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Cellular and humoral host immune response to sand fly saliva

Buněčná a protilátková imunitní odpověď hostitele na sliny flebotomů

Ph.D. thesis/Dizertační práce

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I declare that the data presented in this Ph.D. thesis are results of Tereza Lestinova work. Tereza participated on the projects and experiments ongoing in our laboratory and substantially contributed to the writting of the manuscripts.

Prohlašuji, že data prezentovaná v předkládané dizertační práci jsou výsledky práce Terezy Leštinové. Tereza se významně podílela na projektech probíhajících v naší laboratoři i na sepsání uvedených publikací.

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Table of contents

LIST OF ABBREVIATIONS

- **AMP, ADP, ATP** - adenosine mono/di/tri phosphate
- **CAM** - clasically activated macrophages
- **CCL** - CC chemokine ligand
- **CD** - cluster of differentiation
- **DTH** - delayed type of hypersensitivity
- **ELISA** - enzyme-linked immuno sorbent assay
- **fPPGs** - filamentous proteophosphoglycans
- **GIPLs** - glycoinositolphospholipids
- **gp** - glycoprotein
- **GPI** - glycosylphosphatidylinositol
- **HLA** - human leukocyte antigen
- **IFN-γ** - interferon gamma
- **Ig** - immunoglobulin
- **IL** - interleukin
- **iNOS** - inducible nitric oxide synthase
- **kDNA** - kinetoplast deoxyribonucleic acid
- ***L.*** - *Leishmania*
- **LPG** - lipophosphoglycan
- **LPS** - lipopolysaccharide
- ***Lu.*** - *Lutzomyia*
- **Lufaxin** - *Lutzomyia longipalpis* factor Xa inhibitor
- **Lundep** - *Lutzomyia* netrophil extracellular traps destroying protein
- **MCP** - monocyte chemoattractant protein
- **mRNA** - messenger ribonucleic acid
- **NO** - nitric oxide
- ***P.*** - *Phlebotomus*
- **PBS** - phosphate buffered saline
- **PGE** - prostaglandin
- **PSG** - promastigote secretory gel
- **r** - recombinant form of protein
- **SDS-PAGE** - sodium dodecyl sulfate polyacrylamide gel electrophoresis
- ***Se.*** - *Sergentomyia*
- **SGH** - salivary gland homogenate
- **SGS** - salivary gland sonicate
- **TGF** - transforming growth factor
- **Th** - helper T cell
- **TNF-α** - tumor necrosis factor alpha

ABSTRACT

Sand flies (Diptera: Phlebotominae) are bloodfeeding insects serving as vectors of *Leishmania* parasites (Kinetoplastida). Sand flies possess salivary glands with pharmacologically active molecules that provide them with an effective weapon against the host defence and that play an important role in *Leishmania* infection development. During the bloodfeeding, sand fly saliva is inoculated into the feeding site. Repeated exposures induce saliva-specific immune response, both humoral and cell-mediated. While anti-saliva antibody response correlates with the intensity of exposure and can be used as a marker of exposure, specific cellular immunity provide protection against leishmaniasis in some vector-parasite-host combinations. Sand flies differ in composition of the saliva and thus the elicited immunity is species-specific. This species-specific variability makes difficult the development of one saliva-based vaccine applicable to different *Leishmania*-vector combinations.

However, saliva composition is more conserved among closely-related vector species, which may evoke cross-protection in bitten and subsequently infected hosts. We focused on cross-reactive properties of saliva from *Phlebotomus papatasi* and *Phlebotomus duboscqi*, the two natural vectors of *Leishmania major*. We demonstrated that protection against *Leishmania* infection was observed not only in *P. papatasi*-exposed mice challenged with homologous saliva but also in the group challenged with *P. duboscqi* saliva. These groups did not differ significantly in parasite load, macrophage activity or in the levels of anti-*L. major* and anti-*P. papatasi*/*P. duboscqi* antibodies which indicates cross-protection caused by salivary antigens of these two *Phlebotomus* species.

Although cross-reactivity of salivary proteins among species is advantegous for aforementioned saliva-based vaccine development, it is a disadvantage for their utilization in sand flies exposure testing; cross-reaction among sympatrically occuring species could lead to false positive results. In our study focusing on sero-epidemiology characterization of three endemic foci in Ethiopia, I used murine model to test the specificity of *P. orientalis* SGH against anti-saliva antibodies elicited by sympatrically ocurring sand fly species. Antigen from *P. orientalis* reacted strongly only with anti-*P. orientalis* antibodies, while reactivity with heterologous anti-sand fly saliva antibodies were comparable to those from non-exposed mice. The observed high species-specificity of the reaction indicates similar specificity also for anti-*P. orientalis* antibodies from other host species. Therefore, anti-*P.orientalis* antibody response supports the hypothesis about possible role of domestic animals in the epidemiology of visceral leishmaniasis caused by *L. donovani*.

Studies dealing with the utilization of anti-saliva antibody response for estimating sand fly exposure usually exploit whole salivary gland homogenate as the antigen, however recombinant proteins were suggested as convenient alternative for salivary gland homogenate. In two extensive epidemiological studies, we validated recombinant form of *P. perniciosus* yellow-related protein as a marker of sand fly exposure based on convincingly high correlation between antibodies recognizing recombinant protein and the whole salivary antigen. Moreover, it was shown that this protein depicts the dynamics of antibodies comparatively with saliva and may even be applied as antigen in the distant regions where *P. perniciosus* is the unique or principal vector species.

ABSTRAKT

Krevsající flebotomové (Diptera: Phlebotominae) jsou významnými přenašeči parazitických prvoků rodu *Leishmania* (Kinetoplastida), kteří jsou do hostitele inokulováni společně se slinami vektora. Sliny flebotomů obsahují imunomodulační složky mající vliv na leishmaniovou infekci. U opakovaně pobodaného hostitele vzniká druhově specifická imunitní odpověď, protilátkově i buněčně zprostředkovaná. Zatímco množství protilátek proti slinám flebotomů pozitivně koreluje s intenzitou pobodání, čehož může být využito jako ukazatele kontaktu mezi flebotomy a hostiteli, buněčná imunita zodpovídá v některých kombinacích přenašeč-parazit-hostitel za protekci proti leishmanióze. Nejen samotné složení slin, ale i slinami vyvolaná imunita se ukázala být druhově specifická, což znesnadňuje vyvinutí univerzální vakcíny založené na slinách, která by byla aplikovatelná pro různé kombinace parazit-vektor.

Zároveň bylo popsáno, že složení slinných žláz je více konzervativní mezi fylogeneticky příbuznými druhy flebotomů, což může vyvolat u pobodaného a následně infikovaného hostitele zkříženou protekci. Na základě těchto poznatků jsme se zaměřili na prověření možnosti zkřížené reakce mezi dvěma blízce příbuznými druhy flebotomů *P. papatasi* a *P. duboscqi*, kteří jsou zároveň významnými vektory *L. major*. Protekce myší proti leishmaniím byla pozorována nejen u myší vystavených sání *P. papatasi* a infikovaných v přítomnosti homologního slinného homogenátu, ale také u skupiny myší infikovaných v přítomnosti homogenátu získaného z druhu *P. duboscqi*. Obě zmiňované skupiny se nelišily v množství parazitů, aktivitě makrofágů či v protilátkové odpovědi namířené proti slinám nebo parazitům. Tyto výsledky potvrzují částečnou zkříženou protekci myší proti leishmaniím mezi dvěma příbuznými druhy flebotomů.

Přestože se antigenní podobnost slinných proteinů uplatňující se mezi více druhy flebotomů jeví jako užitečná při konstrukci vakcíny, její nevýhody se odráží ve studiích testující expozici sání. Přítomnost zkřížené reakce mezi sympatricky se vyskytujícími druhy flebotomů může vést k získání falešně pozitivních výsledků. Na myším modelu jsem testovala míru druhové specificity slinného homogenátu *P. orientalis.* Použitý antigen se ukázal být silně rozpoznáván pouze protilátkami z myší poštípaných *P. orientalis*. Míra rozpoznání heterologními protilátkami, tj. z myší pobodaných sympatricky se vyskytujícími druhy flebotomů na území severní Etiopie, dosahovala úrovně negativních kontrol. Tato vysoce druhově specifická reakce myších protilátek proti slinám *P. orientalis* naznačuje podobnou specifitu i u jiných hostitelů. Výsledky serologického testování imunitní odpovědi proti *P. orientalis* podporují hypotézu o úloze domácích zvířat v životním cyklu *L. donovani*.

Dodnes je ve studiích, zabývajících se využitím protilátek proti slinám flebotomů k testování míry pobodání, nejčastěji používaným antigenem slinný homogenát. Vhodnou náhradou by mohly být rekombinantní slinné proteiny. Na vzorcích z rozsáhlých epidemiologických studií jsme potvrdili užití rekombinantního yellow proteinu získaného z *P. perniciosus* jako účinného ukazatele expozice. Korelace vazby IgG protilátek na oba výše zmíněné antigeny byla vysoce signifikantní. Navíc bylo pozorováno, že rekombinantní yellow protein zachycuje dynamiku protilátek srovnatelně s celým homogenátem a je využitelný v celém areálu rozšíření druhu *P. perniciosus*.

INTRODUCTION

Sand flies (Diptera: Phlebotominae) are medically and veterinary important bloodfeeding insects that transmit parasites from the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). These protozan parasites are the causative agents of leishmaniases, neglected infection diseases which affect people in 98 countries. They are manifested by different clinical symptoms, ranging from disfiguring cutaneous and muconasal form to fatal visceral form, if left untreated. The outcome of infection is influenced by the virulence of the parasite strain but also by host´s genetic background and immune status [reviewed in (Antinori et al., 2012; Savoia, 2015)]. The annual incidence was estimated to be approximately 0.2-0.4 and 0.7-1.2 million cases for visceral and cutaneous leishmaniasis, respectively. This burden ranks leishmaniasis to the ninth place of all human infectious diseases e.g. (Hotez et al., 2004; Alvar et al., 2012; WHO, 2014).

The parasites are transmitted to the vertebrate hosts by the bites of female sand flies from the genus *Phlebotomus* and *Sergentomyia* in the Old World or *Lutzomyia* in the New World [reviewed in (Maroli et al., 2013)]. In the gut of the invertebrate vector, *Leishmania* parasites occur in several morphological forms of extracellular flagellated promastigotes [described for example in (Rogers et al., 2002)], while in vertebrate host they occur as immobile amastigotes inside phagocytic cells, mainly macrophages. Macrophages are able to kill or to long-term host intracellular forms of *Leishmania* sp. depending on a balance between the ability of the immune response to sufficiently activate *Leishmania*-infected macrophages and the ability of the parasites to resist cytotoxic macrophage mechanisms [reviewed in (Van Assche et al., 2011; Horta et al., 2012)].

The success of infection by *Leishmania* parasites is a result of a long host-parasite co-evolutionary process and it is linked with the ability of the parasite to manipulate vertebrate host immune system in its favor. Affecting the host immune response occurs not only by means of molecules produced by parasites, but also by vector saliva which are obligately injected into the feeding site during transmission as well as during non-infectious feeding. In the following text, I would like to focus mainly (1) on the role of sand fly saliva as antigenic and immunomodulatory factors in *Leishmania* infection development and (2) on the utilization of host anti-saliva immune response in epidemiological studies.

1 Sand fly saliva

Sand fly salivary apparatus consists of salivary glands (lobes), salivary ducts, a salivary pump and a salivary channel. Salivary glands are a paired, hollow organ surrounded by a single layer of epithelium (Adler and Theodor, 1926; Abdel-Badei et al., 2012). The lobes can be heterogeneous or homogeneous (identical in size and shape) depending on the sand fly species. The bigger, fully inflated gland of *Phlebotomus papatasi* may reach about 180 x 140 μm (Adler and Theodor, 1926; Abdel-Badei et al., 2012). The composition of sand fly saliva differs not only among different species (Volf et al., 2000; Volf and Rohousová, 2001) but the difference can be sometimes detected also among populations originating from distinct geographical areas (Warburg et al., 1994; Lanzaro et al., 1999; Volf et al., 2000; Rohousova et al., 2012b; Ramalho-Ortigão et al., 2015). An important difference was described between blood-feeding females and non-hematophagous males; the concentration of salivary proteins from *P. duboscqi* saliva was almost 30 times higher in case of females compared with males. The number of bands revealed by SDS PAGE also differed considerably between genders; in females, eight major bands were detected whereas just one was observed in males (Volf et al., 2000). Concurrently, the number of salivary proteins grows directly with the female age when a complete SDS PAGE salivary profile has been achieved on day 3 and 5 in females maintained at 26 °C and room temperature, respectively (Volf et al., 2000; Coutinho-Abreu et al., 2010).

During the process of taking a blood meal, the skin of vertebrate hosts is damaged by the proboscis of sand flies. The host fights back by means of 3 effective systems including hemostasis, inflamation and immunity which hinder the successful feeding of the insect. Sand fly saliva is composed of pharmacologically active components called sialogenins with anti-hemostatic, anti-inflammatory and immunomodulatory properties which help to circumvent this inhospitable host environment and to successfully finish the bloodmeal [reviewed in (Francischetti, 2010; Abdeladhim et al., 2014)].

1.1 Saliva in hemostasis and blood feeding

Hemostasis is a physiological process by which the hosts can control the loss of blood after injury, including insect bite. It consists of three phenomena: platelet aggregation, blood coagulation and vasoconstriction, which form the first major barriers for sand flies to successfully obtain blood [reviewed in (Ribeiro and Francischetti, 2003; Abdeladhim et al.,

2014)]. Sand flies circumvent this feeding problem by producing various salivary components that counteract the host´s hemostatic system.

The most common enzyme confirmed in several bloodsucking arthopods [reviewed in (Francischetti, 2010)] is an apyrase which hydrolyzes nucleotide triphosphates (ATP) and diphosphates (ADP) to a monophosphate (AMP) and an inorganic phosphate (Pi). This hydrolytic activity prevents the platelet aggregation which is normally induced by ADP released from damaged cells and activated platelets at the feeding site. Three classes of apyrases have already been characterized: "5´-nucleotidase family" isolated for the first time from salivary glands of *Aedes aegypti* (Champagne et al., 1995); "CD 39 family of nucleotidases" isolated from flea *Xenopsylla cheopis* (Andersen et al., 2007) and "Cimex family" originally identified in the bedbug *Cimex lectularius* (Valenzuela et al., 1998), later discovered in sand flies *Phlebotomus papatasi* (Valenzuela et al., 2001b) and *Lutzomyia longipalpis* (Charlab et al., 1999). To date, apyrase was found also in other species studied: *P. orientalis* (Vlkova et al., 2014), *P. argentipes* and *P. perniciosus* (Anderson et al., 2006), *P. arabicus* (Hostomska et al., 2009), *P. duboscqi* (Kato et al., 2006), *P. sergenti* and *P. tobbi* (Rohousova et al., 2012a), *P. ariasi* (Oliveira et al., 2006), *Lu. intermedia* (de Moura et al., 2013) and *Lu. ayacuchensis* (Kato et al., 2013).

Abundant salivary proteins with anti-hemostatic properties, commonly occurring in all sand fly transcriptomes, are yellow-related proteins serving as the biogenic amine binding proteins [reviewed in (Abdeladhim et al., 2014)]. Xu *et al*. described that yellow proteins from saliva of *Lu. longipalpis* bind amines such as histamine, serotonin or catecholamines with the pro-hemostatic function (Xu et al., 2011).

Another plentiful family of salivary proteins occurring in sandflies is a group of SP15 proteins belonging to odorant-binding proteins [reviewed in (Abdeladhim et al., 2014)]. Two SP15 proteins isolated from *P. duboscqi* (SP15a and SP15b) bind with high affinity to negatively charged surface of polymers including polyphosphate, heparin and dextran sulfate, whereby compete for the binding sites with coagulation factor XII and inhibit coagulation (Alvarenga et al., 2013).

Another compound neutralizing the host´s hemostatic process, isolated from the salivary glands of *Lu. longipalpis*, is a vasodilator peptide named maxadilan, which promotes an increase in blood flow and facilitates feeding (Ribeiro et al., 1986; Lerner et al., 1991). The vasodilator similar to maxadilan was not found in Old World sand fly species but in *P. papatasi* saliva, large amounts of purines 5´AMP and adenosine were revealed (Ribeiro et al., 1999). Adenosine was previously described as a strong platelet aggregation inhibitor (Edlund

et al., 1987; Dionisotti et al., 1992) which incerases concentration of platelet cyclic AMP. Simultaneously, both substances (5´AMP and adenosine) are known for their vasodilatory functions (Collis, 1989).

In *Lutzomyia longipalpis*, anticoagulant named Lufaxin was recently described (Collin et al., 2012). It is a potent inhibitor of factor Xa, which normally plays a key role in the coagulation cascade leading to trombin production and fibrin clot formation. The blockage of this factor prevents blood coagulation in the feeding site (Collin et al., 2012). Homologues of Lufaxin were found in all sand flies studied so far (Valenzuela et al., 2004; Anderson et al., 2006; Kato et al., 2006; Oliveira et al., 2006; Hostomska et al., 2009; Abdeladhim et al., 2012; de Moura et al., 2013; Kato et al., 2013; Martín-Martín et al., 2013; Vlkova et al., 2014) but their function have not yet been confirmed.

Hyaluronidases and endonucleases belong among other commonly occurring salivary components. They are not directly associated with disruption of hemostasis but facilitate feeding [reviewed in (Ribeiro et al., 2010)]. Hyaluronidase is an enzyme that degrades hyaluronan and some other glycosaminoglycans occuring in extracellular matrix of the host skin (Volfova et al., 2008). Enzymatic activity of hyaluronidases seems to be substantial for insects taking blood from superficial hemorrhagic pools, including sand flies. This enzyme is often called "spreading factor" because of the ability to decrease the skin matrix viscosity around feeding site, and hence easily spread other pharmacologically active compounds present in saliva (Volfova et al., 2008). To date, the enzymatic activity of hyaluronidases has been found in all tested *Phlebotomus* and *Lutzomyia* species (Charlab et al., 1999; Cerna et al., 2002; Volfova et al., 2008; Hostomska et al., 2009; Rohousova et al., 2012a; Vlkova et al., 2014).

