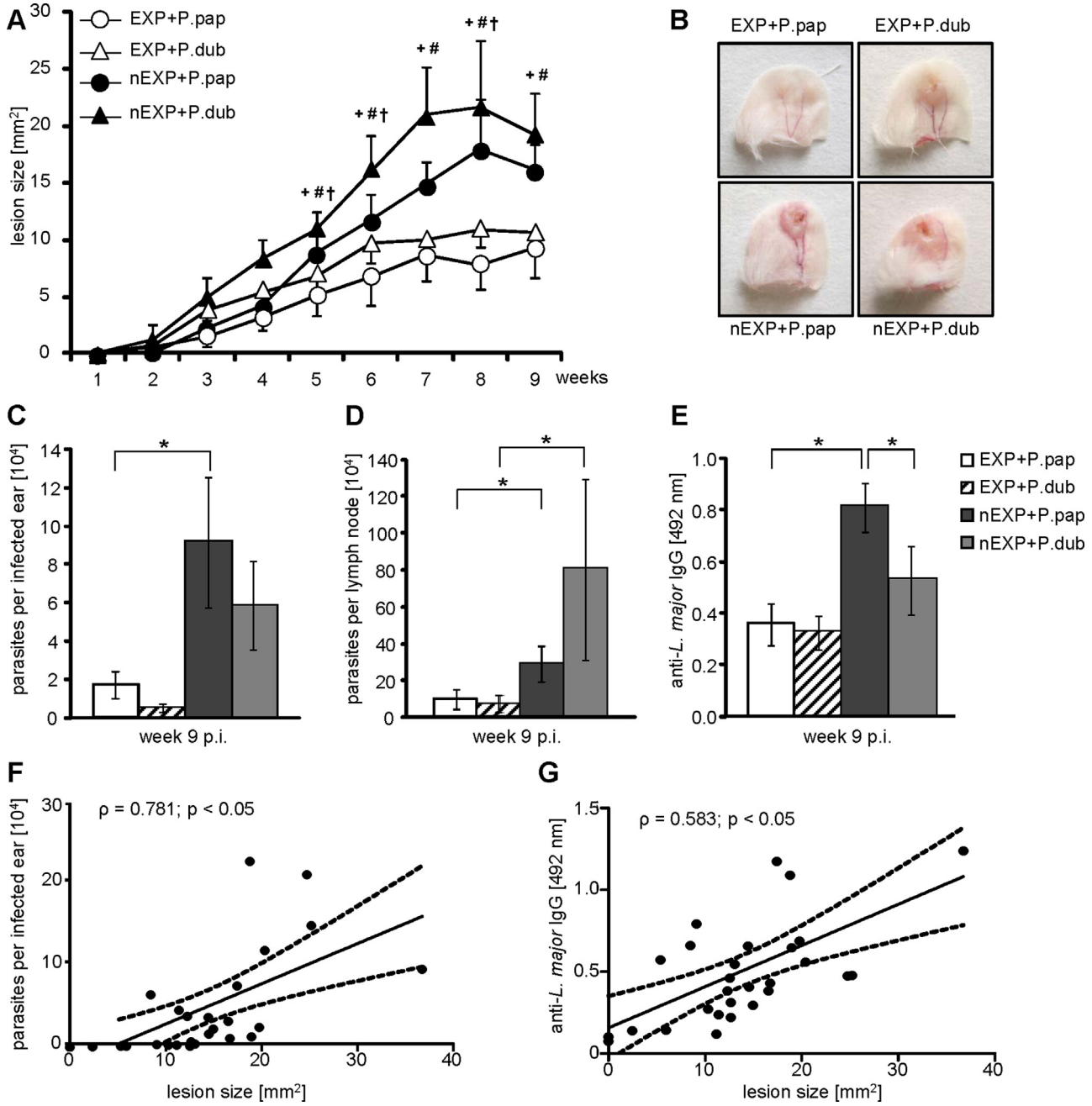

Fig. 2. Development of *Leishmania* infection. Kinetics of *L. major* infection was studied in four groups of BALB/c mice. Two groups of mice immunized by *P. papatasi* bites (EXP, open marks) and two control non-immunized groups (nEXP, full marks) were infected with *L. major* promastigotes along with either *P. papatasi* SGH (EXP + P.pap, nEXP + P.pap; circles) or *P. duboscqi* SGH (EXP + P. dub, nEXP + P.dub; triangles). The development of *Leishmania* lesion size was measured weekly for 9 consecutive weeks (A). The ninth week after the infection, photos of the representative inoculated ears were taken (B), parasite burdens in the inoculated ears (C) as well as in corresponding draining lymph nodes (D) were determined using qPCR, and anti-*L. major* antibodies in the sera were measured using ELISA (E). Data are summarized from three independent experiments. Positive correlation was achieved between the lesion size and the amount of parasites in the inoculated ear (F) and between the lesion size and the levels of anti-*L. major* IgG (G). Graph A: symbols are used as follows: $^{+,\#,\dagger}$ indicate significant difference ($p < 0.05$) in the lesion size in the individual weeks between P.dub groups (EXP + P.dub *versus* nEXP + P. dub), P.pap groups (EXP + P.pap *versus* nEXP + P.pap), and immunized groups (EXP + P.pap *versus* EXP + P.dub), respectively. Significant differences are shown for the week 5 onwards. Graphs C–E: * indicates significant difference ($p < 0.05$) between indicated groups. In all graphs, vertical bars represent standard errors of the means.

Leishmania parasites ($p < 0.05$) (Fig. 4). In the groups challenged together with *P. duboscqi* salivary gland homogenate, the production of TNF-α and IL-10 was comparable (Fig. 4). Significant differences ($p < 0.05$) in tested cytokines were recorded between the non-exposed groups after incubation with *Leishmania* promastigotes; the nEXP + P.dub group produced significantly more TNF-α and significantly less IL-10 compared to group nEXP + P.pap. No differences were found between the exposed groups (EXP + P.pap compared to EXP + P.dub) (Fig. 4).

3.3. Anti-saliva antibody response

As a marker of exposure, the anti-*P. papatasi* and anti-*P. duboscqi* saliva IgG were measured in sera of mice of all four groups.

Two exposures to *P. papatasi* females did not result in significantly increased levels of anti-*P. papatasi* IgG or anti-*P. duboscqi* IgG at T2 (Fig. 5A,B). However, 9 weeks later, at T3, there was a significant increase of anti-*P. papatasi* and anti-*P. duboscqi*

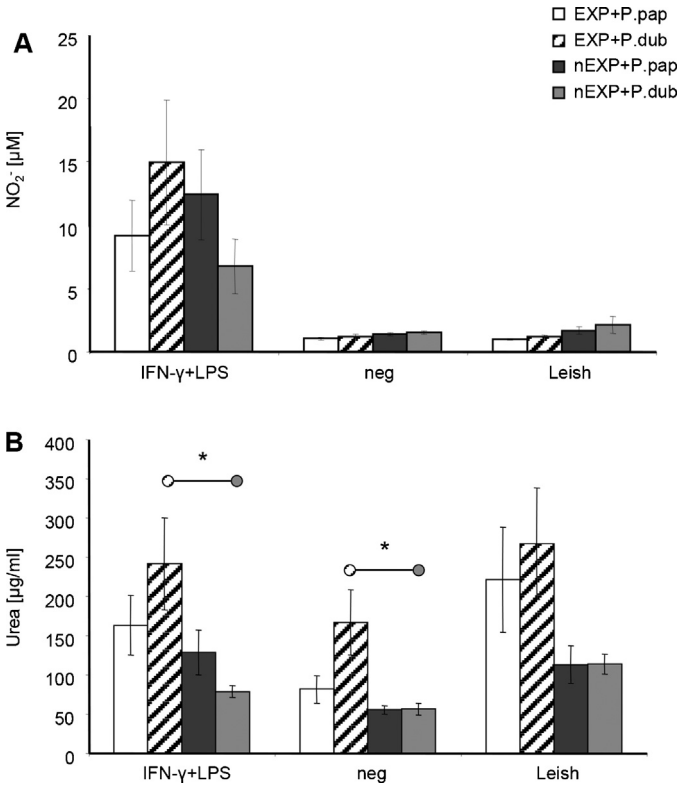

Fig. 3. Effect of sand fly saliva on the macrophages activity. BALB/c mice were exposed to *P. papatasi* bites (EXP) or left non-exposed (nEXP). Mice were then infected with *L. major* promastigotes along with either *P. papatasi* (EXP + P.pap, nEXP + P.pap) or *P. duboscqi* (EXP + P.dub, nEXP + P.dub) SGH. The activity of macrophages obtained by the peritoneal lavage in the ninth week after infection was determined by production of nitric oxide using Griess reaction (A) and by production of urea using Arginase assay (B). Obtained macrophages were incubated alone (neg) or stimulated *ex vivo* by combination of IFN-γ and LPS, or with *Leishmania major* promastigotes (Leish). * Indicates significant difference ($p < 0.05$) between groups. Vertical bars represent standard errors of the means.

antibodies ($p < 0.05$) in both exposed groups (EXP + P.pap and EXP + P.dub) (Fig. 5A,B).

At T3, EXP + P.pap as well as EXP + P.dub mice produced significantly higher levels of anti-*P. papatasi* IgG antibodies ($p < 0.05$, $p < 0.01$, respectively) compared to the relevant non-exposed groups. The levels of specific antibodies in the two exposed groups were comparable throughout whole study. Comparison of the sera of the non-exposed groups from the last sampling point (T3) revealed that nEXP + P.pap presented significantly higher levels of anti-*P. papatasi* IgG ($p < 0.05$) than nEXP + P.dub group (Fig. 5A).

When comparing the production of anti-*P. duboscqi* antibodies between the non-exposed groups in the ninth week after infection, the nEXP + P.dub sera possessed significantly higher levels of specific IgG ($p < 0.05$) than the nEXP + P.pap group (Fig. 5B). No significant difference in the levels of specific antibodies was detected between the exposed groups, although a trend towards higher production in EXP + P.dub group was observed at T3.

A negative correlation was found between the levels of anti-*P. papatasi* IgG at T3 and the number of *L. major* parasites in the draining lymph node ($\rho = -0.548$; $p < 0.01$) as well as between the levels of anti-*P. papatasi* IgG and the size of ear lesion ($\rho = -0.406$; $p < 0.05$) (Table S1).

Furthermore, we also determined specific IgG2a and IgG1 antibodies. Since we did not detect any significant difference in the production of total anti-saliva IgG between pre-immune sera (T1) and sera after the second exposure (T2), the IgG1 and IgG2a antibodies were measured only in T3 samples. Anti-*P. papatasi* IgG1 antibodies were the dominant IgG subclass in exposed groups, whereas IgG2a remained near the background level of the negative control (Fig. 5C). EXP + P.pap as well as EXP + P.dub mice produced significantly higher anti *P. papatasi* IgG1 antibodies ($p < 0.01$) compared to the appropriate non-exposed groups (Fig. 5C). The levels of IgG1 and IgG2a in the two exposed as well as the non-exposed groups were comparable (Fig. 5C).

No significant difference in the production of anti-*P. duboscqi* IgG1 and IgG2a antibodies was detected between the groups, albeit the trend of higher IgG1 production in exposed groups was also observed (Fig. 5D).