The release of host DNA and thereby the lowering of local viscosity is also caused by salivary endonucleases [reviewed in (Ribeiro et al., 2010)], for example by endonuclease described from *Lutzomyia longipalpis* (Valenzuela et al., 2004) named Lundep. The catalytic activity of Lundep is responsible for the destroying of neutrophil extracellular traps, which normally promote thrombus organization and stability, and is also known for anti-coagulant properties (inhibiting the activation of factor XIIa) (Chagas et al., 2014).

A list of above mentioned salivary components is not complete but a more detailed description of functionally known sialogenins is beyond the scope of this thesis. More thorough overview of sand fly salivary coctail is summarized for example in [(Ribeiro et al., 2010; Abdeladhim et al., 2014)].

1.2 Immunomodulating effect of sand fly saliva affecting the macrophage functions

Apart from anti-hemostatic properties, sand fly saliva is chemotactic for different immune cells, thereby modifying inflammatory processes at the feeding site. Saliva of *P. papatasi*, *Lu. longipalpis* and *P. duboscqi* significantly enhanced positive chemotaxis for macrophages thus accelerating the entry of parasites into these cells (Anjili et al., 1995; Zer et al., 2001). In an air pouch model, Teixeira *et al.* observed macrophages influx after an addition of *Lu. longipalpis* saliva in BALB/c and C57BL/6 mice (Teixeira et al., 2005). Salivary gland homogenate (SGH) of *Lu. longipalpis* induced a significant attraction of macrophages in BALB/c strain directly correlating with the higher chemokine expression of CC chemokine ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1). On the other hand, in C57BL/6 mice, expression of these chemokines was weak. It means that the same salivary components can cause different inflammatory effect according to host background (Teixeira et al., 2005).

The effect of sand fly saliva on a variety of macrophage functions was examined in detail especially for Old World species *P. papatasi* and New World sand fly *Lu. longipalpis*. Hall and Titus described that saliva of *P. papatasi* inhibits the ability of interferon gamma (IFN-γ) to activate macrophages to the production of nitric oxide (NO) which facilitates parasites survival (Hall and Titus, 1995). Indeed, an addition of *P. papatasi* salivary gland homogenate to macrophages caused reduction in inducible nitric oxide synthase (iNOS) mRNA expression (Mbow et al., 1998; Waitumbi and Warburg, 1998). Later, the small, ethanol-soluble salivary molecule resistant to boiling was defined to be responsible for this down-regulation of the iNOS gene expression and reduction of NO production through the inhibition of protein phosphatase 1 and protein phosphatase 2A (Waitumbi and Warburg, 1998), two phosphatases with key function in the signaling pathway leading to nitric oxide synthesis (Dong et al., 1995). One year later, this phosphatases inhibitors were revealed as 5´AMP and adenosine (Ribeiro et al., 1999). The adenosine itself was able to reduce the iNOS gene expression to the same degree as *P. papatasi* saliva (Katz et al., 2000).

Moreover, the ability of *P. papatasi* saliva to decrease the secretion of pro-inflammatory cytokines and to enhance the production of anti-inflammatory cytokines which modulate macrophage effector functions was described (Mbow et al., 1998; Rogers and Titus, 2003). Salivary gland lysate of *P. papatasi* inhibited IL-12 and IFN-γ expression, while the expression of IL-4 mRNA was up-regulated in mice (Mbow et al., 1998). The cellular immune response against the saliva of *P. papatasi* in humans naturally exposed to sand fly bites was characterized by high levels of IL-10 (Abdeladhim et al., 2011). The polarization of

immune response towards Th2 was also observed after addition of adenosine alone – the production of IL-12, IFN-γ and TNF-α was decreased (Hasko et al., 1996; Hasko et al., 1998; Link et al., 2000) while IL-10 was increased (Le Moine et al., 1996).

A similar effect of saliva on host immunity was also observed in the case of *Lu. longipalpis*. Saliva induced an increase in IL-6, IL-8 and IL-12p40 production, but decreased TNF-α and IL-10 production by LPS-stimulated human monocytes (Costa et al., 2004). On the contrary, increased level of IL-10 associated with decreased NO production was observed in bone marrow derived macrophages exposed to *Lu. longipalpis* SGH (Norsworthy et al., 2004). Aforementioned observation confirms that genetic differences among hosts may influence the immune responses elicited by salivary proteins from the same sand fly species. In addition, maxadilan itself was described to modulate host immune response to a similar degree as the whole saliva (Morris et al., 2001). Maxadilan upregulates the cytokines associated with a type 2 response such as IL-10, IL-6 and TGF-β but downregulates type 1 cytokines such as IL-12p70, IFN-γ and TNF-α (Soares et al., 1998; Rogers and Titus, 2003; Brodie et al., 2007).

Further, *Lu. longipalpis* saliva was shown to induce lipid body formation and prostaglandin E_2 (PGE_2) production by peritoneal macrophages *ex vivo* and *in vitro* (Araújo-Santos et al., 2010). PGE_2, eicosanoid derived from arachidonic acid, is mostly produced in cytoplasmic organelles called lipid bodies, which are created in leukocytes and other cells in response to inflammatory stimuli [reviewed in (Bozza et al., 2009)]. Prostaglandin contributes to the development of an anti-inflammatory response and has also vasodilatory effect (Araújo-Santos et al., 2010). An increasing production of PGE_2 by macrophages was also shown after addition of maxadilan alone (Soares et al., 1998).

1.3 Effect of saliva on leishmaniasis

If a sand fly delivers *Leishmania* parasite, it will be co-inoculated with saliva to the same skin site. Thereafter, parasites can benefit from this by means of vector saliva-altered site [reviewed in (Abdeladhim et al., 2014)].

The above mentioned chemotactic effect of saliva (see chapter 1.2) was more pronounced when the *Leishmania* parasites were added to inoculum; the greater number of recruited neutrophils and macrophages was observed (Teixeira et al., 2005). The phagocytes influx was benefitial to parasites because of their early entry into these cells. Promastigotes that fail to get internalized into the professional phagocytes are rapidly degraded by cytotoxic

activity of natural killer cells, neutrophils and eosinophils in vertebrate host (Pimenta et al., 1987). Therefore, it is essential for promastigotes to invade macrophages as quickly as possible.

The importance of neutrophils as the first recruited host cells to the feeding site and for the pathogen entry was confirmed by Peters et al., (2008). *Leishmania* can survive temporarily inside neutrophils which protect parasites from hostile extracellular host environment [reviewed in (Ritter et al., 2009)]. The sand fly saliva alone or in a combination with *Leishmania* parasites was described as robust stimulus for an accumulation of neutrophils at the inoculation site in murine or hamster models (Silva et al., 2005; Teixeira et al., 2005; Monteiro et al., 2007; Peters et al., 2008; Vasconcelos et al., 2014). Moreover, Prates *et al.* showed that salivary gland sonicate (SGS) of *Lu. longipalpis* enhance caspase-dependent and Fas ligand-mediated neutrophil apoptosis associated with a enhanced *Leishmania* survival inside these cells. At the same time, neutrophils incubated with *L. chagasi* plus SGS produced significantly higher amounts of MCP-1 (CCL2), a chemokine which attracts number of macrophages for clearance of these recruited infected neutrophils (van Zandbergen et al., 2004; Prates et al., 2011). Van Zandbergen *et al*. suggested that infected apoptotic neutrophils can serve as "Trojan horses" to transfer *Leishmania* silently to macrophages inside apoptotic neutrophils (van Zandbergen et al., 2004). Later, "Trojan rabbit" hypothesis was admitted; in this scenario, *Leishmania* parasites escaped from dying neutrophils before ingestion by macrophages (Peters et al., 2008; Ritter et al., 2009).

Moreover, Theodos and Titus described that presence of saliva may be responsible also for the inhibition of *Leishmania* T-cell specific response because of macrophages inability to present leishmanial antigens to parasite specific T cells (Theodos and Titus, 1993). The decreased capability to present antigen was mediated by changing the costimulatory molecules expression (CD80, HLA-DR) on surface of LPS-treated human macrophages (Costa et al., 2004).

The effect when sand fly saliva exacerbates the infection caused by *Leishmania* sp. is called "enhancing effect". *Leishmania major* co-injected with *Lu. longipalpis* or *P. papatasi* saliva resulted in a more severe disease reflected by a larger lesion when compared with a group of mice receiving parasites alone (Titus and Ribeiro, 1988; Theodos et al., 1991). Co-inoculation of *P. papatasi* SGH with *L. major* even converted naturally resistant mouse strain (C57BL/6) into a non-healing phenotype associated with an early increase of epidermal cells producing type 2 cytokines (Belkaid et al., 1998). To date, saliva-mediated enhancing effect has also been shown for other *Leishmania*-sand fly combinations; e. g. *Lu. longipalpis - L.*

braziliensis (Samuelson et al., 1991), *Lu. longipalpis - L. amazonensis* (Theodos et al., 1991), *Lu. longipalpis - L. chagasi* (Warburg et al., 1994), *Lu. longipalpis - L. mexicana* (Samuelson et al., 1991), *Lu. whitmani - L. braziliensis* (Bezerra and Teixeira, 2001) and *P. duboscqi - L. major* (Rohousova et al., 2011). What is even more important is the fact that enhancing effect is unique only for sand fly saliva. Saliva from *Aedes aegypti*, *Rhodnius prolixus* or *Ixodes scapularis* did not enhance *L. major* infectivity in mice (Titus and Ribeiro, 1988).

Conversely, mice repeatedly exposed to SGH or to uninfected sand flies were protected against *Leishmania* infection (Belkaid et al., 1998; Kamhawi et al., 2000). The reason is that many of the salivary components are able to induce specific immunity - both cellular and humoral as shown in Figure 1. Therefore, the protective immunity was hypothetized to be mediated by neutralizing antibodies, or delayed-type hypersensitivity (DTH) reaction at the bite site formed by a cellular influx as a response to salivary antigens (Belkaid et al., 2000; Kamhawi et al., 2000). Although both possibilities are not mutually exclusive, later studies proved that the protection is due to DTH reaction and enhanced IFN-γ/IL-12 production (Belkaid et al., 1998; Kamhawi et al., 2000; Valenzuela et al., 2001a). This was further confirmed by the experiments conducted on B lymphocytes deficient mice that were also protected after vaccination with saliva-derived plasmid and challenged with *L. major* plus SGH of *P. papatasi* (Valenzuela et al., 2001a). The feeding site may be changed by the presence of DTH and inflammatory cytokines elicited by sand fly salivary antigens which create an inhospitable environment for *Leishmania* parasites (Belkaid et al., 2000). As a bystander effect, this saliva-elicited immunity may even induce protection to *Leishmania* parasites [reviewed in (McDowell, 2015)].

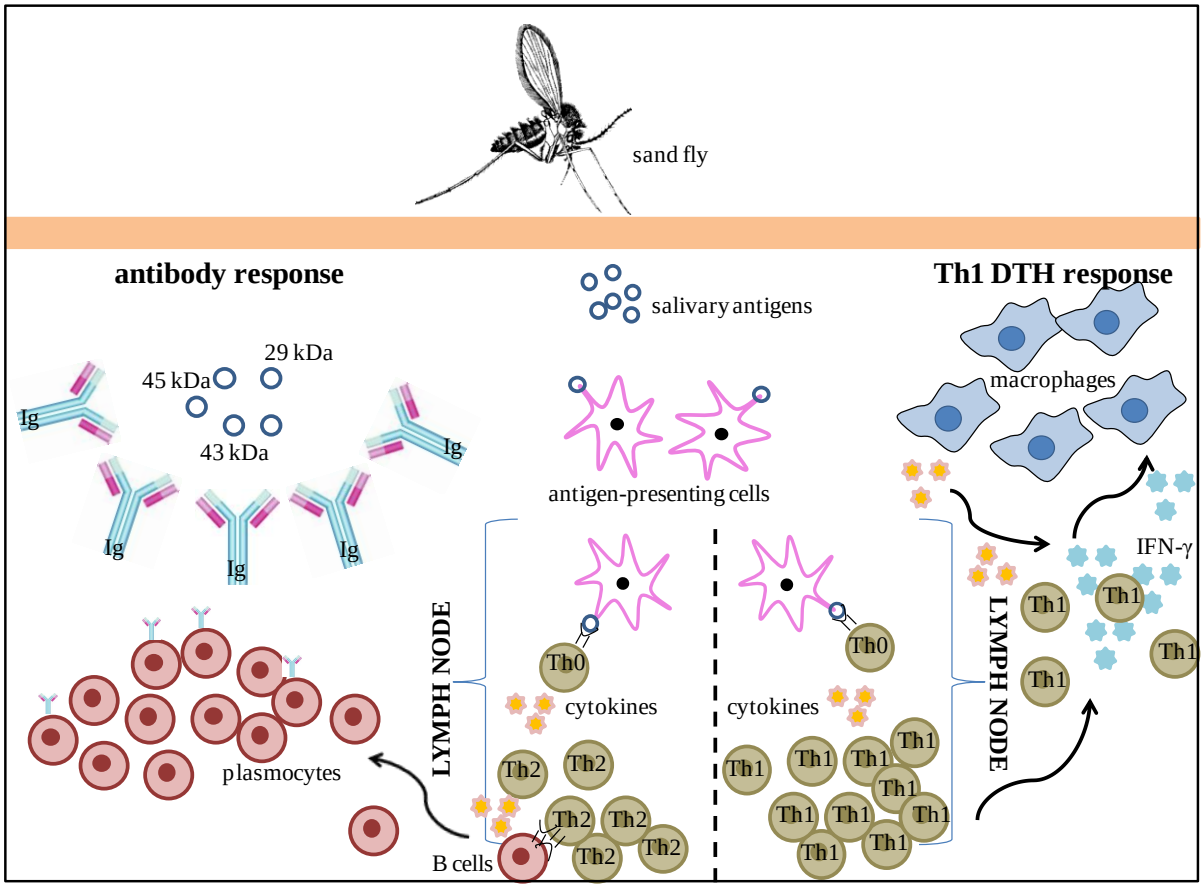

Figure 1: Hypothetical model depicting the immune response in repeatedly exposed host to sand fly bites, modified according to Gomes and Oliveira, 2012; Andrade and Teixeira, 2012.

In laboratory settings, protection against leishmaniasis caused by *L. major*, *L. amazonensis* and *L. braziliensis* due to anti-saliva cellular immunity was well described in rodent models [reviewed in (Gomes and Oliveira, 2012)]. The protectivity was elicited by both injection of *P. papatasi* (Belkaid et al., 1998) and *Lu. longipalpis* SGH (Thiakaki et al., 2005; Tavares et al., 2011; Xu et al., 2011) or by exposure to *P. papatasi* (Kamhawi et al., 2000) and *P. duboscqi* (Rohousova et al., 2011) bites. The protective effect of saliva was demonstrated as smaller lesion size correlated with the decreased parasite burden (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005; Rohousova et al., 2011; Tavares et al., 2011; Xu et al., 2011). Moreover, pre-exposure to SGH/saliva of sand flies shifted immune response toward Th1 responsiveness characterized by incerased IFN-γ and IL-12 production (Kamhawi et al., 2000; Xu et al., 2011) or by higher IFN-γ /IL-4 ratio (Belkaid et al., 1998) compared with non-immunized group. It may activate infected macrophages for killing during the early phase of infection and may also promote faster *Leishmania*-specific T helper cell type 1 response. On the other hand, type 2 cytokines such as IL-4 (Belkaid et al., 1998), IL-10 and TGF-β (Tavares et al., 2011) were reduced in pre-exposed mice.

In some experimental models, the protective effect of pre-exposure to sand fly saliva or SGH was not pronounced. Exposure to *Lu. intermedia* SGH shifted the immune response to unprotective Th2 type in BALB/c mice (de Moura et al., 2007). SGH immunized mice developed larger lesions that retained for a longer period when compared with PBS-inoculated mice (de Moura et al., 2007).

It was shown that immunization of the host with individual salivary molecules may have diverse effect on *Leishmania* infection contrary to whole saliva. Oliveira *et al.* showed that although the immunization of mice with *P. papatasi* SGH protected mice from *L. major* infection (Belkaid et al., 1998), some salivary proteins from this species contributed to contrasting outcomes of *L. major* infection (Oliveira et al., 2008). While mice immunized with PpSP15 showed persistent protection, immunization with PpSP44 enhanced the infection; the outcome of infection was associated with an anti-*Leishmania* Th1 and Th2 immune response, respectively (Oliveira et al., 2008). In the model of *Lu. intermedia* - BALB/c - *L. braziliensis*, the plasmid coding for a Linb-11 protein was shown as a potent inducer of a cellular immune response conferring protection against *L. braziliensis* infection (de Moura et al., 2013), contrary to the exacerbating effect of whole saliva (de Moura et al., 2007).

The protective effect against leishmaniasis was demonstrated also for several individual peptids and proteins of *Lu. longipalpis* saliva (Morris et al., 2001; Gomes et al., 2008; Xu et al., 2011). CBA mice injected with synthetic maxadilan were partly protected against challenge with *L. major* plus SGH from *Lu. longipalpis* (Morris et al., 2001). Cutaneous lesions were severalfold smaller, healing by day 50 of infection and parasite burden was reduced in a vaccinated group. Simultaneously, an addition of maxadilan to lymph node cells *in vitro* caused a release of IFN-γ and NO (Morris et al., 2001). DNA plasmid coding for LJM19, belonging to odorant-binding proteins group, protected hamsters against infection of *L. infantum* mixed with SGH of *Lu. longipalpis* (Gomes et al., 2008). The protection was demonstrated by increased IFN-γ/TGF-β ratio and iNOS expression in the spleen and liver till 5 months post infection when compared with control group (Gomes et al., 2008). Immunization with the yellow-related protein LJM11 or with plasmid coding for LJM11 protected mice against *L. major* infection (Xu et al., 2011; Gomes et al., 2012). The increased production of IFN-γ in splenocytes after stimulation with LJM11 showed that immunity to this protein is Th1-based which was reflected in a smaller lesion size and lower parasite burden (Xu et al., 2011). This long-lasting immunity resulted in protection against *L.*

major and was observed when parasite was incorporated into hosts by needle injection but also when transmitted by vector bites (Gomes et al., 2012).