3.4. Cross-reactivity of *P. papatasi* and *P. duboscqi* salivary antigens

The protein profile of *P. papatasi* and *P. duboscqi* salivary glands was studied using SDS–PAGE. In *P. papatasi* and *P. duboscqi* saliva, 11 and 13 prominent protein bands, respectively, were visualized using silver staining. Comparison of both salivary profiles revealed a significant difference particularly within the 10–50 kDa range (Fig. 6A).

To test the cross-reactivity of anti-*P. papatasi* and anti-*P. duboscqi* antibodies, Western blot analysis was performed using

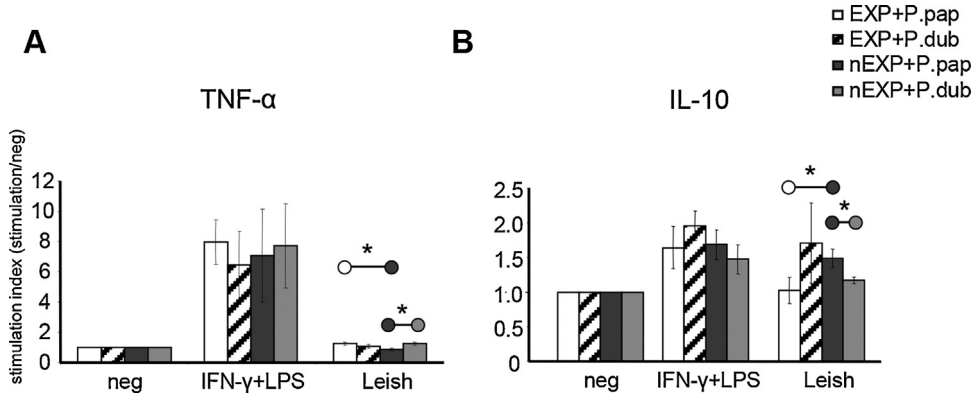

Fig. 4. Cytokine production of the peritoneal macrophages. BALB/c mice were exposed to *P. papatasi* bites (EXP) or left non-exposed (nEXP). Mice were then infected with *L. major* promastigotes along with either *P. papatasi* (EXP + P.pap, nEXP + P.pap) or *P. duboscqi* (EXP + P.dub, nEXP + P.dub) SGH. Macrophages were obtained by the peritoneal lavage in the ninth week after infection and stimulated by combination of IFN-γ and LPS (IFN-γ + LPS) or *Leishmania major* promastigotes (Leish). Macrophages without any stimulation were used as the negative control (Neg). The production of TNF-α (A) and IL-10 (B) was determined from the supernatant collected after 72 h of incubation. Data are stated in the stimulation index form; the cytokine absorbance was divided by the negative control value.* indicates significant difference ($p < 0.05$) between groups. Vertical bars represent standard errors of the means.