Protection caused by salivary proteins was also described for Old World species, *P. papatasi* and *P. duboscqi* (Valenzuela et al., 2001a; Oliveira et al., 2015). Vaccination with PpSP15 isolated from *P. papatasi* affected disease progression caused by *L. major* in mice; lesion size and parasite load were significantly smaller compared with controls (Valenzuela et al., 2001a). Nonhuman primates (rhesus macaques) immunized by the homologue of aforementioned PpSP15, isolated from *P. duboscqi* (PdSP15), were protected against *L. major* transmitted by infected sand fly bites (Oliveira et al., 2015). Protection correlated with an early appearance of *Leishmania*-specific $CD4^{+}IFN\text{-}\gamma^{+}$ lymphocytes which was reflected in reduced parasite burden compared to controls. Moreover, the immunogenicity of recombinant PdSP15 was tested in inhabitants living in endemic area of Mali. The ability of SP15 to recall a pro-inflammatory response in humans naturally exposed to *P. duboscqi* bites was shown (Oliveira et al., 2015). When human peripheral blood mononuclear cells were stimulated by SGH or recombinant PdSP15, the significant levels of IFN-γ, IL-10 and IL-17 were produced compared to medium. Actually, rSP15 was able to induce release of IFN-γ to similar degree as the whole SGH, inferring rPdSP15 as a potent Th1-inducing salivary protein in humans and therefore a promising vaccine candidate against human cutaneous leishmaniasis (Oliveira et al., 2015).

However, sand fly vectors differ in composition of the saliva [reviewed in (Ribeiro et al., 2010)] and the protection elicited by salivary proteins was shown to be species-specific (Thiakaki et al., 2005). *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). Nevertheless, the saliva-based vaccine could be cross-protective between phylogenetically related vector species with more conserved salivary proteins and as such, it could be applicable in more endemic foci. Sharing similar salivary antigens is one of the conditions required for the successful cross-protection against *Leishmania* infection. For the first time, the cross-protective effect was demonstrated between the *Lutzomyia* species; *Lu. longipalpis* and *Lu. intermedia*, vectors of *L. brasiliensis* (Tavares et al., 2011). 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 demonstrated by reduced number of parasites in the inoculated ear and in the draining lymph node (Tavares et al., 2011). As was shown, SDS-PAGE salivary profiles obtained from both species display bands migrating at similar

molecular weight (de Moura et al., 2007). Up to date, similar cross-protective study was not performed for *Phlebotomus* species.

1.4 Antibody response to saliva and their utilization to estimate sand fly exposure

Repeated exposures to sand fly bites or saliva elicite both arms of adaptive immunity; antibody- and cell-mediated. Antibodies have been characterized after sand fly bites or injection of saliva in humans and several animal models in laboratory settings as well as in endemic areas [reviewed in (Andrade and Teixeira, 2012; Gomes and Oliveira, 2012)]. Examples of studies testing the presence of antibodies are given in the following table (Table 1).

Table 1: Example of studies showing the presence of anti-sand fly saliva antibodies in repeatedly exposed humans and animals

type of exposure			exposure to			
experimental	natural	sand fly sp.	saliva	SGH	host sp.	reference
√		*Lu. longipalpis*	√		dogs	Collin *et al*. 2009
√		*Lu. longipalpis*	√		dogs	Hostomska *et al*. 2008
√		*Lu. longipalpis*	√		mice	Rohousova et.al. 2005, Thiakaki *et al*. 2005
√		*Lu. longipalpis*	√		mice	Silva *et al*. 2005
√		*Lu. longipalpis*	√		human	Vinhas *et al*. 2007
	√	*Lu. longipalpis*	√		human	Aquino *et al*. 2010
	√	*Lu. longipalpis*	√		dogs	Bahia *et al*. 2007
	√	*Lu. longipalpis*	√		human	Barral *et al*. 2000
	√	*Lu. longipalpis*	√		human	Gomes *et al*. 2002
	√	*Lu. longipalpis*	√		foxes	Gomes *et al*. 2007
	√	*Lu. longipalpis*	√		chicken	Soares *et al*. 2013
	√	*Lu. longipalpis*	√		human	Souza *et al*. 2010
√		*Lu. intermedia*		√	mice	de Moura *et al*. 2007
	√	*Lu. intermedia*	√		human	
√		*P. papatasi*	√		mice	Rohousova et.al. 2005, Thiakaki *et al*. 2005
	√	*P. papatasi*	√		human	Rohousova *et al*. 2005
	√	*P. papatasi*	√		human	Clements *et al*. 2010
	√	*P. papatasi*	√		human	Marzouki *et al*. 2011
√		*P. perniciosus*	√		mice, rabbits	Martín-Martín *et al*. 2015
√		*P. perniciosus*	√		dogs	Vlkova *et al*. 2011
	√	*P. perniciosus*	√		dogs	
	√	*P. perniciosus*	√		leporids, dogs	Martín-Martín *et al*. 2014
√		*P. argentipes*	√		hamsters	Ghosh and Mukhopadhyay 1998
	√	*P. argentipes*	√		human	Clements *et al*. 2010
√		*P. sergenti*	√		mice	Rohousova et.al. 2005, Thiakaki *et al*. 2005
√		*P. sergenti*	√		mice	Drahota *et al*. 2009
	√	*P.sergenti*	√		human	Rohousova *et al*. 2005
√		*P. duboscqi*	√		mice	Rohousova *et al*. 2011
√		*P. ariasi*		√	mice	Oliveira *et al*. 2006

In mice, repeated exposure to sand fly bites or salivary gland homogenate resulted in increased level of anti-saliva IgG antibodies represented mainly by the IgG1 subclass (Silva et al., 2005; Oliveira et al., 2006; de Moura et al., 2007; Vlkova et al., 2012; Martín-Martín et al., 2015). In sera of immunized dogs, a significant increase of anti-saliva IgG and IgE antibodies was observed after exposure to *Lu. longipalpis*. However, only IgG (and IgG2 subclass) correlated with sand fly exposure intensity (Hostomska et al., 2008; Collin et al., 2009). Anti-saliva IgG and IgG2 were observed also in canine sera from dogs exposed to *P. perniciosus* bites (Vlkova et al., 2011). Individuals living in area endemic for *Lu. longipalpis* or volunteers exposed to uninfected laboratory-reared females of *Lu. longipalpis* developed predominantly IgG1 and IgE anti-saliva antibodies (Gomes et al., 2002; Vinhas et al., 2007). On the other hand, antibody response to the saliva of *P. papatasi* in children living in Tunisia was prominently of IgG4 isotype and at a lesser extent of the IgG2 and IgG1 subclasses (Marzouki et al., 2011). These results show that in humans, antibody response to sand fly saliva may differ among genetically variable host populations and it could be also influnced by sand fly species.

In endemic areas, sand fly population fluctuates seasonally [reviewed in (Maroli et al., 2013)] which may influence host anti-saliva antibody response. There are several studies focusing on the long term kinetics of anti-saliva antibodies in mice (Vlkova et al., 2012; Martín-Martín et al., 2015), dogs (Hostomska et al., 2008; Vlkova et al., 2011), humans (Vinhas et al., 2007; Clements et al., 2010) or rabbits (Martín-Martín et al., 2015). In humans repeatedly bitten by *P. argentipes*, specific antibodies significantly declined within 30 days of sand fly-free period, although they have persisted in low levels for 5 months after the last sand flies exposure (Clements et al., 2010). An increased specific anti-saliva antibody response was still detected in dogs and mice after 6 months bitting-free period of *Lu. longipalpis* and *P. papatasi*, respectively (Hostomska et al., 2008; Vlkova et al., 2012). However, a rapid antibody decrease in canine sera was observed already one week after last *P. perniciosus* exposure (Vlkova et al., 2011) reflecting changes in the vector exposure intensity. Importantly, after the six-month or one-year bites-free period further re-exposure with *P. argentipes* or *Lu. longipalpis* bites caused significant increase of antibody levels in humans (Vinhas et al., 2007; Clements et al., 2010), which indicates an antibody memory response to saliva for both sand fly species. An effective recall immune response was observed also in mice and rabbits bitten by *P. perniciosus* (Vlkova et al., 2012; Martín-Martín et al., 2015).

Antibody response elicited by sand fly salivary proteins was shown to be species-specific, e.g. (Volf and Rohousová, 2001; Rohousova et al., 2005; Thiakaki et al., 2005; de

Moura et al., 2007; Gomes et al., 2007; Teixeira et al., 2010; Marzouki et al., 2012). Mice exposed individually to *P. papatasi*, *P. sergenti* or *Lu. longipalpis* produced antibodies specific to the respective species (Thiakaki et al., 2005). Sand fly species-specific salivary antigen was also observed among *P. perniciosus*, *P. halepensis* and *P. papatasi* (Volf and Rohousová, 2001). Even though salivary profile of *Lu. longipalpis* and *Lu. intermedia* is similar, their antigenic properties seemed to be different (de Moura et al., 2007); serum samples from mice immunized with SGS of *Lu. intermedia* recognized only one *Lu. longipalpis* SGS protein of about 45kDa (de Moura et al., 2007). Moreover, the antigenicity of salivary proteins is also host species-specific (Rohousova et al., 2005; Martín-Martín et al., 2012; Martín-Martín et al., 2014). Several differences in the recognition pattern were observed between hamster and murine anti-*P. perniciosus* antibodies. While yellow-related proteins and apyrases were recognized by both rodents, D7- related proteins were found to be antigenic only for hamster sera (Martín-Martín et al., 2012). Interestingly, some *P. perniciosus* salivary antigens were specifically recognized solely by hare or rabbit anti-*P. perniciosus* antibodies, while some salivary antigens were common to those host species, despite the intensity of reaction showing individual pattern (Martín-Martín et al., 2014). Main salivary bands identified in *P. papatasi* and *P. sergenti* saliva reacted with mouse as well as with human sera, nevertheless differences were observed in the intensity of reaction (Rohousova et al., 2005). In summary, salivary proteins antigenic for broad spectrum of bitten hosts belong to yellow-related proteins, apyrases, D7-related proteins and antigen 5-related proteins. The comprehensive summary of these immunogenous salivary proteins is shown in Table 2.

While cellular immunity is responsible rather for protection against leishmaniasis (Belkaid et al., 1998; Kamhawi et al., 2000; Valenzuela et al., 2001a), anti-saliva antibodies correlate well with the intensity of exposure (Hostomska et al., 2008; Vlkova et al., 2011; Vlkova et al., 2012; Martín-Martín et al., 2015) and thus can be used in epidemiological studies, e.g. to measure the effectiveness of vector control programmes (Clements et al., 2010; Gidwani et al., 2011). To this date, a significant correlation between levels of specific IgG anti-saliva antibodies and intensity of exposure was documented in mice (Vlkova et al., 2012; Martín-Martín et al., 2015), dogs (Hostomska et al., 2008; Vlkova et al., 2011), humans (Clements et al., 2010) as well as in leporids (Martín-Martín et al., 2015).

Table 2: The most antigenic salivary protein families recognized by sera of repeatedly bitten hosts

protein family	sand fly species	host species	reference
yellow-related	*Lu. longipalpis*	humans	(Gomes et al., 2002)
		mice	(Rohousova et al., 2005)
		dogs	(Bahia et al., 2007)
		dogs	(Hostomska et al., 2008)
		foxes, dogs	(Gomes et al., 2007)
		chickens	(Soares et al., 2013)
	P. arabicus	mice	(Hostomska et al., 2009)
	P. papatasi	mice	(Rohousova et al., 2005)
		mice	(Vlkova et al., 2012)
		humans	(Marzouki et al., 2011)
	P. perniciosus	mice, hamsters	(Martin-Martin et al., 2012)
		mice, rabbits	(Martin-Martin et al., 2015)
		hares, rabbits	(Martin-Martin et al., 2014)
		dogs	(Vlkova et al., 2011)
	P. tobbi	rabbits	(Rohousova et al., 2012a)
apyrase	*Lu. longipalpis*	humans	(Gomes et al., 2002)
		dogs	(Hostomska et al., 2008)
		mice	(Rohousova et al., 2005)
	P. arabicus	mice	(Hostomska et al., 2009)
	P. papatasi	humans	(Rohousova et al., 2005)
		mice	(Vlkova et al., 2012)
		humans	(Marzouki et al., 2011)
	P. perniciosus	mice	(Martin-Martin et al., 2012)
		mice, rabbits	(Martin-Martin et al., 2015)
		hares, rabbits	(Martin-Martin et al., 2014)
		dogs	(Vlkova et al., 2011)
	P. tobbi	rabbits	(Rohousova et al., 2012a)
D7-related	*Lu. longipalpis*	dogs	(Bahia et al., 2007)
		dogs	(Hostomska et al., 2008)
	P. papatasi	humans	(Rohousova et al., 2005)
		mice	(Vlkova et al., 2012)
		humans	(Marzouki et al., 2011)
	P. perniciosus	dogs	(Vlkova et al., 2011)
		hares, rabbits	(Martin-Martin et al., 2014)
		hamsters	(Martin-Martin et al., 2012)
		mice, rabbits	(Martin-Martin et al., 2015)
	P. tobbi	rabbits	(Rohousova et al., 2012a)
antigen-5	*P. papatasi*	mice	(Vlkova et al., 2012)
	P. perniciosus	dogs	(Vlkova et al., 2011)
	P. tobbi	rabbit	(Rohousova et al., 2012a)
SP-15	*P. papatasi*	mice	(Vlkova et al., 2012)
		humans	(Marzouki et al., 2011)
	P. perniciosus	dogs	(Vlkova et al., 2011)
		mice, rabbits	(Martin-Martin et al., 2015)
	P. tobbi	rabbits	(Rohousova et al., 2012a)

Beside serving as a marker of exposure, anti-saliva antibodies specific to *P. sergenti*, *Lu. intermedia* or *P. papatasi* can be also utilized as a risk marker of cutaneous leishmaniasis (Rohousova et al., 2005; de Moura et al., 2007; Marzouki et al., 2011). The higher titer of anti-saliva antibodies suggests more frequent contact with sand flies, thus increasing probability to encounter infected bite (Rohousova et al., 2005; de Moura et al., 2007; Marzouki et al., 2011). Moreover, anti-saliva antibodies were significantly associated with the disease development (de Moura et al., 2007; Marzouki et al., 2011). Therefore it can be used as a marker of risk for *Leishmania* transmission and for development of cutaneous leishmaniasis.

On the other hand, a different scenario seems to be valid for vectors of *Leishmania* causing visceral leishmaniasis [reviewed in (Andrade and Teixeira, 2012; Gomes and Oliveira, 2012)]. In this case, the co-occurrence of anti-saliva antibodies and anti–*Leishmania* DTH reaction was observed in humans (Barral et al., 2000; Gomes et al., 2002; Aquino et al., 2010), suggesting that immune response against SGS correlates with a protective response against leishmaniasis. Moreover, individuals that did not recognize salivary proteins developed anti-*Leishmania* antibodies generally associated with disease progress (Gomes et al., 2002; Miles et al., 2005). However, further studies are needed to validate this hypothesis.

Concurrently, the use of anti-saliva antibodies is a novel approach which can indicate an important blood source for sand flies or parasite hosts and putative reservoirs. The existence of a sylvatic cycle independent on previously well-known domestic cycle was confirmed by using this approach in Brazil (Gomes et al., 2007) and in Spain (Martín-Martín et al., 2014). In both cases, dogs were expected to be the main reservoir hosts of *L. chagasi* and *L. infantum*, respectively [reviewed in (Loría-Cervera and Andrade-Narváez, 2014)]. In Brazil, sylvatic cycle of *L. chagasi* has been revealed among wild foxes (*Cerdocyon thous*) (Gomes et al., 2007); high levels of anti-*Lu. longipalpis* SGH antibodies were found among local foxes but not among those living in region where *Lu. longipalpis* is absent. Infection by *Leishmania* parasites was even detected in three foxes (Gomes et al., 2007). A new wild reservoir of visceral lesihmaniasis was also confirmed in Spain (Molina et al., 2012). Hares (*Lepus granatensis*) caught in periurban green park situated in south-west of Madrid showed higher anti-saliva antibody levels compared to hares from a non-endemic region, indicating frequent contact with vector´s bites (Martín-Martín et al., 2014). Furthermore, these hares were able to infect *P. perniciosus* sand flies with *L. infantum*, which has been demonstrated by xenodiagnostic transmission (Molina et al., 2012). Additionally, anti-saliva antibodies can be also used to monitor sand flies distribution in endemic regions [as mentioned in (Souza et

al., 2010)]. In summary, monitoring anti-saliva antibodies could help to better focus anti-vector campaigns and to design better strategies for the control of leishmaniasis in the spreading foci.

Anti-saliva antibody response could be also used as a marker of cell-mediated immune response. Reciprocal regulatory interaction between T cells subsets has potent effects on B cell differentiation. In a murine model, secreted cytokines of distinct T cell populations such as IFN-γ and IL-4, were shown to promote expression of specific Ig subtypes. While Th1 cell-derived cytokine IFN-γ stimulates expression of the IgG2a isotypes, Th2 cell-derived IL-4 promotes switching to IgG1 and IgE expression (Snapper and Paul, 1987). The type of immune response was assayed in mice immunized with several DNA plasmids encoding salivary proteins of *P. ariasi*, which previously induced either strong antibody or strong antibody immune response together with cellular one (Oliveira et al., 2006). Interestingly, it was shown that sera of mice immunised with the strongest inducer of DTH and antibody response (ParSP25) produced significantly higher levels of IgG1 (Th2 phenotype) antibodies when compared with the IgG2a subclass (Th1 phenotype). On the other hand, LJL143 and LJM17 plasmids from *Lu. longipalpis* triggered cellular response as well as dominant IgG2 antibodies in immunized dogs, indicating Th1 profile (Collin et al., 2009). Thus, characterization of anti-saliva IgG subclassess might help e.g. in selecting candidate proteins for anti-*Leishmania* vaccine.

As mentioned above, the antigenic property of sand fly saliva provides a possibility for its utilization as indicator of close contact between a host and a sand fly. In such studies, possible cross-reactivity between various sand fly species occuring on tested locality or even between sand flies and other blood-feeding insects should be excluded since the cross-reaction could lead to false positive results [reviewed in (Andrade and Teixeira, 2012)].

So far, in most studies the anti-saliva antibodies were detected using whole salivary gland homogenate. The advantage is that it represents the complete repertoire of secreted salivary proteins which are in native forms. However, this approach has also some limitations. Closely related sand fly species have higher probability of shared salivary antigens, thus a utilization of total saliva as antigen might reduce the specificity of anti-saliva antibody detection. Another limitation for large-scale serological studies is the maintainance of the sand fly colony for salivary gland dissection which is economically demanding and extremely time consuming. Additionally, the proteins composition and quantity in obtained salivary gland extract may vary even in long-term established colonies due to differences in sand fly physiological factors such as age and diet (Volf et al., 2000; Prates et al., 2008).