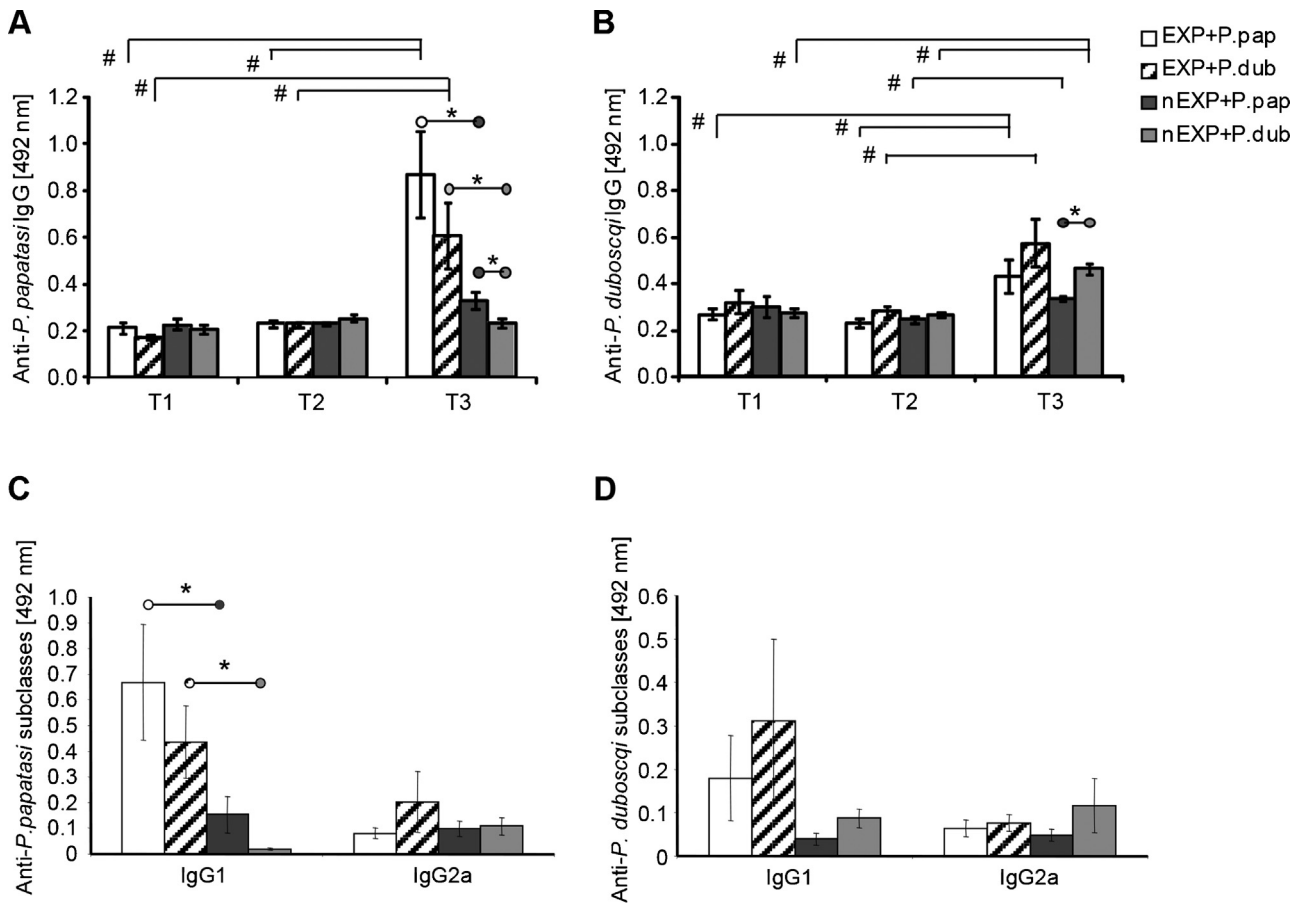

Fig. 5. Anti-sand fly saliva antibody response. BALB/c mice were exposed to *P. papatasi* bites (EXP) or left non-exposed (nEXP). Mice were then infected with *L. major* promastigotes along with either *P. papatasi* (EXP + P.pap, nEXP + P.pap) or *P. duboscqi* (EXP + P.dub, nEXP + P.dub) SGH. IgG against saliva of *P. papatasi* (A) and *P. duboscqi* (B) were measured in sera of mice at three time points: before sand fly exposure (T1), after the last sand fly exposure (T2), and in the ninth week post infection (T3). IgG1 and IgG2a against saliva of *P. papatasi* (C) and *P. duboscqi* (D) were measured at T3. Symbols are used as follows: # indicates significant difference ($p < 0.05$) in production of specific antibodies within the group at different time points; * indicates significant difference ($p < 0.05$) between groups within the same time point. Vertical bars represent standard errors of the means.

sera of mice repeatedly bitten by either *P. papatasi* or *P. duboscqi*. In both sand fly species, strong reaction was observed in the samples tested against homologous antigen (Fig. 6B). The sera of mice bitten by *P. papatasi* recognized 8–11 *P. papatasi* salivary proteins within 10–50 kDa range. Similar reactivity was observed in sera of mice bitten by *P. duboscqi*; they also reacted with 8–11 *P. duboscqi* salivary antigens of the same molecular weights (Fig. 6B). Substantial cross-reactivity was observed among both sand fly species when tested by reaction with heterologous antigen; however the reactions were less intense compared to homologous antigen. Sera of mice bitten by *P. duboscqi* recognized 6 out of 11 *P. papatasi* protein bands (of approximately 12, 22, 23, 28, 29, and 32 kDa) (Fig. 6B), while sera of mice bitten by *P. papatasi* recognized only three *P. duboscqi* antigens, of about 15, 23, and 38 kDa (Fig. 6B).

4. Discussion

This study demonstrates for the first time the cross-protective effect between saliva of two closely related *Phlebotomus* species: *P. papatasi* and *P. duboscqi*, both the natural vectors of *L. major*. In accordance with previous studies (Belkaid et al., 1998; Kamhawi et al., 2000), mice immunized by *P. papatasi* saliva were protected against *L. major* infection co-inoculated with *P. papatasi* salivary gland homogenate. It was reflected in significantly smaller ear lesion size and lower number of *Leishmania* parasites in the inoculated ear as well as in the draining lymph node. The course of infection in mice exposed to *P. papatasi* sand flies but infected together with *P. duboscqi* SGH was similar. Herein, the cross-protective effect was demonstrated by significantly smaller ear lesion size which corresponded to lower number of *Leishmania* parasites in the draining lymph node with trend to lower number of parasites also in the inoculated ear. Importantly, when compared to *P. papatasi*-challenged group, there was no difference in parasite load in the inoculated ear and draining lymph node or anti-*Leishmania* IgG level.

It has been shown that the prior exposure of mice to *P. papatasi* saliva attracts several immune cells to the bite site and promotes the Th1-derived immune milieu capable of effective defense against *L. major* co-inoculated with sand fly saliva or transmitted by sand fly bite (Belkaid et al., 1998; Kamhawi et al., 2000). On the other hand, the Th2 type inducing saliva, for example of *Lu. intermedia*, failed to protect against challenge comprised of *L. brasiliensis* and homologous saliva (de Moura et al., 2007). Therefore, in addition to the course of infection, we also tested several aspects of cellular and humoral immunity in the infected mice.

The peritoneal macrophages were employed to study the systemic immune response. Although the protective effect elicited by the *P. papatasi* bite was observed in both exposed groups, the cytokine profile of peritoneal macrophages (PMΦ) was affected only in the group challenged in the presence of homologous antigen. In this group, the production of the proinflammatory cytokine TNF-α, was enhanced, whereas the production of IL-10, the