To overcome these limitations, the identification of a single, specific salivary protein was initiated [reviewed in (Andrade and Teixeira, 2012; Gomes and Oliveira, 2012)]. The main intention was to express salivary antigens in recombinant forms in controlled quality and large quantity for use in large epidemiological studies. Several studies showed that anti-*Lu.longipalpis* antibodies in human or animal serum recognize specific salivary proteins of different molecular weight (Barral et al., 2000; Gomes et al., 2002; Silva et al., 2005; Bahia et al., 2007; Gomes et al., 2007; Vinhas et al., 2007; Hostomska et al., 2008; Soares et al., 2013). Proteins frequently recognized by humans (Gomes et al., 2002; Teixeira et al., 2010) and canids such as foxes and dogs (Gomes et al., 2007; Teixeira et al., 2010) from endemic place in Brazil were selected. Nine of those most antigenic salivary proteins recognized by human, canine or fox sera were produced in mammalian expression system (Teixeira et al., 2010). Among the best candidates were LJM17 (45 kDa yellow-related protein) recognized by sera from all three aforementioned hosts and LJM11 (43 kDa yellow-related protein) recognized by human and dog serum samples (Teixeira et al., 2010). These recombinant proteins were further tested in a large scale study using 1077 individuals from place endemic for visceral leishmaniasis (Souza et al., 2010). Human sera, which recognized *Lu. longipalpis* saliva in ELISA, also recognized the mixture of rLJM17 and rLJM11 proteins. The detection of seroconversion was extensively improved by using a combination of both proteins (Souza et al., 2010). These two molecules were also used in monitoring of chicken exposure to phlebotomine bites in Brazil (Soares et al., 2013); results obtained with SGS significantly positively correlated with those obtained with rLJM11 but not with rLJM17 (Soares et al., 2013) highlighting host-specificity of anti-saliva antibody response.

Antibodies from humans and animals exposed to *P. papatasi* bites recognized mainly proteins of 42, 36 and 30 kDa (Rohousova et al., 2005; Marzouki et al., 2011; Vlkova et al., 2011). The last one was prepared in a recombinant form in mammalian expression system and further tested with sera of humans naturally exposed to *P. papatasi* in Tunisia and Saudi Arabia, areas endemic for cutaneous leishmaniasis (Marzouki et al., 2012; Marzouki et al., 2015; Mondragon-Shem et al., 2015). A study conducted in Tunisia described rPpSP32 as the immunodominant antigen, able to act as an alternative marker to saliva for screening of sandfly exposure (Marzouki et al., 2012; Marzouki et al., 2015). Moreover, the binding of human IgG antibodies to native PpSP32 was inhibited by pre-incubation of serum samples with recombinant form of PpSP32 proving similarities between recombinant and native form of this protein (Marzouki et al., 2012). In addition, human and canine sera immunized by *P. perniciosus* bites, a species widely present in Tunisia, did not react with rPpSP32, confirming

absence of cross-reaction between these two sympatric species (Marzouki et al., 2012; Marzouki et al., 2015).

The reactivity of sera with four *P. papatasi* recombinant proteins coding for yellow-related proteins (PpSP44 and PpSP42) and D7-related proteins (PpSP30 and PpSP28) was tested in BALB/c mice repeatedly exposed to *P. papatasi* bites (Vlkova et al., 2012). Each serum reacted with at least one of the tested recombinant protein, although none of the recombinant proteins were recognized by all sera. Therefore, the mixture of several recombinant proteins could eventually replace whole salivary gland homogenate for this sand fly-host combination (Vlkova et al., 2012).

Recent studies showed that dogs experimentally bitten by *P. perniciosus* females recognized with the highest affinity proteins of 42, 38, 33 and 29 kDa described as yellow-related protein, two apyrases and an antigen 5 protein, respectively (Vlkova et al., 2011). From the total number of six bacterially expressed salivary proteins of *P. perniciosus*, recombinant yellow-related protein (rSP03B) and two apyrases (rSP01 and rSP01B) were chosen as the best candidates for the exposure assessment in mice and dogs experimentally bitten with *P. perniciosus* females (Drahota et al., 2014). The antibody response targeting these three recombinant proteins correlated well with the anti-SGH antibody response not only in experimentally exposed hosts (Drahota et al., 2014) but also in naturally bitten dogs and hares (Martín-Martín et al., 2014). *P. perniciosus* recombinant yellow-related protein rSP03B showed the best correlation scores for hares and rabbits compared with SGH. Moreover, it seems to be the best marker of canine exposure because it presents the lowest data dispersion (Martín-Martín et al., 2014).

Recently, recombinant apyrase (rSP01B) and D7-related protein (rSP04) from *P. perniciosus* were tested with serum samples obtained from laboratory-exposed mice (Martín-Martín et al., 2015). While, anti-saliva antibodies showed similar reactivity to rSP01B and SGH, they exhibited highly variable reactivity to rSP04. Therefore rSP01B seems to be the best candidate for marker of *P. perniciosus* exposure in this sand fly-host model (Martín-Martín et al., 2015).

In summary, the utilization of recombinant salivary proteins of *Lu. longipalpis*, *P. papatasi* and *P. perniciosus* as markers of exposure to sand fly bites was confirmed and fully validated not only in experimental models, but more importantly also in field settings for both humans and animals (Souza et al., 2010; Marzouki et al., 2012; Soares et al., 2013; Martín-Martín et al., 2014; Marzouki et al., 2015).

2 Other factors affecting development of *Leishmania* infection in vertebrate host

To complete the list of known components participating in the *Leishmania* transmission it is necessary to mention also parasite-derived factors such as promastigotes secretory gel (PSG), surface molecules and material contained in secreted vesicles.

Promastigote secretory gel is a dense, gel-like material consisting of filamentous proteophosphoglycans (fPPGs), the largest molecules produced by parasites, which engulf the promastigote bodies (Stierhof et al., 1999; Rogers et al., 2002). This promastigote secretory gel is formed in culture as well as in the anterior midgut and stomodeal valve of infected sand fly females [reviewed in (Rogers, 2012)]. The peak of fPPG accumulation in *Lu. longipalpis* gut corresponds with leptomonads occurrence and at a later phase with metacyclogenesis (Rogers et al., 2002). Secreted fPPG can affect insect vectors as well as the mammalian host. The latter ability will be described in more detail below.

Promastigote secretory gel was confirmed as a potent exacerbative factor in cutaneous infection caused by *L. mexicana* (Rogers et al., 2004) in BALB/c as well as in CBA/Ca mice, representing susceptible and resistant phenotype for *Leishmania* infection, respectively. In both strains co-injected with PSG, higher parasitemia was manifested, lesions appeared earlier and were larger in size, when compared with respective controls. The main bioactive component of PSG with enhancing effect is fPPG, more specifically glycan moieties of proteophosphoglycan (Rogers et al., 2004). Later, the enhancing role of PSG was demonstrated for another causative agents of both cutaneous (*L. major*, *L. tropica* and *L. braziliensis*) and visceral leishmaniasis (*L. infantum*) (Rogers, 2012).

The exacerbative activity of PSG is probably related to its modulation of macrophages activity (Rogers et al., 2009). Two main type of macrophages contribute to either kill or to host intracellular forms of *Leishmania* sp. In the anti-inflammatory milieu, Th2 cytokines (e.g. IL-4, IL-10, IL-13) alternatively activate macrophages (AAM) towards the production of urea and L-ornithine [reviewed in (Arango Duque and Descoteaux, 2014)]. The latter is a building element for synthesis of polyamines which are beneficial for *Leishmania* intra-macrophage growth (Kropf et al., 2005). On the other hand, in the pro-inflammatory environment, macrophages are classically activated (CAM) by Th1 cytokines (e.g. IFN-γ, TNF-α). As a result, CAM produces NO and other toxic intermediates, leading to the destruction of *Leishmania* parasites (Murray and Cartelli, 1983; Green et al., 1990). Experiments performed by group of Dr. Rogers showed that PSG isolated from *Lu. longipalpis* promoted survival of *L. mexicana* amastigotes in unstimulated, alternatively as well as in classically stimulated macrophages (Rogers et al., 2009). PSG did not affect

classical activation in macrophages, instead, it has been found that PSG enhance alternative way of activation ensuring more nutrients for parasites (Rogers et al., 2009).

Moreover, PSG is chemotactic for macrophages to the transmission site as demonstrated in an air pouch model (Rogers et al., 2009); the number of recruited macrophages was about 100-fold higher in favor of PSG-injected mice over saline-injected individuals. Synergistic effect was demonstrated using PSG together with saliva; this attracted 224-fold more macrophages compared to control group (Rogers et al., 2009). This confirms that the infective inoculum considerably modifies the feeding site for the favor of parasite development during the early phase of infection.

Another component influencing infection development in the vertebrate host is *Leishmania* surface glycocalyx, composed of glycoconjugates attached by glycosylphosphatidylinositol (GPI) anchors. This thesis is not aimed on this subject, thus these molecules will be discussed only briefly.

Multivirulence factor such as lipophosphoglycan (LPG) is primarily expressed in promastigotes while the expression massively decreases in the intracellular amastigotes [reviewed in (de Assis et al., 2012; Forestier et al., 2014)]. LPG is able e.g. to protect parasite against complemetnt lysis or against oxidative burst during the phagocytosis (Puentes et al., 1989; Puentes et al., 1990; Späth et al., 2003; Lodge et al., 2006), to inhibit IL-12 secretion and iNOS expression in macrophages (Proudfoot et al., 1996; Feng et al., 1999; Piedrafita et al., 1999), or to postpone phago-endosomal fusion giving *Leishmania* more time for differentiation from promastigote to amastigote form (Desjardins and Descoteaux, 1997).

Another important GPI-anchored molecules at parasites surface are GIPLs, which were shown as an essential virulence component for *Leishmania* (Ilgoutz et al., 1999; Yoneyama et al., 2006). However, very little is known about their function affecting *Leishmania* biology. In contrast with LPG, GIPLs remain highly expressed throughout the parasite life cycle [reviewed in (Novozhilova and Bovin, 2010; Forestier et al., 2014)]. It was shown that GIPLs are able to inhibit nitric oxide synthesis and oxidative burst (Proudfoot et al., 1995; Assis et al., 2012). Moreover, an involvement of GIPL in penetration into macrophages was confirmed (ligand for macrophage mannose-fucose receptor) (Tachado et al., 1997).

The zinc-dependent matalloprotease, gp63 or leishmanolysin, is abundant in promastigotes but down-regulated in amastigotes [reviewed in (Novozhilova and Bovin, 2010; Isnard et al., 2012; Olivier et al., 2012). Despite its lower number at surface of intracellular forms, gp63 was shown as a potent modulator protecting amastigotes from the

harsh macrophages environment e.g. (Chaudhuri et al., 1989; Seay et al., 1996; Chen et al., 2000;). Glycoprotein 63 provides protection against lysis mediated by complement (Brittingham et al., 1995), encourages internalization to macrophages e.g. (Liu and Chang, 1992; Brittingham et al., 1999), promotes survival in phagolysosomes e.g. (Chaudhuri et al., 1989) or facilitates parasite migration through the extracellular matrix components such as collagen IV and fibronectin (McGwire et al., 2003).

Those glycoconjugates are important virulence factors, however, their exact properties and function are *Leishmania* species-specific and therefore above described functions can not be universally declared for all parasite-host combinations e.g. (Ilg, 2000; Späth et al., 2003). For more comprehensive review of this topic, refer to (Novozhilova and Bovin, 2010; de Assis et al., 2012; Olivier et al., 2012).

Finally, *Leishmania* parasites are capable to secret vesicles into the extracellular milieu as well as into the cytoplasmic compartment of an infected host´s cell. The microvesicles, which fall into two broad categories of exosomes and secreted vesicles, can participate in pathogenesis, as inner cargo mediates immunomodulation in a pro-parasitic manner and functionally primes host cells for *Leishmania* invasion [reviewed in (Silverman and Reiner, 2011a)]. *Leishmania* exosomes were found to contain for example soluble as well as membrane-bound virulence factors such as elongation factor 1-α, adolase, acid phosphatase and metalloprotease gp63 or well known T cell antigen, kinetoplast membrane protein 11, with immunosupresive properties [reviewed in (Silverman and Reiner, 2011b)]. Importantly, it was demonstrated that exosomes were secreted by either *L. infantum* or *L. major* promastigotes within *Lu. longipalpis* midgut. Midgut-secreted exosomes were proven as a part of the sand fly inocula and were able to regulate the immune system and exacerbate disease outcome when co-inoculated alongside parasites (Atayde et al., 2015). Therefore, it is necessary to add these vesicles to the list of virulence factors involved in vector-transmitted infections.

OBJECTIVES

During the bloodfeeding process, sand fly saliva is obligatorily inoculated into the feeding site. Repeated exposures to sand fly bites or salivary proteins induce saliva-specific immune response, both humoral and cell-mediated. While anti-saliva antibody response correlates with the intensity of exposure and can be used as a reliable epidemiological tool, specific cellular immunity is responsible for protection against leishmaniasis in some vector-parasite-host combination and may be useful in the search for and identification of vaccine candidates.

The immunity elicited by sand fly salivary proteins as well as the protection was shown to be sand fly species-specific. This attribute is very useful in sand flies exposure testing where species-specific reaction eliminates false positive results. On the other hand, species-specific protection obstructs development of universal saliva-based vaccine.

Until recently, most of the works dealing with the utilization of anti-saliva antibody response for estimating sand fly exposure used whole salivary gland homogenate as the antigen. However, recombinant proteins as an alternative have several advantages; among others they allow better proteins quality and quantity control and hence standardize results from large-scale serological studies.

Thus, the main objectives of my thesis were as follows:

Determination of host´s immune response specificity to sand fly saliva

a) to determine the cross-protective properties of saliva from *P. papatasi* and *P. duboscqi*, the two natural vectors of *L. major*
b) to determine serological cross-reactivity between *P. orientalis*, the vector of *L. donovani*, and other sand fly species with sympatric distribution (*P. papatasi*, *P. duboscqi*, *P. arabicus*, *Se. schwetzi*)

Utilization of anti-saliva antibodies for estimating the sand fly exposure in dogs

a) to test salivary recombinant proteins of *P. perniciosus* as a valid alternative to salivary gland homogenate
b) to test salivary recombinant yellow-related protein of *P. perniciosus* as a universal antigen within the area of this sand fly species distribution

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- **Lestinova T.**, Vlkova M., Votypka J., Volf P., Rohousova I. (2015)
  Phlebotomus papatasi exposure cross-protects mice against *Leishmania major* co-inoculated with *Phlebotomus duboscqi* salivary gland homogenate.
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  The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* in distant foci of canine leishmaniasis.
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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

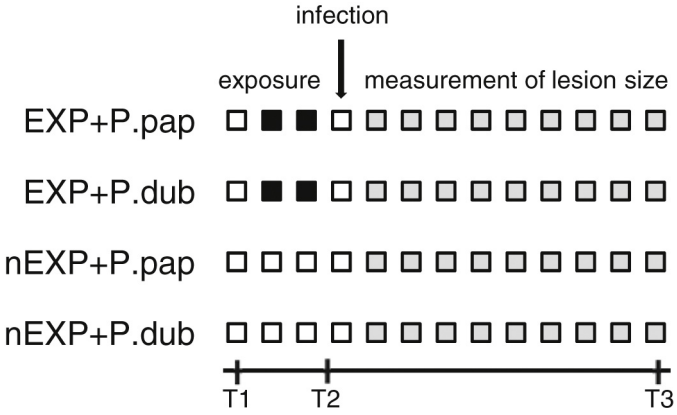

Fig. 1. Timeline of the experiment. Mice were divided into four groups. Two groups of mice were exposed to *Phlebotomus papatasi* bites (black squares, EXP), two groups remained unexposed (white squares, nEXP). One week after the second exposure, all mice were intradermally challenged with 10^4 *L. major* 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

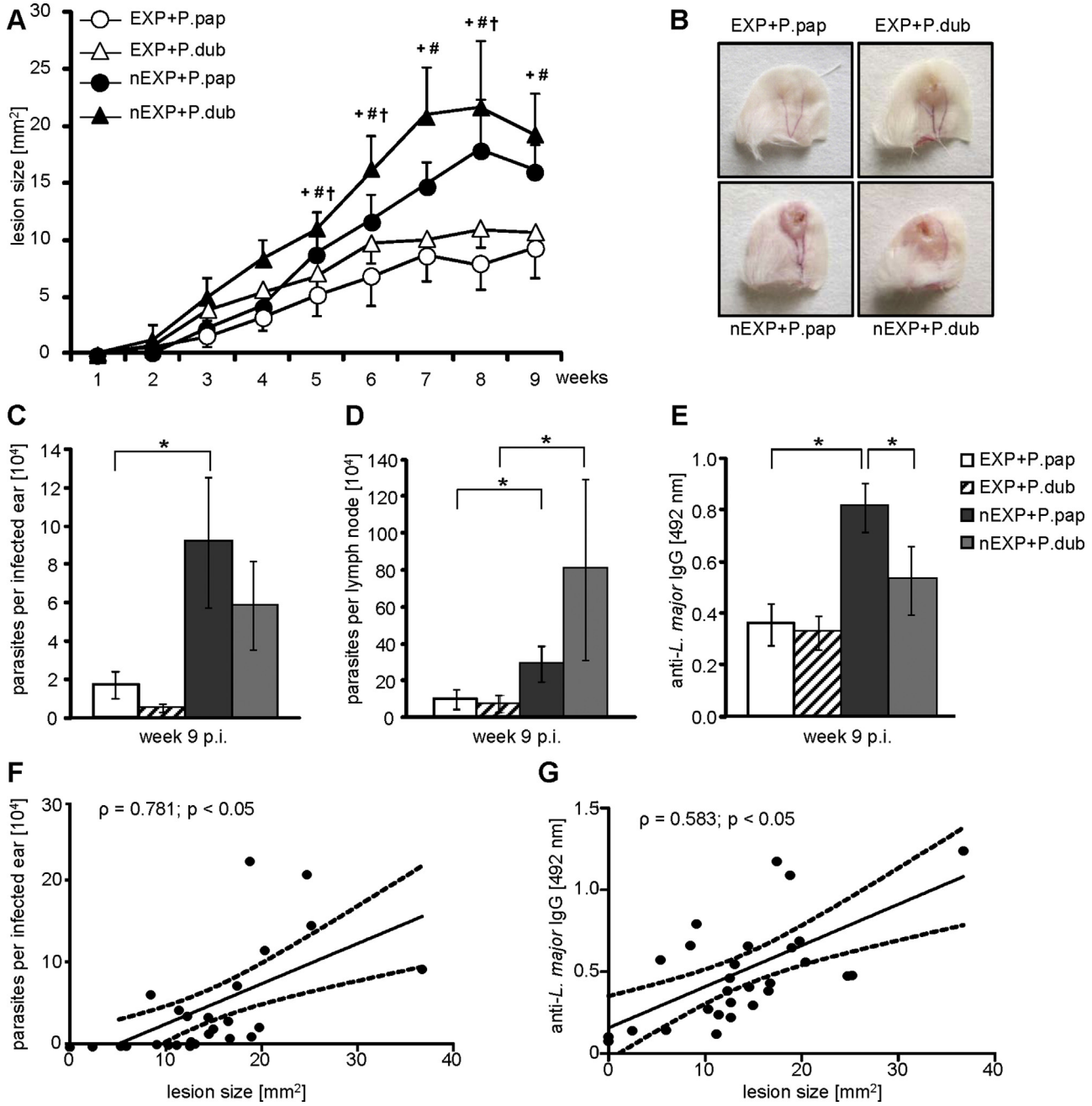

Fig. 2. Development of *Leishmania* infection. Kinetics of *L. major* infection was studied in four groups of BALB/c mice. Two groups of mice immunized by *P. papatasi* bites (EXP, open marks) and two control non-immunized groups (nEXP, full marks) were infected with *L. major* promastigotes along with 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*