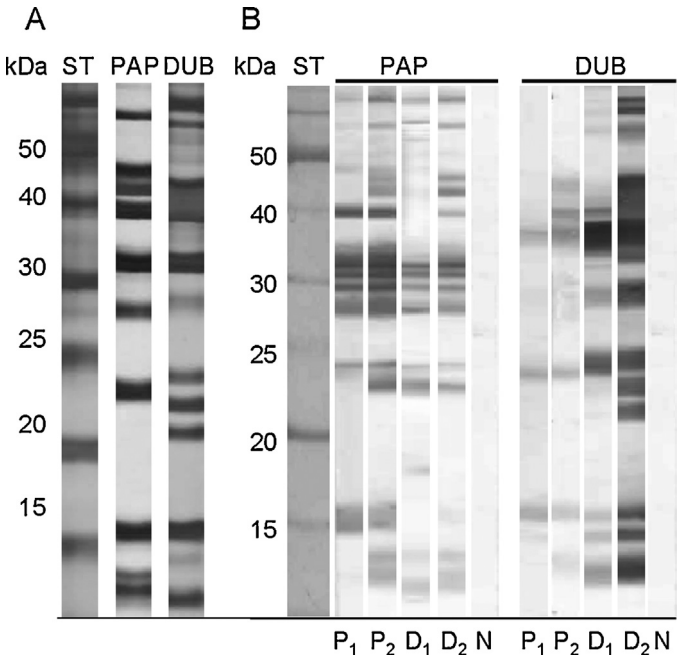

Fig. 6. Cross-reactivity of *P. papatasi* and *P. duboscqi* salivary antigens. Salivary proteins of *P. papatasi* (PAP) and *P. duboscqi* (DUB) were separated under non-reducing conditions by SDS–PAGE electrophoresis and silver staining was used to compare the salivary profiles of both species (A). Western blot analysis (B) was performed with sera of BALB/c mice experimentally bitten by *P. papatasi* (P1, P2) or by *P. duboscqi* (D1, D2). Serum from a naive mouse was used as the negative control (N). Molecular weight standard (ST), stained by silver, for protein profile and by amido black for western blot analysis, labeled with the corresponding molecular weights are indicated (kDa).

anti-inflammatory cytokine, was significantly suppressed. These data suggest similar polarization of the cytokine production by PMΦ as described for dermal macrophages isolated from protected mice (Belkaid et al., 1998). The absence of such polarization in the exposed group challenged together with *P. duboscqi* SGH might be explained by the fact that the immunity elicited by the *P. papatasi* bites was probably not fully boosted by *P. duboscqi* whose salivary proteins might have different T-cell epitopes. Nonetheless, further studies are needed to clarify this phenomenon.

Besides the cytokine expression, macrophages also play a crucial role in the establishment of *Leishmania* infection by processing L-arginine to the nitric oxide, the molecule produced by classically activated macrophages necessary to kill *Leishmania* amastigotes (Green et al., 1990). Alternatively, L-arginine is processed to the ornithine and urea, the molecules produced by alternatively activated macrophages that support *Leishmania* proliferation (reviewed in Horta et al. (2012)). Seemingly contradictory results were obtained in our study. The production of nitric oxide was not increased in the protected group when compared to the control one, but the opposite trend was observed. Conversely, the urea production was enhanced in exposed group. To our best knowledge, there are no comparable studies describing the effect of *P. papatasi* saliva on nitric oxide and urea production in repeatedly exposed host, however, several studies were performed on naive mice (Hall and Titus, 1995; Katz et al., 2000; Mbow et al., 1998; Waitumbi and Warburg, 1998). Mbow et al. (1998) described the association between the course of *L. major* infection and the expression of iNOS. They showed that the enhancing effect observed in naive mice infected together with *P. papatasi* saliva corresponds to the inhibition of iNOS gene expression but only until day 28 p.i. Since then, the expression of iNOS mRNA corresponded with the amount of *Leishmania* parasites in tissues (Mbow et al., 1998). Furthermore, Blos et al. (2003) demonstrated that iNOS dependent macrophage cytotoxicity is utilized mainly in the early phase of *Leishmania* infection, whereas in the chronic phase the defense of macrophages relies mostly on NADPH oxidase-catalysed reactions. Our results are in accordance with these findings; as the PMΦ were taken at week 9 post infection we suppose that the higher production of urea and decreased production of NO in both groups of protected mice might be ascribed to the sampling time of PMΦ. Although the time point was chosen with respect to the *Leishmania* lesion development, to better elucidate the association between protective effect and macrophage activity, it might be beneficial to focus further studies on early phase of *Leishmania* infection.

Conversely to the cellular immunity, the anti-*Leishmania* antibody response is likely not employed in the protection against leishmaniasis; however, the antibodies can serve as the indicator of infection severity (Miles et al., 2005; Rohousova et al., 2011). The production of anti-*Leishmania* IgG is linked with the formation of immune complexes responsible for the aggravation of *Leishmania* infection (Kima et al., 2000; Miles et al., 2005) as well as with the production of IL-10 (Miles et al., 2005). This complies well with our results as anti-*Leishmania* IgG levels were lower in exposed groups compared to non-exposed ones and positively correlated with the *Leishmania* lesion size as well as with the amount of parasites in the lesion. In accordance with previous studies (Ebrahimpoor et al., 2013), we showed the dominancy of IgG1 over the IgG2a subclass in BALB/c mice, indicating ongoing Th2 immune response; no differences between the groups were observed.

Sand fly salivary antigens are known to induce specific antibody response in experimentally bitten animals (Volf and Rohousová, 2001), as well as in humans (Gomes et al., 2002; Rohousova et al., 2005) and wild animals (Gomes et al., 2007; Martín-Martín et al., 2014). This antibody response can be utilized as a marker of exposure (reviewed in Gomes and Oliveira (2012)). In our experiments, we did not detect any significant difference in production of anti-*P. papatasi* saliva IgG between pre-immune sera and sera obtained after the second exposure. In contrast, in the ninth week post infection, both exposed groups produced around 2.5-fold more anti-*P. papatasi* IgG compared to non-exposed groups and over threefold higher levels than the pre-immune sera. These findings are in accordance with our recent study (Vlkova et al., 2012), where the antibody levels did not significantly increase till the fourth week since the first exposure but once elevated, they persisted in the murine sera for more than 20 weeks after the last exposure (Vlkova et al., 2012).

Similar to other studies (Silva et al., 2005; Vlkova et al., 2012), IgG1 was the dominant IgG subclass in exposed groups; however only anti-*P. papatasi* antibodies were found significantly elevated compared to non-exposed groups, suggesting certain level of antigenic species-specificity between *P. papatasi* and *P. duboscqi* salivary proteins. IgG1 is also dominant in mice immunized with salivary gland homogenate (de Moura et al., 2007; Oliveira et al., 2006), indicating that saliva and SGH elicit similar type of antibody response in immunized mice.

Salivary antigens of sand flies are mostly species-specific with possible cross-reactions occurring only between closely related species (Drahota et al., 2009; Rohousova et al., 2005; Thiakaki et al., 2005; Volf and Rohousová, 2001). However, sharing similar salivary antigens is one of the conditions required for the successful cross-protection against *Leishmania* infection. Thus *Lutzomyia longipalpis* saliva did not mediate cross-protection against *L. amazonensis* challenge together with saliva of phylogenetically distant species, *P. papatasi* and *P. sergenti* (Thiakaki et al., 2005). In contrast, the cross-protective effect was demonstrated between the *Lutzomyia* species; *Lu. longipalpis* and *Lu. intermedia*, vectors of *L. brasiliensis*. The golden hamsters immunized with *Lu. longipalpis* SGH or with a DNA plasmid coding LJM19 salivary protein were protected against *L. brasiliensis* infection in the presence

of *Lu. intermedia* saliva with reduced number of parasites in the inoculated ear and in the draining lymph node (Tavares et al., 2011). In accordance with the aforementioned rules for effective cross-protectivity, we observed cross-reactivity between *P. papatasi* and *P. duboscqi* salivary antigens using sera of hyper-immunized mice. This cross-reactivity was observed despite the differences in the saliva composition between both species (Kato et al., 2006; Volf et al., 2000), but was likely efficient enough to provide the protective effect to the mice infected together with heterologous antigen.

In conclusion, this is the first study showing the cross-protection in *P. papatasi*-exposed mice challenged with *L. major* in the presence of *P. duboscqi* saliva. The cross-protective effect suggests that the anti-*Leishmania* vaccine based on *P. papatasi* salivary proteins could be applicable also in sub-Saharan endemic areas where *L. major* is transmitted by *P. duboscqi*. Moreover, similar cross-protection might be possible also between other closely related sand fly species such as *Larroussius* species responsible for transmission of *L. infantum* in Mediterranean basin. We would like to further analyze immune mechanisms of this cross-protective phenomenon using a sand fly bite challenge model that mimic more closely the natural way of *Leishmania* transmission.

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References

Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., Ribeiro, J., Sacks, D.L., 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J. Exp. Med. 188, 1941–1953.

Blos, M., Schleicher, U., Soares Rocha, F.J., Meissner, U., Röllinghoff, M., Bogdan, C., 2003. Organ-specific and stage-dependent control of *Leishmania major* infection by inducible nitric oxide synthase and phagocyte NADPH oxidase. Eur. J. Immunol. 33, 1224–1234.

Clements, M.F., Gidwani, K., Kumar, R., Hostomska, J., Dinesh, D.S., Kumar, V., Das, P., Müller, I., Hamilton, G., Volfova, V., Boelaert, M., Das, M., Rijal, S., Picado, A., Volf, P., Sundar, S., Davies, C.R., Rogers, M.E., 2010. Measurement of recent exposure to *Phlebotomus argentipes*, the vector of Indian visceral leishmaniasis, by using human antibody responses to sand fly saliva. Am. J. Trop. Med. Hyg. 82, 801–807.

da Silva, R.A., Tavares, N.M., Costa, D., Pitombo, M., Barbosa, L., Fukutani, K., Miranda, J.C., de Oliveira, C.I., Valenzuela, J.G., Barral, A., Soto, M., Barral-Netto, M., Brodskyn, C., 2011. DNA vaccination with KMP11 and *Lutzomyia longipalpis* salivary protein protects hamsters against visceral leishmaniasis. Acta Trop. 120, 185–190.

de Moura, T.R., Oliveira, F., Novais, F.O., Miranda, J.C., Clarêncio, J., Follador, I., Carvalho, E.M., Valenzuela, J.G., Barral-Netto, M., Barral, A., Brodskyn, C., de Oliveira, C.I., 2007. Enhanced *Leishmania braziliensis* infection following pre-exposure to sandfly saliva. PLoS Negl. Trop. Dis. 1, e84.

Drahota, J., Lipoldová, M., Volf, P., Rohousová, I., 2009. Specificity of anti-saliva immune response in mice repeatedly bitten by *Phlebotomus sergenti*. Parasite Immunol. 31, 766–770.

Ebrahimpoor, S., Pakzad, S.R., Ajdary, S., 2013. IgG1 and IgG2a profile of serum antibodies to *Leishmania major* amastigote in BALB/c and C57BL/6Mice. Iran J. Allergy Asthma Immunol. 12, 361–367.

Gidwani, K., Picado, A., Rijal, S., Singh, S.P., Roy, L., Volfova, V., Andersen, E.W., Uranw, S., Ostyn, B., Sudarshan, M., Chakravarty, J., Volf, P., Sundar, S., Boelaert, M., Rogers, M.E., 2011. Serological markers of sand fly exposure to evaluate insecticidal nets against visceral leishmaniasis in India and Nepal: a cluster-randomized trial. PLoS Negl. Trop. Dis. 5, e1296.

Gomes, R., Oliveira, F., 2012. The immune response to sand fly salivary proteins and its influence on leishmania immunity. Front. Immunol. 3, 110.