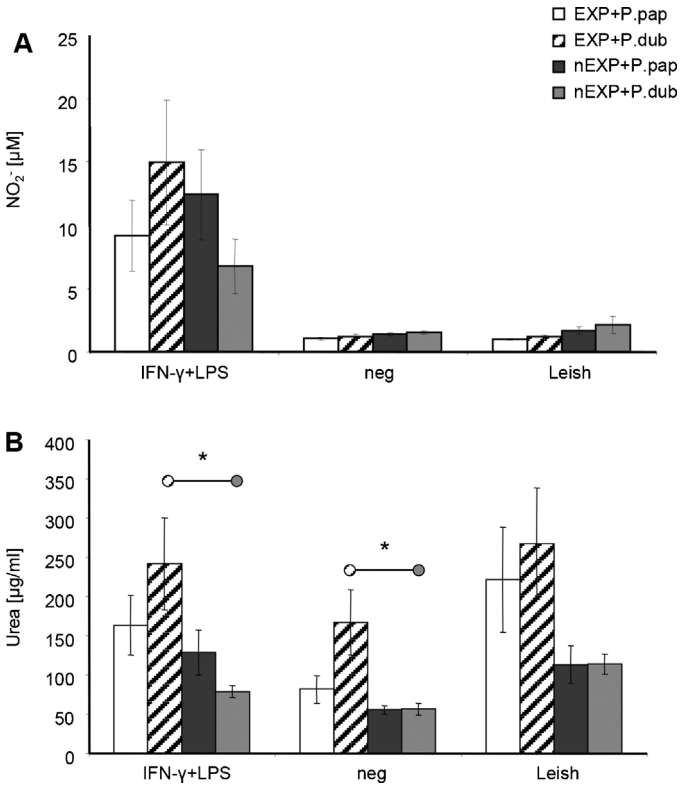

Fig. 3. Effect of sand fly saliva on the macrophages activity. BALB/c mice were exposed to *P. papatasi* bites (EXP) or left non-exposed (nEXP). Mice were then infected with *L. major* promastigotes along with either *P. papatasi* (EXP + P.pap, nEXP + P.pap) or *P. duboscqi* (EXP + P.dub, nEXP + P.dub) 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

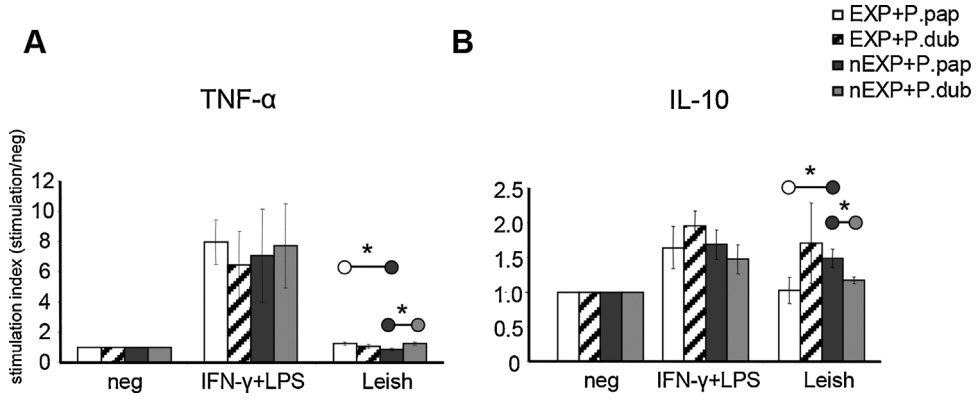

Fig. 4. Cytokine production of the peritoneal macrophages. BALB/c mice were exposed to *P. papatasi* bites (EXP) or left non-exposed (nEXP). Mice were then infected with *L. major* promastigotes along with either *P. papatasi* (EXP + P.pap, nEXP + P.pap) or *P. duboscqi* (EXP + P.dub, nEXP + P.dub) SGH. 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.

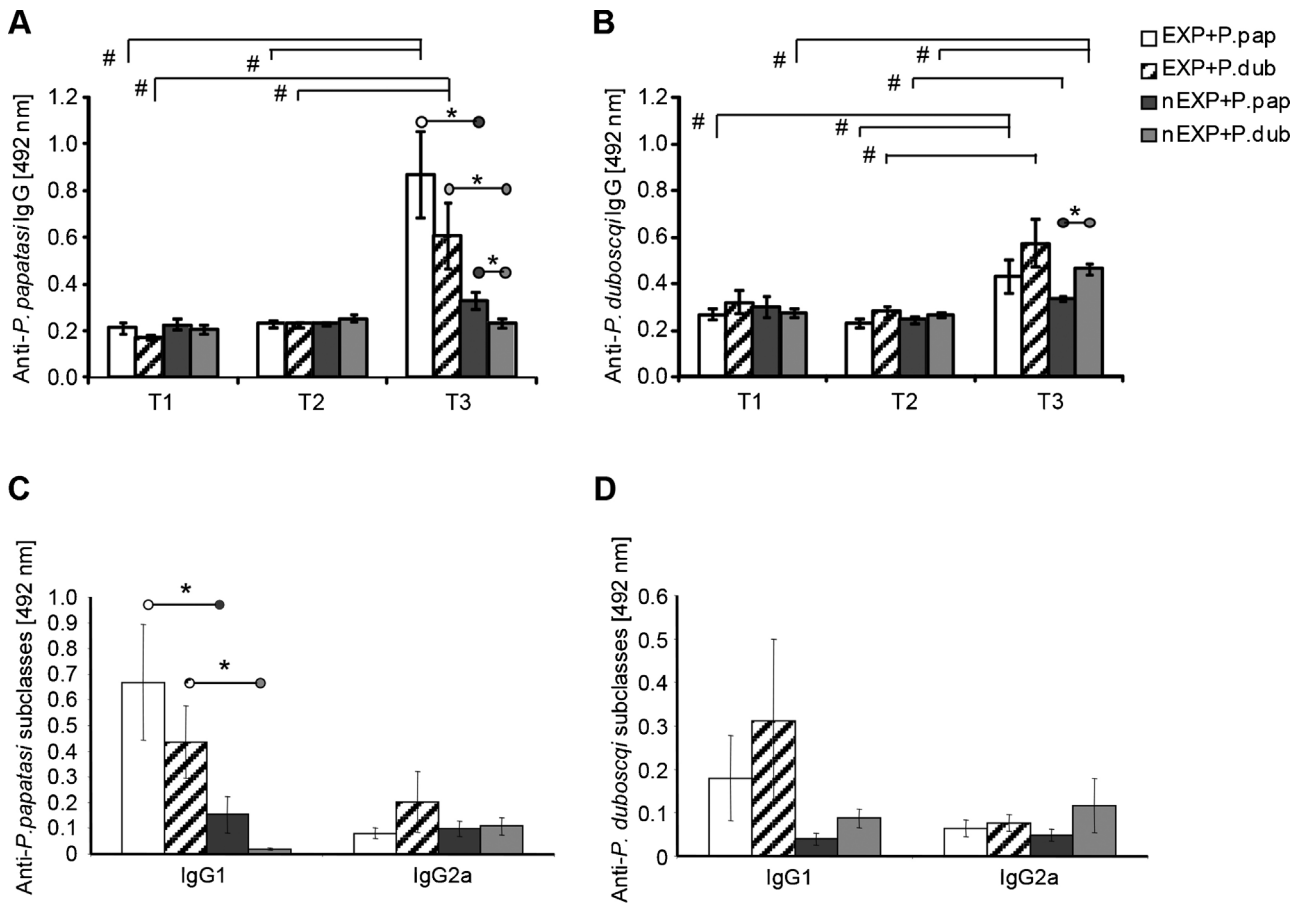

Fig. 5. Anti-sand fly saliva antibody response. BALB/c mice were exposed to *P. papatasi* bites (EXP) or left non-exposed (nEXP). Mice were then infected with *L. major* promastigotes along with either *P. papatasi* (EXP + P.pap, nEXP + P.pap) or *P. duboscqi* (EXP + P.dub, nEXP + P.dub) SGH. IgG against 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

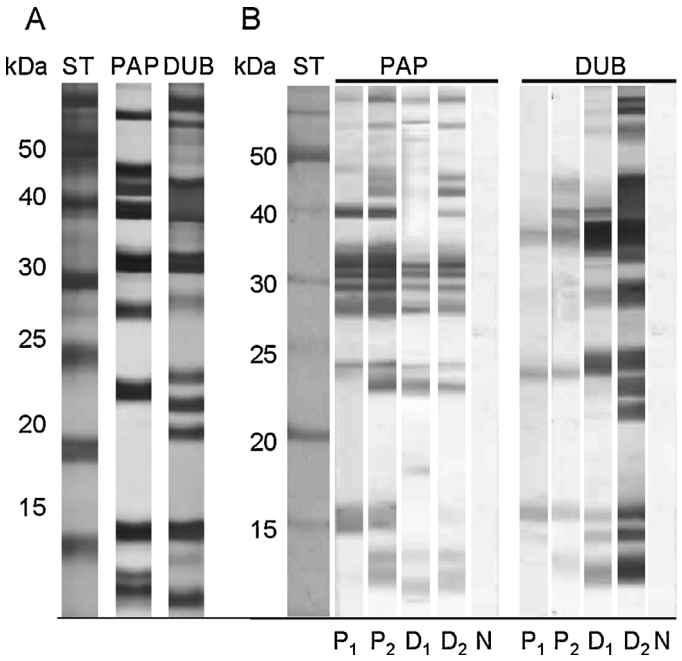

Fig. 6. Cross-reactivity of *P. papatasi* and *P. duboscqi* salivary antigens. Salivary proteins of *P. papatasi* (PAP) and *P. duboscqi* (DUB) were separated under non-reducing conditions by SDS–PAGE electrophoresis and silver staining was used to compare the salivary profiles of both species (A). Western 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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Exposure to *Leishmania* spp. and sand flies in domestic animals in northwestern Ethiopia

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RESEARCH Open Access

Exposure to *Leishmania* spp. and sand flies in domestic animals in northwestern Ethiopia

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Abstract

Background: Human visceral leishmaniasis caused by *Leishmania donovani* is considered an anthroponosis; however, *Leishmania*-infected animals have been increasingly reported in *L. donovani* foci, and the role of these animals as reservoirs for human *L. donovani* infection remains unclear.

Methods: We conducted a study of domestic animals (goats, sheep, cows, dogs, and donkeys) in three *L. donovani* foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and for anti-*L. donovani* IgG. Serum anti-sand fly saliva antibodies were used as a marker of exposure to the vector sand fly, *Phlebotomus orientalis*.

Results: Of 546 animals tested, 32 (5.9 %) were positive for *Leishmania* DNA, with positive animals identified among all species studied. Sequencing indicated that the animals were infected with parasites of the *L. donovani* complex but could not distinguish between *L. infantum* and *L. donovani*. A total of 18.9 % of the animals were seropositive for anti-*L. donovani* IgG, and 23.1 % of the animals were seropositive for anti-*P. orientalis* saliva IgG, with the highest seroprevalence observed in dogs and sheep. A positive correlation was found between anti-*P. orientalis* saliva and anti-*L. donovani* IgGs in cows, goats, and sheep.

Conclusions: The detection of *L. donovani* complex DNA in the blood of domestic animals, the reported seroprevalence to the *L. donovani* antigen, and the widespread exposure to sand fly saliva among domestic animals indicate that they are frequently exposed to *Leishmania* infection and are likely to participate in the epidemiology of *Leishmania* infection, either as potential blood sources for sand flies or possibly as parasite hosts.

Keywords: Visceral leishmaniasis, Ethiopia, Domestic animals, Serology, PCR, *Phlebotomus orientalis*, *Leishmania donovani*, Sand fly saliva

Background

Leishmaniasis, a protozoan disease that is transmitted by sand flies (Diptera: Phlebotominae) and caused by parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), is a neglected tropical and subtropical disease endemic to 98 countries worldwide. In East Africa, life-threatening human visceral leishmaniasis (VL) is caused by *Leishmania donovani* and primarily affects the poor due to the lack of preventive measures and reduced access to health care facilities [1].

The optimal strategy for controlling this disease depends on understanding the epidemiology of VL, including its local transmission cycles. Leishmaniasis caused by *L. donovani* is believed to be an anthroponosis. However, in Latin America and the Mediterranean Basin, the closely related species *L. infantum* causes a zoonosis for which canids are the main reservoirs [2]. Controlling zoonoses involving domestic or sylvatic transmission requires a more complex intervention than would be necessary if humans were the only hosts. Several

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Leishmania-infected animals have been previously reported in *L. donovani* foci, including wild and domestic animals [3–5]. However, the role of these animals as parasite hosts or, possibly, as reservoirs for human *L. donovani* VL remains unclear and requires further examination.

Our study focused on the detection of *Leishmania* infections in domestic animals in three VL foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and anti-*L. donovani* IgG in their peripheral blood to detect infection and exposure to *Leishmania*, respectively. Additionally, anti-sand fly saliva antibodies were used as a marker of exposure [6] to *Phlebotomus orientalis*, the suspected vector of *L. donovani* in northwestern Ethiopia [7, 8]. The findings from this study could be used to further study the involvement of domestic animals in the transmission cycle of VL.

Methods

Study sites and sample collection

Animal blood and serum samples were collected in Addis Zemen, Humera, and Sheraro, three localities in northwestern Ethiopia endemic to human VL. In the Humera district (Tigray region), several outbreaks of VL have been recorded since 1970. Addis Zemen (Amhara region) and Sheraro (Tigray region) are sustained VL foci characterized by a local transmission cycle supported by migrant agricultural laborers returning from Humera [1].

Animal surveys were conducted during two field studies. In October 2010, 266 samples were collected in Addis Zemen and Sheraro, and in November 2010, an additional 280 samples were obtained in Humera (Table 1). For DNA extraction, samples of whole blood (with anticoagulant) were transported to the Hebrew University of Jerusalem (Israel), where extraction was performed. For serological testing, serum samples treated with a 1 % azide solution were transported to Charles University in Prague (the Czech Republic) and stored at −70 °C.

Table 1 Serum samples collected from October to November 2010 in Ethiopian VL foci

	Addis Zemen	Sheraro	Humera	Total
Cow	62	26	16	104
Dog	19	7	8	34
Donkey	3	11	6	20
Goat	0	106	133	239
Sheep	27	5	117	149
Total	111	155	280	546

Ethical approval

The study was approved by the Ethiopian National Research Ethics Review Committee (NRERC), under approval no. 3.10/3398/04. Consent was obtained from the owners of the domestic animals for the collection of blood samples by a veterinarian. International animal experimentation guidelines were followed.

DNA extraction and PCR amplification

DNA was extracted from whole blood using the guanidine thiocyanate technique [9]. DNA was tested for *Leishmania* spp. infection via kDNA real-time PCR as previously described [10, 11]. Samples that tested positive were further tested by *Leishmania* internal transcribed spacer 1 (ITS1) real-time PCR and high-resolution melt analysis (ITS1-HRM PCR) [12]. Samples that tested positive by ITS1-HRM PCR were further assessed via conventional PCR to amplify a larger segment of ITS1 [13]. All samples were tested in duplicate, and the results were compared with positive controls: *L. infantum* (MCAN/IL/2002/Skoshi), *L. tropica* (MHOM/IL/2005/ LRC-L1239), and *L. major* (MHOM/TM/1973/5ASKH) promastigotes. The negative controls included blood samples obtained from five Israeli dogs that had tested negative for *Leishmania* by PCR. All positive PCR products were submitted for DNA sequencing to the Center for Genomic Technologies at the Hebrew University of Jerusalem. The derived DNA sequences were compared with sequences in GenBank using the NCBI BLAST program (www.ncbi.nlm.nih.gov/BLAST). The percentage of positive animals for each species was calculated based on positive kDNA PCR results followed by sequencing. Samples were considered positive for *Leishmania* only if their kDNA sequence demonstrated the closest BLAST match to *Leishmania* and was at least 80 % identical. A species was considered to be identified only when its ITS1 sequence shared 99 to 100 % identity with an existing GenBank sequence.

Discrimination between *Leishmania infantum* and *Leishmania donovani*

As ITS1-HRM PCR does not discriminate between *L. infantum* and *L. donovani* infections [12], samples that tested positive for the *L. donovani* complex were further evaluated using conventional PCR to determine the species. Two independent PCR assays were carried out to amplify fragments of the *Leishmania* cysteine protease B (CPB) gene [14, 15]. Furthermore, amplification of the heat shock protein 70 (HSP70) gene, followed by restriction fragment length polymorphism analysis was also attempted for species discrimination [16]. The same positive and negative controls used for ITS1-HRM PCR were employed.