Gomes, R., Teixeira, C., Teixeira, M.J., Oliveira, F., Menezes, M.J., Silva, C., de Oliveira, C.I., Miranda, J.C., Elnaiem, D.E., Kamhawi, S., Valenzuela, J.G., Brodskyn, C.I., 2008. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. Proc. Natl. Acad. Sci. U. S. A. 105, 7845–7850.

Gomes, R.B., Brodskyn, C., de Oliveira, C.I., Costa, J., Miranda, J.C., Caldas, A., Valenzuela, J.G., Barral-Netto, M., Barral, A., 2002. Seroconversion against *Lutzomyia longipalpis* saliva concurrent with the development of anti-*Leishmania chagasi* delayed-type hypersensitivity. J. Infect. Dis. 186, 1530–1534.

Gomes, R.B., Mendonça, I.L., Silva, V.C., Ruas, J., Silva, M.B., Cruz, M.S., Barral, A., Costa, C.H., 2007. Antibodies against *Lutzomyia longipalpis* saliva in the fox *Cerdocyon thous* and the sylvatic cycle of *Leishmania chagasi*. Trans. R. Soc. Trop. Med. Hyg. 101, 127–133.

Green, S.J., Meltzer, M.S., Hibbs, J.B., Nacy, C.A., 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J. Immunol. 144, 278–283.

Hall, L.R., Titus, R.G., 1995. Sand fly vector saliva selectively modulates macrophage functions that inhibit killing of *Leishmania major* and nitric oxide production. J. Immunol. 155, 3501–3506.

Horta, M.F., Mendes, B.P., Roma, E.H., Noronha, F.S., Macêdo, J.P., Oliveira, L.S., Duarte, M.M., Vieira, L.Q., 2012. Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. J. Parasitol. Res. 2012, 203818.

Hostomska, J., Rohousova, I., Volfova, V., Stanneck, D., Mencke, N., Volf, P., 2008. Kinetics of canine antibody response to saliva of the sand fly *Lutzomyia longipalpis*. Vector Borne Zoonotic Dis. 8, 443–450.

Kamhawi, S., Belkaid, Y., Modi, G., Rowton, E., Sacks, D., 2000. Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. Science 290, 1351–1354.

Kato, H., Anderson, J.M., Kamhawi, S., Oliveira, F., Lawyer, P.G., Pham, V.M., Sangare, C.S., Samake, S., Sissoko, I., Garfield, M., Sigutova, L., Volf, P., Doumbia, S., Valenzuela, J.G., 2006. High degree of conservancy among secreted salivary gland proteins from two geographically distant *Phlebotomus duboscqi* sandflies populations (Mali and Kenya). BMC Genomics 7, 226.

Katz, O., Waitumbi, J.N., Zer, R., Warburg, A., 2000. Adenosine, AMP, and protein phosphatase activity in sandfly saliva. Am. J. Trop. Med. Hyg. 62, 145–150.

Killick-Kendrick, R., 1999. The biology and control of phlebotomine sand flies. Clin. Dermatol. 17, 279–289.

Kima, P.E., Constant, S.L., Hannum, L., Colmenares, M., Lee, K.S., Haberman, A.M., Shlomchik, M.J., McMahon-Pratt, D., 2000. Internalization of *Leishmania mexicana* complex amastigotes via the Fc receptor is required to sustain infection in murine cutaneous leishmaniasis. J. Exp. Med. 191, 1063–1067.

Kimblin, N., Peters, N., Debrabant, A., Secundino, N., Egen, J., Lawyer, P., Fay, M.P., Kamhawi, S., Sacks, D., 2008. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. Proc. Natl. Acad. Sci. U.S.A. 105, 10125–10130.

Kropf, P., Baud, D., Marshall, S.E., Munder, M., Mosley, A., Fuentes, J.M., Bangham, C.R., Taylor, G.P., Herath, S., Choi, B.S., Soler, G., Teoh, T., Modolell, M., Müller, I., 2007. Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. Eur. J. Immunol. 37, 935–945.

Kropf, P., Fuentes, J.M., Fahnrich, E., Arpa, L., Herath, S., Weber, V., Soler, G., Celada, A., Modolell, M., Muller, I., 2005. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. FASEB J. 19, 1000–1002.

Maia, C., Seblova, V., Sadlova, J., Votypka, J., Volf, P., 2011. Experimental transmission of *Leishmania infantum* by two major vectors: a comparison between a viscerotropic and a dermotropic strain. PLoS Negl. Trop. Dis. 5, e1181.

Martín-Martín, I., Molina, R., Rohoušová, I., Drahota, J., Volf, P., Jiménez, M., 2014. High levels of anti-*Phlebotomus perniciosus* saliva antibodies in different vertebrate hosts from the re-emerging leishmaniosis focus in Madrid, Spain. Vet. Parasitol. 202, 207–216.

Mary, C., Faraut, F., Lascombe, L., Dumon, H., 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J. Clin. Microbiol. 42, 5249–5255.

Mbow, M.L., Bleyenberg, J.A., Hall, L.R., Titus, R.G., 1998. *Phlebotomus papatasi* sand fly salivary gland lysate down-regulates a Th1, but up-regulates a Th2, response in mice infected with *Leishmania major*. J. Immunol. 161, 5571–5577.

Miles, S.A., Conrad, S.M., Aves, R.G., Jeronimo, S.M.B., Mosser, D.M., 2005. A role for IgG immune complexes during infection with the intracellular pathogen *Leishmania*. J. Exp. Med. 201, 747–754.

Morris, R.V., Shoemaker, C.B., David, J.R., Lanzaro, G.C., Titus, R.G., 2001. Sandfly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. J. Immunol. 167, 5226–5230.