A phylogenetic analysis was carried out using Kalign (www.ebi.ac.uk/tools/msa/kalign/) and BioEdit softwares. Only well-defined ITS sequences that were unambiguously assigned to the species *L. donovani* or *L. infantum* were downloaded from the GenBank database and used in the analysis (Additional file 1). The final alignment included 286 characters and is available upon request. Phylogenetic analyses of the ITS datasets were performed with PhyML for maximum likelihood (ML); the best-fitting model [GTR + *I* + Γ] of sequence evolution was assessed using Modeltest 3.7 software and bootstrapped with 1000 replicates.

Anti-*Leishmania donovani* IgG antibodies

An ELISA was used to measure specific anti-*L. donovani* IgG. Wells (CovaLink NH, Nunc) were coated with *L. donovani* promastigotes (Ethiopian strain MHOM/ET/67/HU3, 10^5 cells per well) in 20 mM carbonate-bicarbonate buffer (pH 9.25) overnight at 4 °C and incubated with 6 % blocking solution for 60 min at 37 °C. Serum samples were diluted in 2 % blocking solution and incubated in duplicate for 60 min at 37 °C. Thereafter, peroxidase-conjugated secondary antibodies were added, followed by 45 min of incubation at 37 °C. For details on the blocking solutions, sample dilutions, and conjugates employed in these assays, see Additional file 2. Absorbance was measured using a Tecan Infinite M200 microplate reader (Schoeller) at 492 nm.

Hyperimmune sera from laboratory-bred mice experimentally infected with *L. donovani* served as positive controls. Negative serum samples were obtained from healthy cattle (n = 33), horses (as controls for the donkeys; n = 9), goats (n = 21), and sheep (n = 32) from the Czech Republic, which is a sand fly- and *Leishmania*-free country. Canine-negative (n = 15) and canine-positive (n = 2) control sera were obtained during a previous study [17] from laboratory-bred beagles with no history of exposure to sand flies or *Leishmania* or from *Leishmania*-positive dogs, respectively.

Anti-sand fly saliva IgG antibodies

To estimate the exposure of domestic animals to *P. orientalis*, anti-saliva IgG antibodies were measured via ELISA. The same protocol applied for anti-*Leishmania donovani* IgG was used, with the following modifications: wells were coated with a salivary gland homogenate (corresponding to 0.2 gland/well, prepared as previously described [18]), and serum samples were incubated in duplicate for 90 min at 37 °C. Hyperimmune sera from laboratory-bred mice exposed solely to *P. orientalis* served as a positive control. The same negative controls employed for the anti-*L. donovani* ELISA were also used here.

To assess the possible cross-reactivity of *P. orientalis* salivary gland homogenate with IgG antibodies against the saliva of other sand fly species, sera from mice and dogs that were experimentally exposed to a single sand fly species were used. Canine sera positive for anti-*P. perniciosus* and anti-*L. longipalpis* IgG antibodies were available from previous experiments in laboratory-bred beagles exposed solely to *P. perniciosus* [17] and *L. longipalpis* [18], respectively, the two proven vectors of *L. infantum*. The ELISA protocol described in Additional file 2 was applied with one modification: the sera were diluted 1:500. For the murine sera, the applied ELISA protocol was modified as follows: low-fat, dry milk (Bio-Rad) was used as a blocking solution and diluent for the serum samples (1:200), and goat anti mouse IgG:HRP (AbD SEROTEC, STAR120P) diluted 1:1000, was used as a secondary antibody. The serum samples were obtained from BALB/c mice subjected to more than ten repeated exposures solely to *P. orientalis* (Ethiopia), *P. papatasi* (Turkey), *P. duboscqi* (Senegal), *P. arabicus* (Israel), or *Sergentomyia schwetzi* (Ethiopia). The experiments were approved by the Committee on the Ethics of Animal Experiments of Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under a Certificate of Competency (Registration Number: CZU 934/05), in accordance with an Examination Order approved by the Central Commission for Animal Welfare of the Czech Republic.

Statistical analysis

For seroprevalence, cut-off values were calculated by the addition of three standard deviations to the mean optical density (OD) of the control sera. The differences in antibody levels between localities were analyzed using the nonparametric Wilcoxon Rank-Sum Test for Differences in Medians. Spearman's rank correlation matrix was used to assess the correlation between the variables. Statistical analyses were performed using NCSS 6.0.21 software, and the p-value was set at 0.05.

Results

Prevalence of *Leishmania* infection

The overall prevalence of *Leishmania* DNA detected via PCR was 5.9 % (32/546) (Table 2, Additional file 3). None of the 546 tested domestic animals presented visible clinical signs associated with leishmaniasis. Of the 32 animals that tested positive by kDNA PCR, nine were also positive for ITS1 PCR (Table 2, Additional file 3). The majority of *Leishmania*-positive animals (30 out of 32) were found in Humera, with the highest prevalence observed in cows (18.8 %). At the other localities, only one donkey in Sheraro and one dog in Addis Zemen were found to be positive for *Leishmania* (Table 2, Additional file 3).

Table 2 *Leishmania* PCR positivity in samples from Ethiopian animals

	Leishmania kDNA positive/total animals sampled (% positive)				*Leishmania* ITS1 positive (% positive)
Species	Addis Zemen	Sheraro	Humera	Total	Total
Cow	0/62	0/26	3/16 (18.8 %)	3/104 (2.9 %)	1 (1 %)
Dog	1/19 (5.3 %)	0/7	1/8 (12.5 %)	2/34 (5.9 %)	1 (2.9 %)
Donkey	0/3	1/11 (9.1 %)	1/6 (16.7 %)	2/20 (10.0 %)	0
Goat	0/0	0/106	16/133 (12.0 %)	16/239 (6.7 %)	3 (1.3 %)
Sheep	0/27	0/5	9/117 (7.7 %)	9/149 (6.0 %)	4 (2.7 %)
Total	1/111 (0.9 %)	1/155 (0.6 %)	30/280 (10.7 %)	32/546 (5.9 %)	9 (1.6 %)

A total of nine ITS1 DNA sequences, 265 bp long and 99 % identical to *L. infantum*/*L. donovani* sequences, were obtained via ITS1-HRM-PCR. None of the animal samples yielded positive PCR results when targeting the CPB and HSP70 genes. A DNA sequence was obtained for only a single longer ITS1 amplicon from one sheep originating in Humera. This sequence (314 bp, [GenBank:KJ010540]) shares 100 % identity with sequences from both *L. infantum* and *L. donovani* with 100 % coverage, and its phylogeny did not permit discrimination between these two closely related species (Fig. 1).

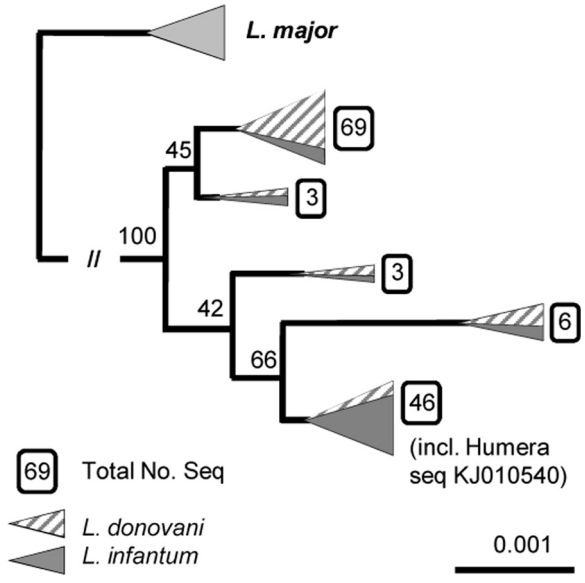

Fig. 1 Phylogenetic analysis of the *Leishmania* ITS1 sequence in a sheep from Humera, Ethiopia. Maximum likelihood (ML) phylogenetic analysis of a 286 bp *Leishmania* sequence amplified from the blood of a sheep from Humera, Ethiopia [GenBank:KJ010540]. Only well-defined ITS sequences that could be unambiguously assigned to a species (*L. donovani* or *L. infantum*) were downloaded from the GenBank database and used for the following analysis (Additional file 1). The ITS sequences were aligned using Kalign (www.ebi.ac.uk/Tools/msa/kalign/), and the resulting alignments were edited manually using the BioEdit software program to remove unambiguous positions. The final alignment included 286 characters and is available upon request. Phylogenetic analyses of the ITS datasets were performed with PhyML for ML (the best-fitting model [GTR + *I* + Γ] of sequence evolution was assessed using Modeltest 3.7 and bootstrapped with 1000 replicates; likelihood: loglk = −404.139)

Anti-*Leishmania donovani* IgG antibodies

Seropositive animals were found for every species tested. The overall seroprevalence of anti-*L. donovani* IgG in the Ethiopian samples was 18.9 % (103/546) (Table 3; Fig. 2). Across all localities tested, the highest seropositivity was observed in dogs (overall 55.9 %) and the lowest in cows and donkeys (Table 3). Of the 32 animals that tested positive for *Leishmania* DNA, 12 animals also demonstrated seropositivity for the *L. donovani* antigen: 1 donkey, 3 goats, and 8 sheep (Additional file 3).

Apart from the cows, all of the Ethiopian animal species exhibited significantly higher levels of anti-*L. donovani* IgG compared with control animals (Fig. 2). Geographically, significantly higher levels of anti-*L. donovani* IgG were observed in all animal species from Humera and in dogs, goats, and sheep from the other localities tested, when compared with control animals (Fig. 2).

Anti-*Phlebotomus orientalis* saliva IgG antibodies

The seroprevalence of anti-*P. orientalis* IgG in Ethiopian animals was 23.1 % (126/546) (Table 4). Seropositive animals were identified for every species and at every locality tested. In Addis Zemen and Sheraro, the highest seroprevalence was observed in dogs (57.9 and 57.1 %, respectively), whereas in Humera, the highest seroprevalence was among donkeys, dogs, and sheep (66.7, 62.5, and 57.3 %, respectively) (Table 4).

Apart from cows, all of the animal species from Ethiopia exhibited significantly ($p < 0.05$) higher anti-*P. orientalis* IgG seroreactivity compared with control animals (Fig. 2). Geographically, elevated levels of anti-*P. orientalis* IgG were observed in dogs, donkeys, and sheep from Humera and in dogs, donkeys, and goats from Sheraro. In Addis Zemen, only dogs exhibited significantly higher seroreactivity than control animals. The seroreactivities in the bovine

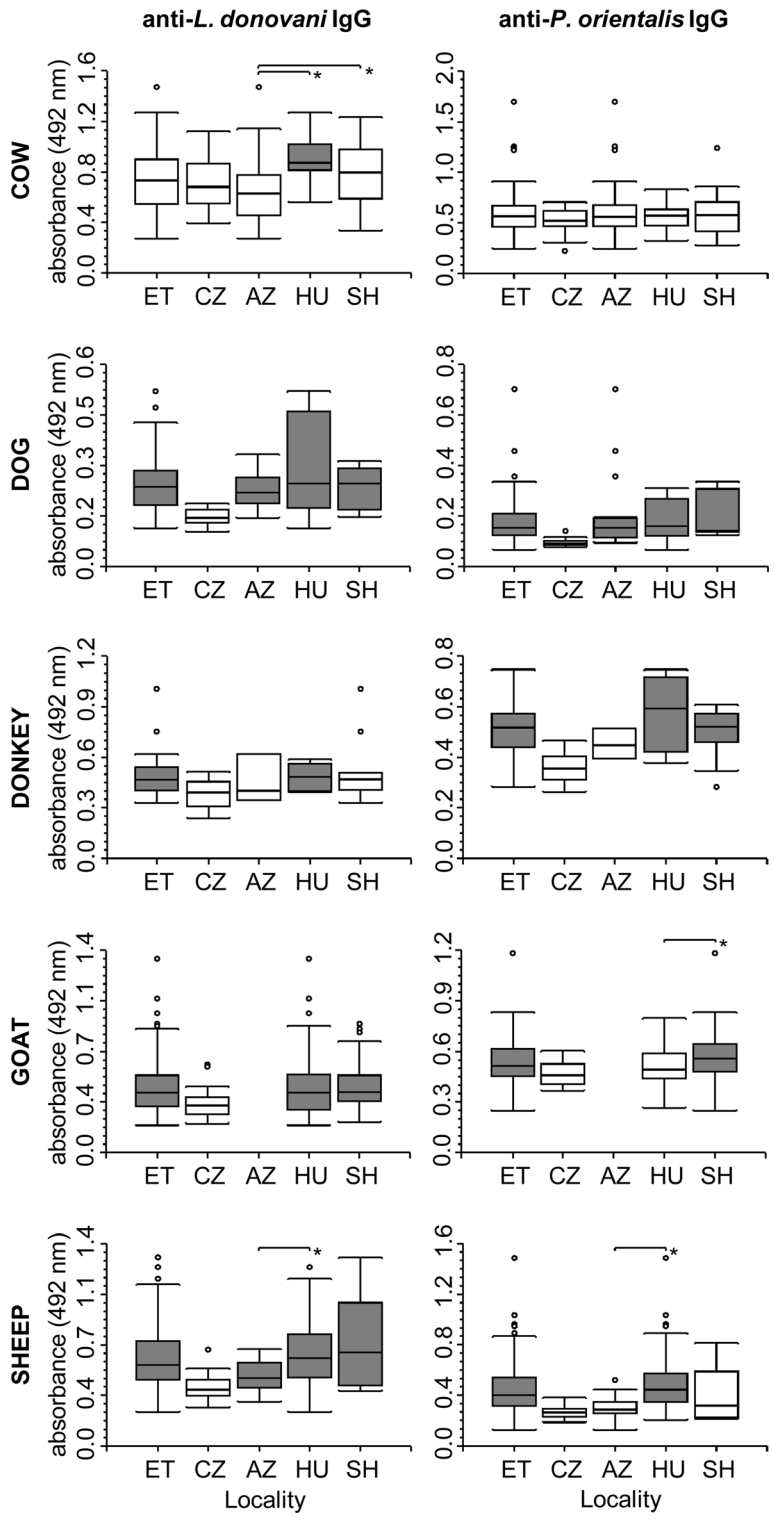

Fig. 2 Serological survey of domestic animals in Ethiopia. IgG antibodies against *Leishmania donovani* promastigotes or *Phlebotomus orientalis* saliva in all serum samples collected from domestic animals in Ethiopia (ET) from Addis Zemen (AZ), Humera (HU), and Sheraro (SH). Significant differences compared with the Czech controls (CZ) are highlighted in gray. The asterisk represents differences between the 3 localities (AZ, HU, and SH) in Ethiopia

Table 3 Seropositivity of Ethiopian animals for *Leishmania donovani* IgG. The cut-off value was calculated as the mean optical density in the control animals plus 3 standard deviations (details provided in the Methods)

		Anti-*L. donovani* IgG positive/total animals sampled (% seropositive)			
Species	Cut-off	Addis Zemen	Sheraro	Humera	Total
Cow	1.298	1/62 (1.6 %)	0/26 (0 %)	0/16 (0 %)	1/104 (1.0 %)
Dog	0.223	9/19 (47.4 %)	5/7 (71.4 %)	5/8 (62.5 %)	19/34 (55.9 %)
Donkey	0.652	0/3 (0 %)	2/11 (18.2 %)	0/6 (0 %)	2/20 (10.0 %)
Goat	0.675		10/106 (9.4 %)	15/133 (11.3 %)	25/239 (10.5 %)
Sheep	0.648	1/27 (3.7 %)	3/5 (60.0 %)	52/117 (44.4 %)	56/149 (37.6 %)
Total		11/111 (9.9 %)	20/155 (12.9 %)	72/280 (25.7 %)	103/546 (18.9 %)

samples were similar to those in control animals, regardless of the locality (Fig. 2).

To verify the specificity of the anti-*P. orientalis* saliva antibodies we used sera from dogs and mice that had been experimentally exposed to a single sand fly species. In dogs, the reactivity of anti-*P. perniciosus* and anti-*Lutzomyia longipalpis* sera against *P. orientalis* salivary gland homogenate (SGH) was similar to that for sera from non-exposed dogs (Fig. 3a). However, all of the selected canine sera of Ethiopian origin reacted strongly to *P. orientalis* SGH (Fig. 3a). In mice, the *P. orientalis* salivary antigen reacted strongly only to the homologous IgGs (Fig. 3b). The reactivities of all heterologous antigen-antibody combinations were similar to those for sera from non-exposed mice (Fig. 3b).

Correlation analysis of serological results

A positive correlation was found between the levels of anti-*P. orientalis* and anti-*L. donovani* IgG in Ethiopian cows ($\rho = 0.37$, $p = 0.0001$), goats ($\rho = 0.37$, $p < 0.0001$), and sheep ($\rho = 0.65$, $p < 0.0001$) (Table 5). This correlation remained significant even when the locality was considered, except for the cows from Humera, for which the correlation was only slightly outside of the level of significance ($\rho = 0.48$, $p = 0.057$). No significant correlation was found for the canine and donkey sera (Table 5).

Discussion

Visceral leishmaniasis is considered to be an anthroponosis in northwestern Ethiopia, but in nearby Sudanese foci, zoonotic transmission has also been suspected, with dogs and mongooses serving as possible reservoirs [3–5, 19]. With regard to domestic animals, sleeping near dogs, cattle, goats, or donkeys has been associated with an increased risk of VL in migrants and residents of Humera [20]. Understanding the mode of disease transmission, whether anthroponotic or zoonotic, is critical for the planning and implementation of effective VL control programs. Thus, one of the main goals of our study was to screen domestic animals for *Leishmania* DNA and discuss their possible involvement in the epidemiology of VL in Ethiopia as possible parasite hosts.

We evaluated two parameters associated with the ability of an animal to be a host for *Leishmania* parasites [21, 22]: (1) exposure to a sand fly vector as a source of blood and (2) the presence of *Leishmania* DNA in the animal's peripheral blood.