Murray, H.W., Cartelli, D.M., 1983. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes–evidence for oxygen-dependent and oxygen-independent leishmanicidal activity. J. Clin. Invest. 72, 32–44.

Myskova, J., Votypka, J., Volf, P., 2008. *Leishmania* in sand flies: Comparison of quantitative polymerase chain reaction with other techniques to determine the intensity of infection. J. Med. Entomol. 45, 133–138.

Oliveira, F., Kamhawi, S., Seitz, A.E., Pham, V.M., Guigal, P.M., Fischer, L., Ward, J., Valenzuela, J.G., 2006. From transcriptome to immunome: identification of DTH inducing proteins from a *Phlebotomus ariasi* salivary gland cDNA library. Vaccine 24, 374–390.

Oliveira, F., Lawyer, P.G., Kamhawi, S., Valenzuela, J.G., 2008. Immunity to Distinct Sand Fly Salivary Proteins Primes the Anti-*Leishmania* Immune Response towards Protection or Exacerbation of Disease. PLoS Negl. Trop. Dis. 2, e226.

Ready, P.D., 2013. Biology of phlebotomine sand flies as vectors of disease agents. Annu. Rev. Entomol. 58, 227–250.
Ribeiro, J.M., Mans, B.J., Arcà, B., 2010. An insight into the sialome of blood-feeding Nematocera. Insect Biochem. Mol. Biol. 40, 767–784.
Rohousova, I., Ozensoy, S., Ozbel, Y., Volf, P., 2005. Detection of species-specific antibody response of humans and mice bitten by sand flies. Parasitology 130, 493–499.
Rohoušová, I., Hostomská, J., Vlková, M., Kobets, T., Lipoldová, M., Volf, P., 2011. The protective effect against *Leishmania infection* conferred by sand fly bites is limited to short-term exposure. Int. J. Parasitol. 41, 481–485.
Rohoušová, I., Volfová, V., Nová, S., Volf, P., 2012. Individual variability of salivary gland proteins in three *Phlebotomus* species. Acta Trop. 122, 80–86.
Silva, F., Gomes, R., Prates, D., Miranda, J.C., Andrade, B., Barral-Netto, M., Barral, A., 2005. Inflammatory cell infiltration and high antibody production in BALB/c mice caused by natural exposure to *Lutzomyia longipalpis* bites. Am. J. Trop. Med. Hyg. 72, 94–98.
Tavares, N.M., Silva, R.A., Costa, D.J., Pitombo, M.A., Fukutani, K.F., Miranda, J.C., Valenzuela, J.G., Barral, A., de Oliveira, C.I., Barral-Netto, M., Brodskyn, C., 2011. *Lutzomyia longipalpis* saliva or salivary protein LJM19 protects against *Leishmania braziliensis* and the saliva of its vector, *Lutzomyia intermedia*. PLoS Negl. Trop. Dis. 5, e1169.
Thiakaki, M., Rohousova, I., Volfova, V., Volf, P., Chang, K.P., Soteriadou, K., 2005. Sand fly specificity of saliva-mediated protective immunity in *Leishmania amazonensis*-BALB/c mouse model. Microbes Infect. 7, 760–766.
Titus, R.G., Ribeiro, J.M.C., 1988. Salivary gland lysates from the sand lfy *Lutzomyia longipalpis* enhance *Leishmania* infectivity. Science 239, 1306–1308.
Valenzuela, J.G., Belkaid, Y., Garfield, M.K., Mendez, S., Kamhawi, S., Rowton, E.D., Sacks, D.L., Ribeiro, J.M.C., 2001. Toward a defined anti-*Leishmania* vaccine targeting vector antigens: Characterization of a protective salivary protein. J. Exp. Med. 194, 331–342.
Vlkova, M., Rohousova, I., Drahota, J., Stanneck, D., Kruedewagen, E.M., Mencke, N., Otranto, D., Volf, P., 2011. Canine antibody response to *Phlebotomus perniciosus* bites negatively correlates with the risk of *Leishmania infantum* transmission. PLoS Negl. Trop. Dis. 5, e1344.
Vlkova, M., Rohousova, I., Hostomska, J., Pohankova, L., Zidkova, L., Drahota, J., Valenzuela, J.G., Volf, P., 2012. Kinetics of Antibody Response in BALB/c and C57BL/6 Mice Bitten by *Phlebotomus papatasi*. PLoS Negl. Trop. Dis. 6, e1719.
Volf, P., Rohousová, I., 2001. Species-specific antigens in salivary glands of phlebotomine sandflies. Parasitology 122 Pt 1, 37–41.
Volf, P., Tesarova, P., Nohynkova, E., 2000. Salivary proteins and glycoproteins in phlebotomine sandflies of various species, sex and age. Med. Vet. Entomol. 14, 251–256.
Volf, P., Volfova, V., 2011. Establishment and maintenance of sand fly colonies. J. Vector Ecol. 36, S1–S9.
Waitumbi, J., Warburg, A., 1998. *Phlebotomus papatasi* saliva inhibits protein phosphatase activity and nitric oxide production by murine macrophages. Infect. Immun. 66, 1534–1537.
Warburg, A., Schlein, Y., 1986. The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. Am. J. Trop. Med. Hyg. 35, 926–930.
Xu, X., Oliveira, F., Chang, B.W., Collin, N., Gomes, R., Teixeira, C., Reynoso, D., My Pham, V., Elnaiem, D.E., Kamhawi, S., Ribeiro, J.M., Valenzuela, J.G., Andersen, J.F., 2011. Structure and function of a yellow protein from saliva of the sand fly *Lutzomyia longipalpis* that confers protective immunity against *Leishmania major* infection. J. Biol. Chem. 286, 32383–32393.