In northwestern Ethiopia, the sand fly vector species of *L. donovani* has not yet been identified. However, *Phlebotomus orientalis* is the most probable vector given that it has been found to be infected with *L. donovani* in nearby Sudanese foci [7] and its susceptibility to this *Leishmania* species has been demonstrated experimentally [8]. Exposure to *P. orientalis* was assessed using anti-sand fly saliva antibodies as a marker [6]. Anti-

Table 4 Seropositivity of Ethiopian animals for *Phlebotomus orientalis* saliva IgG. The cut-off value was calculated as the mean optical density in the control animals plus 3 standard deviations (details provided in the Methods)

		Anti-*P. orientalis* IgG positive/total animals sampled (% seropositive)			
Species	Cut-off	Addis Zemen	Sheraro	Humera	Total
Cow	0.876	4/62 (6.5 %)	1/26 (3.8 %)	0/16 (0 %)	5/104 (4.8 %)
Dog	0.143	11/19 (57.9 %)	4/7 (57.1 %)	5/8 (62.5 %)	20/34 (58.8 %)
Donkey	0.550	0/3 (0 %)	3/11 (27.3 %)	4/6 (66.7 %)	7/20 (35.0 %)
Goat	0.685		17/106 (16.0 %)	6/133 (4.5 %)	23/239 (9.6 %)
Sheep	0.410	3/27 (11.1 %)	1/5 (20.0 %)	67/117 (57.3 %)	71/149 (47.7 %)
Total		18/111 (16.2 %)	26/155 (16.8 %)	82/280 (29.3 %)	126/546 (23.1 %)

saliva IgG antibodies were found in all of the animal species tested, which is indicative of the opportunistic feeding behavior of *P. orientalis* [23], thus meeting one criteria for the possible zoonotic transmission of *L. donovani*. Feeding preferences, together with other ecological constraints such as the localization of vector breeding sites [24] or vector susceptibility to harboring *Leishmania* infection [8] may help us to understand the complex picture of the ecology and transmission dynamics of VL in Ethiopia.

The presence of *Leishmania* DNA in animal peripheral blood and *Leishmania* seropositivity serve as reliable epidemiological markers for assessing infection. PCR positivity indicates the presence of the parasite [25, 26]. Although this technique cannot prove the intact integrity of the parasite, viability of the detected *Leishmania* is highly probable given that its DNA degrades shortly after parasite death [27]. Seropositivity, on the other hand, is considered a marker of exposure to *Leishmania* infection [28]. The majority of *Leishmania*-positive animals were found in Humera, indicating dynamic transmission to domestic animals in this well-known active focus. However, many *L. donovani*-seropositive animals were found in all the three surveyed localities, suggesting that exposure to *Leishmania* parasites also occurred in the foci of Addis Zemen and Sheraro.

The fact that only one-third of the PCR-positive animals were positive for both kDNA and ITS1-HRM PCR, is not surprising because the ITS1 region has a considerably lower copy number [11, 12]. Due to the small amount of parasite DNA available in blood samples, distinguishing between the closely related species *L. donovani* and *L. infantum* is notoriously difficult [15]. Moreover, distinction within the *L. donovani* complex in East Africa is controversial; strains that were previously split into *L. donovani*, *L. archibaldi* or *L. infantum* have now been classified into one group: *L. donovani* s.s. [29].

The most suspected animal reservoirs for *L. donovani* are dogs, which are known to play a key role as reservoir hosts in the transmission cycle of the closely related *L. infantum* [2, 30]. Several authors have reported PCR-positivity or seropositivity of dogs in *L. donovani* foci [3, 4, 19, 31–35], including Humera and Addis Zemen in Ethiopia [36–39]. In the present study, dogs demonstrated the highest *Leishmania* seroprevalence out of all the species tested at all study sites, with two PCR-positive dogs identified in Humera and Addis Zemen. As a suspected reservoir species, dogs are also highly attractive to the vector [35], which is supported by our findings that dogs exhibited the highest seroprevalence of anti-*P. orientalis* antibodies among the tested animal species. Most importantly, the same *Leishmania* strains have been recovered from dogs and VL patients [3, 4, 19] and have been shown to persist in dogs for years [19]. Dogs have been recognized as a risk factor for human VL [20, 37, 39], and as the most probable reservoir hosts, their involvement in disease transmission should be addressed in control strategies for VL caused by *L. donovani*.

Almost 38 % of *Leishmania*-positive animals have also been found to be seropositive, indicating these domestic animals (donkeys, goats, sheep) as putative host species in local VL foci. Nevertheless, it is important to mention that neither PCR-positivity nor seropositivity indicates that an animal is able to maintain the parasite for a long period of time. This must be primarily demonstrated by the follow-up of infected animals. Several studies of naturally or experimentally infected non-canine domestic animals have demonstrated their different capabilities to maintain *Leishmania* infection. Cerqueira *et al*. [40] experimentally infected four donkeys with *L. chagasi* (syn. *L. infantum*). These donkeys remained seropositive until the end of the study, which lasted 12 months; however, the donkeys were able to overcome the infection and failed to infect the vector [40]. A PCR survey reported by Bhattarai *et al*. indicated that *Leishmania* infection in goats can persist for at least seven months [41]. On the other hand, *L. donovani* infection in sheep is likely time-limited because only one out of six experimentally infected sheep was shown to develop measurable amounts of anti-*L. donovani* antibodies and the transient presence of amastigotes in sampled tissue in a study that included 244 days of monitoring [42]. Thus, the 37.6 % seropositivity detected in our study may indicate a high infection rate among Ethiopian sheep, further supported by the significantly higher levels of anti-*L. donovani* IgG antibodies among *Leishmania*-positive sheep (Additional files 3 and 4).

The fact that many animals were seropositive for *Leishmania* while PCR-negative in the blood, and, on the other hand, that out of 32 PCR-positive animals, 20 animals were seronegative, could be explained by several possible mechanisms. Seropositivity and PCR-negativity might be attributable to infection in hosts that have resolved the infection but retain high titers of specific antibodies [40, 43]. Another possibility is that seropositive animals might carry the infection in their tissues without parasitemia and are therefore negative according to blood PCR [44]. The reverse situation with PCR-positivity and seronegativity could be attributable to the delayed development of a detectable antibody response in early infection [45], or due to an infection in animals whose B-cells are unresponsive to *Leishmania* antigens, as found in some asymptomatic hosts [2, 43, 44].

The role of other domestic animals as hosts or potential reservoirs for *L. donovani* is still unclear. The

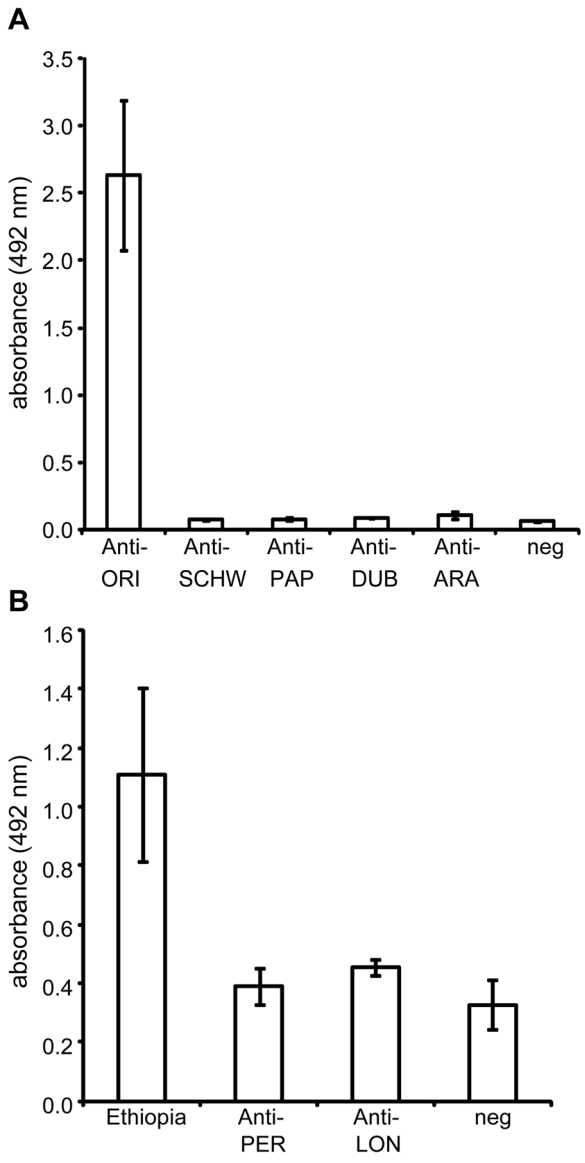

Fig. 3 Specificity of the anti-sand fly saliva IgG antibody response. The reactivity of *Phlebotomus orientalis* salivary gland homogenates (SGH) with sera from mice (**a**) and dogs (**b**) repeatedly exposed to a single sand fly species was measured via ELISA. In graph A, SGH was incubated with sera from mice exposed to *P. orientalis* (Anti-ORI), *Sergentomyia schwetzi* (Anti-SCHW), *P. papatasi* (Anti-PAP), *P. duboscqi* (Anti-DUB), or *P. arabicus* (Anti-ARA). Each bar represents the mean for two serum samples ± the standard error. The values for the positive controls (the sera of mice incubated with homologous antigen) were as follows: *S. schwetzi* = 1.48 ± 0.43, *P. papatasi* = 2.38 ± 0.32, *P. duboscqi* = 2.87 ± 0.60, and *P. arabicus* = 1.86 ± 0.24. In graph B, the SGH was incubated with the sera from dogs exposed to *P. perniciosus* (Anti-PER) or *Lutzomyia longipalpis* (Anti-LON). Seropositive Ethiopian dogs (Ethiopia) and dogs that had never been exposed to sand flies (neg) were used as positive and negative controls, respectively. Each bar represents the mean of five serum samples ± the standard error. The absorbencies of the sera incubated with the homologous antigen were 2.42 ± 0.06 for *L. longipalpis* and 1.73 ± 0.13 for *P. perniciosus*

Table 5 Correlation analysis of serological results

Species		Ethiopia	Addis Zemen	Sheraro	Humera
Cow	ρ	0.37***	0.38**	0.43*	0.48
	n	104	62	26	16
Dog	ρ	0.12	0.15	−0.46	0.36
	n	34	19	7	8
Donkey	ρ	0.31	0.50	0.52	−0.03
	n	20	3	11	6
Goat	ρ	0.37***		0.36***	0.37***
	n	239		106	133
Sheep	ρ	0.65***	0.67***	1.00***	0.61***
	n	149	27	5	117

Results from the Spearman-Rank Correlation Matrix test for anti-*Leishmania donovani* IgG and anti-*Phlebotomus orientalis* saliva IgG
ρ correlation coefficient, *n* number of serum samples tested
Asterisk (*) indicate significant correlations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

present study is the first to report PCR-positive cattle, donkeys, goats, and sheep in Ethiopia. These animals, especially cattle, serve as sources of blood for *L. donovani* vectors [23, 46]. Even if these species do not serve as reservoir hosts for the parasite, they still attract large numbers of blood-questing female sand flies and may, therefore, act as a protective barrier in the case of resistant or refractory mammal species or as a risk factor in the case of susceptible species [30, 37, 47]. Prediction of the role of domestic animals in the amplification or dilution of VL risk might be possible using a recently described mathematical model for multi-host infectious diseases by applying relevant data [48].

In addition to the maintenance of persistent infection, the transmissibility competence, e.g. infectivity for the sand fly vector, is an important prerequisite for any mammal to serve as a *Leishmania* reservoir [28, 49]. These two criteria, among other aspects, can distinguish between a reservoir host and an incidental host that is not capable of infecting the vector [25]. Validation of these prerequisites for domestic animals in northwest Ethiopia, however, requires further investigation.

Conclusions

In conclusion, leishmaniasis caused by *L. donovani* is traditionally considered to be an anthroponosis in East Africa. However, the present study revealed widespread exposure to *L. donovani* and sand fly vector bites among domestic animals. The possible involvement of domestic animals as sources of blood for vector sand flies should therefore be considered in VL control strategies. However, the direct involvement of domestic animals in the transmission cycle of *L. donovani* warrants further

investigation, most importantly by xenodiagnosis to determine their transmissibility competence.

Additional files

Additional file 1: Accession numbers for *Leishmania* ITS sequences downloaded from the GenBank database and used for the phylogenetic analysis presented in Fig. 1.

Additional file 2: Details of the ELISA methods.

Additional file 3: Detailed list of Ethiopian animals positive for *Leishmania* DNA.

Additional file 4: Differences in the levels of anti-*Leishmania donovani* IgG and anti-*Phlebotomus orientalis* saliva IgG between *Leishmania*-positive (full circle) and *Leishmania*-negative (open circle) animals in the Humera region (the majority of PCR-positive animals are from this locality: 30 out of 32). Significant differences are marked by the probability level on the X-axis.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay; ITS1: Internal transcribed spacer 1; kDNA: Kinetoplast deoxyribonucleic acid; *L.*: *Leishmania* or *Lutzomyia*; OD: Optical density; *P.*: *Phlebotomus*; PBS: Phosphate-buffered saline; PBS-Tw: Phosphate-buffered saline with Tween; PCR: Polymerase chain reaction; SGH: Salivary gland homogenate; VL: Visceral leishmaniasis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IR participated in the study design and field sample collection, carried out *Leishmania* serology, performed the statistical analysis, and drafted and finalized the manuscript. DTF and DYL carried out the *Leishmania* PCR and sequencing. TK, NP, and TL performed sand fly serology. AK, CM, RK, CLJ, and AW participated in field sample collection. JV carried out the sequence alignment and phylogenetic analysis. JV, AW, AH, and PV participated in the study design and coordination. GB conceived and designed the study, coordinated and participated in field work, and drafted and finalized the manuscript. DTF and TK contributed equally to the paper. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Canine Antibodies against Salivary Recombinant Proteins of *Phlebotomus perniciosus*: A Longitudinal Study in an Endemic Focus of Canine Leishmaniasis

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Abstract

Background

Phlebotomine sand flies are vectors of *Leishmania* parasites. During blood feeding, sand flies deposit into the host skin immunogenic salivary proteins which elicit specific antibody responses. These anti-saliva antibodies enable an estimate of the host exposure to sand flies and, in leishmaniasis endemic areas, also the risk for *Leishmania* infections. However, the use of whole salivary gland homogenates as antigen has several limitations, and therefore, recombinant salivary proteins have been tested to replace them in antibody detection assays. In this study, we have used for the first time sand fly salivary recombinant proteins in a longitudinal field study on dogs.

Methodology/Principal Findings

Sera from dogs naturally exposed to *P. perniciosus* bites over two consecutive transmission seasons in a site endemic for canine leishmaniasis (CanL) were tested at different time points by ELISA for the antibodies recognizing whole saliva, single salivary 43 kDa yellow-related recombinant protein (rSP03B), and a combination of two salivary recombinant proteins, 43 kDa yellow-related protein and 35.5 kDa apyrase (rSP01). Dogs were also tested for *Leishmania infantum* positivity by serology, culture, and PCR and the infection status was evaluated prospectively. We found a significant association between active CanL infection and the amount of anti-*P. perniciosus* saliva antibodies. Importantly, we detected a high correlation between IgG antibodies recognizing rSP03B protein and the whole salivary antigen. The kinetics of antibody response showed for both a whole saliva and rSP03B a similar pattern that was clearly related to the seasonal abundance of *P. perniciosus*.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

These results suggest that *P. perniciosus* rSP03B protein is a valid alternative to whole saliva and could be used in large-scale serological studies. This novel method could be a practical and economically-sound tool to detect the host exposure to sand fly bites in CanL endemic areas.

Author Summary

Canine leishmaniasis (CanL) is a widespread zoonosis caused by protozoan parasite *Leishmania infantum*. CanL is endemic in more than 70 countries, including regions of south-western Europe, where the main vector is *Phlebotomus perniciosus*. Saliva inoculated into the host during blood feeding provoke species-specific antibody response. They could be used as a serological marker of exposure to sand flies and, in leishmaniasis endemic areas, also as an indication of *Leishmania* infection risk. However, in case of large-scale serological studies, it is difficult to obtain appropriate amounts of crude salivary antigen from laboratory-reared sand flies. Therefore, we studied if specific salivary recombinant proteins could be suitable to replace whole saliva as an antigen for serological assays. Antibodies recognizing recombinant salivary protein in naturally bitten dogs were highly correlated with antibody response against whole saliva. Seasonal exposure of dogs to sand flies led to antibody response fluctuations related to the period of activity and abundance of vectors. In a context of high CanL endemicity, we found a significant association between progressive CanL infections and antibodies against sand fly saliva. Thus, salivary recombinant proteins could be used as suitable tool for investigations host exposure to sand flies in endemic settings.

Introduction

Canine leishmaniasis (CanL), caused by protozoan parasite *Leishmania infantum*, is a systemic and potentially fatal disease [reviewed in [1, 2]]. It may affect any organ or body fluid [reviewed in [1]] and can manifest variable clinical signs [reviewed in [2, 3]]. However, the majority of infected dogs do not develop any clinical signs. Importantly, *L. infantum* is also a causative agent of human visceral leishmaniasis and both symptomatic and asymptomatic dogs have a crucial role in the epidemiology of this disease, serving as reservoirs [reviewed in [2]]. CanL is endemic in many regions of southern Europe and Latin America, however, climate changes and socioeconomic factors, particularly increased travelling of dogs between endemic and non-endemic areas, led to changes in the distribution of CanL in both continents [reviewed in [1, 2, 4]].

Diagnosis of CanL should be based on an integrated approach considering signalment, history, clinical findings, and results of basic laboratory analyses that detect the parasite or evaluate the immune response in the host [reviewed in [3]]. The commonly used diagnostic methods include direct detection of the parasite by culture or polymerase chain reaction (PCR) and indirect techniques for detection of antibodies against *Leishmania*, such as immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) [5–8]. However, there is still lack of practical methods for detection of the risk of *Leishmania* transmission according to the exposure of dogs to sand fly vectors.

Results from studies on human and canine antibody response against sand fly saliva revealed high immunogenicity of sand fly salivary proteins as well as high specificity of anti-

saliva IgG [9–12]. Studies based on dynamics of IgG response in dogs experimentally exposed to the main *L. infantum* vectors, *Lutzomyia longipalpis* and *Phlebotomus perniciosus* [12, 13], suggest that monitoring canine antibody response to sand fly saliva could be a useful epidemiological tool in CanL foci. However, the use of whole sand fly saliva in such studies is limited by time-consuming salivary gland dissection and complicated by potential cross-reactivity with saliva from sand fly species with no role in *Leishmania* transmission [11] or from other blood sucking insects [14]. These problems could be overcome by using specific immunogenic sand fly salivary recombinant proteins [reviewed in [15]]. The use of recombinant proteins was already shown for *P. papatasi* [16, 17], *Lu. longipalpis* [18, 19] and *P. perniciosus* [20, 21]. Although these studies confirmed the advantages of salivary recombinant proteins, most of them were tested on small sets of samples and never in association with naturally transmitted *Leishmania* infections.

Previous studies on different hosts (dogs, foxes, and humans) bitten by *P. perniciosus* or *Lu. longipalpis* revealed high antigenicity of salivary yellow-related proteins and apyrases [12, 13, 19]. Apyrases are enzymes with potent anti-hemostatic activity hydrolyzing the platelet activator ADP [22]. The role of yellow-related proteins is less clear [23] but most probably they act as a histamin-binding molecules [24]. Recent work using a low number of sera from dogs experimentally exposed to *P. perniciosus* showed that recombinant forms of these proteins could be used as potential candidates for markers of canine exposure to sand flies [20]. Therefore, herein we used the recombinant 43 kDa yellow-related protein (rSP03B) from *P. perniciosus* and its combination with recombinant *P. perniciosus* 35.5 kDa apyrase (rSP01) to study the specific antibody response in a large number of dogs naturally exposed to *P. perniciosus* over two years in a focus endemic for *L. infantum*.

Methods

Ethical statement

The technical protocol for the investigation of natural canine *Leishmania* infections performed in the frame of experimental drug trials was approved by the Veterinary Board of the Italian Ministry of Health (authorization no.4051/P) following the European Directive 86/609/EEC, adopted by the Italian Government with the Law 116/1992.

Experimental design and background

A longitudinal study including two sand fly seasons was performed on 56 Beagle dogs housed in the same open-air kennel sited in a rural municipality of the Naples province (Campania region, southern Italy) where both human visceral leishmaniasis and CanL are highly endemic. Here, *P. perniciosus* is the only *Leishmania* vector, whose activity period ranges from the end of May through late October [25]. *Leishmania infantum* infection rates in dissected sand flies were found to range from 2.8% to 6.2% in places not far from the kennel [26]. In cohorts of naïve dogs previously exposed in the same kennel, the annual incidence of CanL infection and clinical disease was reported to average about 40% and 20%, respectively [8]. The dogs included in our study were part of experimental trials of pharmacological products against CanL. The animals were born in a breeding facility sited in a non-endemic area of northern Europe, and were confirmed to be CanL-free at the time they were moved to the study site when they were about 6 months old. Once housed in the open-air kennel, the use of topical or environmental insecticides was avoided to allow natural exposure of dogs to sand fly bites in the warm season.

Antibodies against sand fly salivary proteins were analyzed retrospectively in dog sera taken for routine clinical examination and periodical CanL serology, and that were stored frozen. Available samples included sera obtained shortly before the dogs were transferred to the

endemic area in July (first year), and they served as pre-immune sera (n = 56). Responses to salivary antigens were then analyzed on selected samples collected at four-time points in the first year [August (n = 53), September (n = 54), October (n = 34), December (n = 54)] and at five occasions during the second year [January (n = 56), March (n = 56), July (n = 56), August (n = 56), September (n = 54)]. As per established protocols [5], follow-up analyses for the detection and classification of natural CanL infections were performed at the beginning of the study in the first year in July (n = 56), twice in the second year [March (n = 56) and July (n = 56)] and once at the beginning of the third year [March (n = 53)].

Analysis of *Leishmania* infection

Dogs were examined by serology, culture, and PCR. Detection of anti-*Leishmania* IgG antibodies was performed by an in-house IFAT assay using *L*. *infantum* promastigotes as antigen and following the protocol recommended by the Office International des Epizooties [27]. The cut-off dilution for *Leishmania* exposure was set at 1:40 (i.e. the upper part of the so-called "IFAT grey zone") [3]. Bone-marrow aspirate material was examined by nested-PCR assay as previously described [28]. Lymph-node aspirate material was cultured from each popliteal node in Evans' Modified Tobie's medium and cultures were periodically examined for promastigotes growth during one month.

At each assessment, the dogs were classified as follows with regards to the infection status: i) "*Leishmania* negative"if found negative by all assays, ii)"*Leishmania* exposed"if tested positive by IFAT at low titers and negative by other tests, iii) having a"subpatent *Leishmania* infection" if bone-marrow PCR tested positive, IFAT was either negative or positive at low titers, and lymph node culture was negative, iv) having an"active *Leishmania* infection" if both bone-marrow PCR and lymph node culture were tested positive. In this infection stage, IFAT can be found negative or positive at low titer initially, but converts shortly to very elevated titers. Once established, active infections do not regress spontaneously towards negative or subpatent conditions and dogs invariably progress to clinical disease [8].

Sand flies and salivary proteins

A colony of *P*. *perniciosus* was reared under standard conditions as described in [29]. Salivary glands, dissected from 4–6 day old female sand flies, were pooled in 20 mM Tris buffer with 150 mM NaCl and stored at -20°C. Recombinant salivary proteins from *P*. *perniciosus*, 35.5 kDa apyrase (rSP01, Genbank accn. DQ192490) and 43 kDa yellow-related protein (rSP03B, Genbank accn. DQ150622) were obtained from Apronex s.r.o. (Prague) as mentioned in [20]. The concentrations of these proteins were quantified by the Lowry method (Bio-Rad) following the manufacturer´s protocol.

Detection of anti- *P*. *perniciosus* IgG

Anti-*P*. *perniciosus* IgG were measured by enzyme-linked immunosorbent assay (ELISA) as described in [12] with minor modifications. Briefly, microtiter plates were coated either with salivary gland homogenate (SGH) (0.2 salivary gland per well) or with rSP03B (5μg/ml) or with rSP03B+rSP01 (5μg/ml of each protein) in 20 mM carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. The plates were incubated with blocking solution, 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine sera were diluted 1:200 for SGH and 1:100 for recombinant proteins in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, Bethyl laboratories) were diluted 1:9000 in PBS-Tw. Absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). Each serum was tested in duplicate and the experiment was repeated twice.

Statistical analysis

Antibodies against sand fly saliva are reported as optical densities (OD) with subtracted blanks and multiplied by 100 for easier readability. In case of linear mixed models, ODs were log transformed (OD value+1).

Statistical analyses were carried out using R software (http://cran.r-project.org/). Repeatability defined as the degree of agreement between repeated OD measures on the same samples was estimated within and between plates by calculating the concordance correlation coefficient (CCC) that ranges from 0 (no concordance) to 1 (perfect concordance) [30].

Antibodies against sand fly saliva and *Leishmania* infection status frequency distributions were analysed over time. Proportions and medians between time points were compared using McNemar's chi-square test for paired data and Wilcoxon signed rank sum test, respectively. Correlations were analysed using Spearman rank correlation test [31].

The "nlme" package [32] was then used to develop multilevel linear regression models to investigate the relationship between IgG anti-saliva antibodies as continuous dependent variable, and sampling months and *L. infantum* infection status (as described above) included as categorical predictor variables, taking into account the correlation between repeated measures of the same dogs over time. Two hierarchical levels were considered in the analysis with repeated measures within individual dogs as the level-1 units and individual dogs as the level-2 units. The random variation at the dog level was examined at both the intercept and at the slopes and significance was assessed testing the -2 log likelihood ratios between the model with and the model without the random effect [33]. The correlation between level 1 units was considered as having a compound-symmetry (CS) or autoregressive with a lag of 1 (AR-1) structure or as unstructured (UN) and Akaikes's Information Criterion (AIC) was used to compare the goodness of fit of models with different correlation structures, selecting those with the smallest values of these statistics [33, 34]. Parameter estimates for fixed effects were estimated using restricted maximum likelihood estimation (REML) and significance was assessed using conditional t-tests and F-tests, and alpha was taken at the 5% ($p<0.05$) level for a two-tailed test.

Results

Dynamics of *L. infantum* infection status

The percentage of dogs classified as negative, *Leishmania* exposed, subpatently and actively infected is presented in Fig 1.

At the beginning of the study (July in the first year) all dogs were negative by all tests, thereafter, the proportion of *L. infantum* infected dogs increased throughout the trial ($p<0.05$) (Fig 1). In the following sampling, in March, 32% of dogs were classified as *Leishmania* exposed, while subpatent and active infections were found in 2% and 4% of dogs, respectively. The percentage of dogs in each category was similar 3 months later in July (Fig 1). In contrast, by the end of the study in March of the third year, the percentage of subpatently and actively infected dogs sharply increased to 17% and 13%, respectively. By this time, 36% of dogs were classified as *Leishmania* exposed and 34% as negative ($p<0.05$) (Fig 1).

Distribution and dynamics of antibody response against salivary proteins using SGH, rSP03B, and rSP03B+rSP01 as antigens

The overall median values of ELISA ODx100 using SGH, rSP03B, and rSP03B+rSP01 antigens were 10 (range: 2–194), 24 (1–234) and 37 (11–189), respectively ($p<0.05$). However, the median increased significantly with time, following a similar pattern for all three antigens

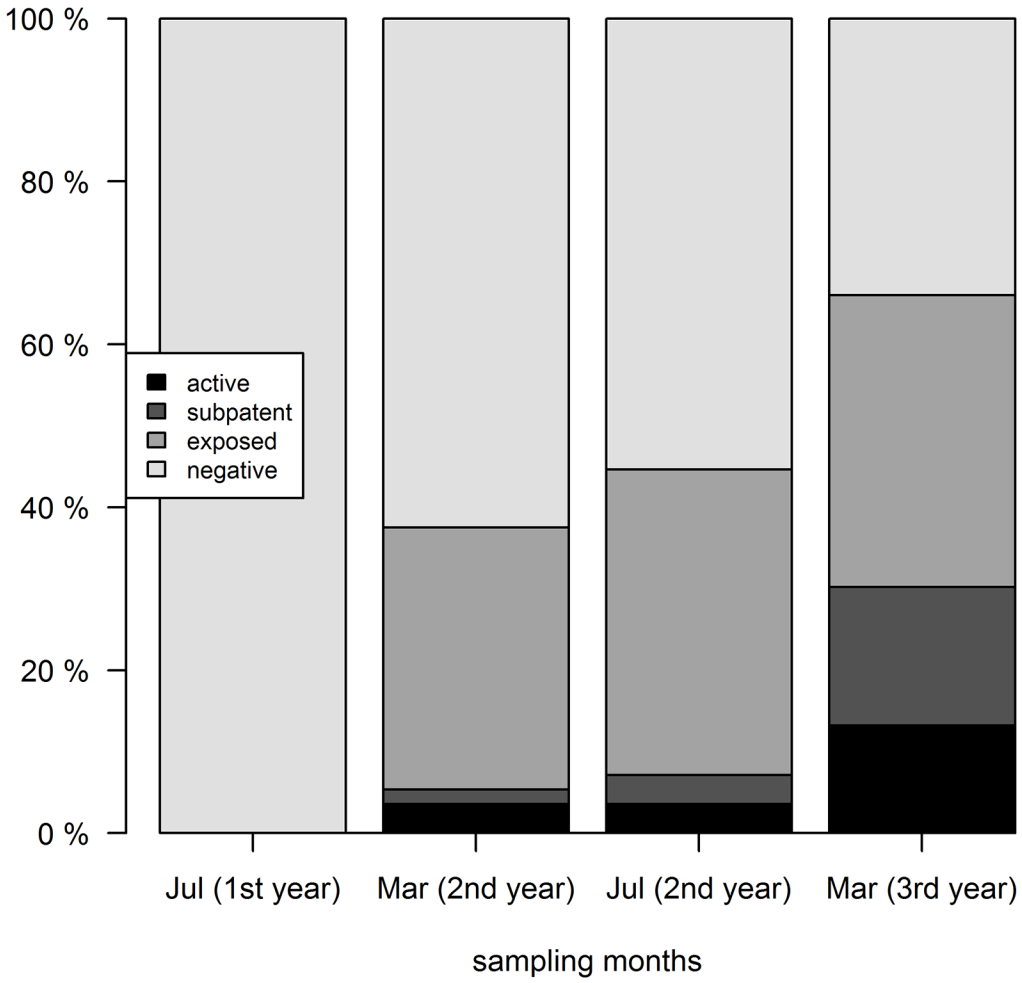

Fig 1. Dynamics of *L. infantum* infection statuses in dogs from a CanL endemic site in southern Italy. Dogs were screened for *L. infantum* infection on four occasions, 56 dogs in July first year, 56 dogs in March the second year, 56 dogs in July the second year and 53 dogs in March the third year.

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tested, most significantly for SGH and rSP03B (Fig 2). For example, the median ODx100 for SGH was 5 at the baseline in July of the first year, increased significantly to 11 through October, decreased thereafter to 8 in January, increased again up to a peak of 24 detected in July of the second year, and remained similar until September (Table 1). The sharp OD increase observed in summer of the second year for SGH and rSP03B was less pronounced for rSP03B+rSP01 (Table 1). Moreover, correlation of antibody response between SGH and rSP03B was stronger (r = 0.77) than between rSP03B+rSP01 and SGH (r = 0.65) (Fig 3). In addition, we detected high correlation between antibodies recognizing rSP03B and antibodies recognizing combination of rSP03B+rSP01 (r = 0.75) (Fig 3).

Within plate repeatability was high for each antigen and the CCC varied from 0.94 (95% CI: 0.93–0.95) to 0.99 (95% CI: 0.98–0.99), depending on the antigen. However, the CCC between plate was moderately high for whole SGH (0.77, 95% CI: 0.73–0.8) and rSP03B (0.88, 95% CI: 0.87–0.9) and low for the combination of rSP03B+ rSP01 (0.53, 95% CI: 0.47–0.59).

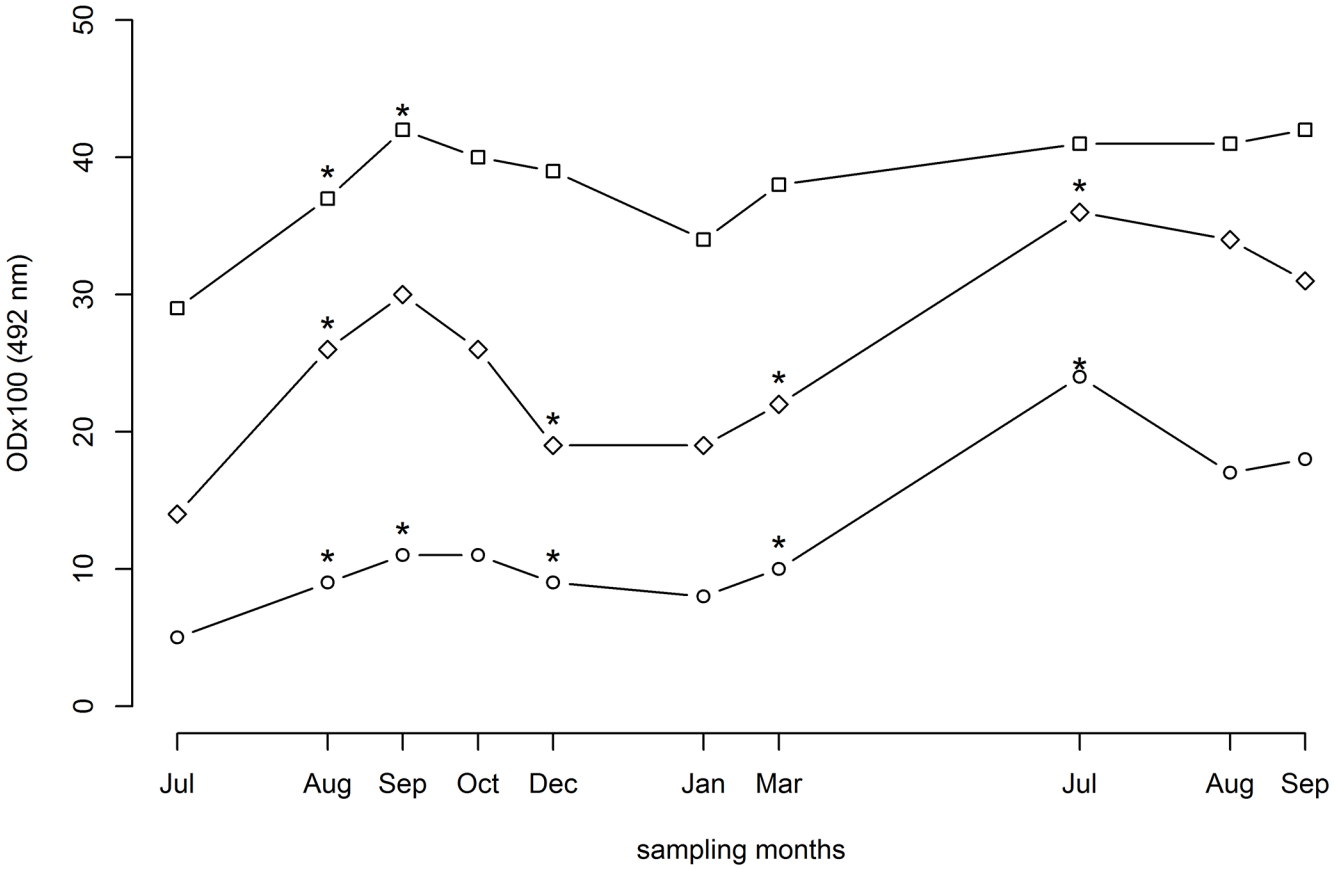

Fig 2. Dynamics of IgG antibody response against sand fly salivary proteins in dogs naturally exposed to *P. perniciosus* over two years in endemic foci. Canine sera were tested by ELISA for the antibodies recognizing SGH (open circle), rSP03B protein (open diamond) and combination of rSP03B+rSP01 proteins (open square). Data are presented as median values for each sampling month. Asterisk represents significant change in the median compared to previous sampling. ODx100 = optical density multiplied by 100.

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Table 1. Median and range antibody OD values (multiplied by 100) for each used antigen according sampling month.

Study year	Sampling month		SGH	rSP03B	rSP03B+rSP01
		n	Median(range)	Median(range)	Median(range)
First	July	56	5 (3–12)	14 (7–36)	29 (19–49)
	August	53	9 (4–42)*	26 (14–75) *	37 (16–71) *
	September	54	11 (4–56) *	30 (11–165)	42 (19–116)*
	October	34	11 (3–59)	26 (11–93)	40 (17–85)
	December	54	9 (4–50) *	19 (10–81) *	39 (21–100)
Second	January	56	8 (2–49)	19 (1–69)	34 (19–93)
	March	56	10 (4–62)*	22 (9–89)*	38 (11–117)
	July	56	24 (6–161) *	36 (9–223) *	41 (17–177)
	August	56	17 (5–129)	34 (11–154)	41 (18–157)
	September	54	18 (6–194)	31 (11–234)	42 (18–189)
	Total		10 (2–194)	24 (1–234)	37 (11–189)

n = number of dogs

* significant change in the median compared to previous sampling

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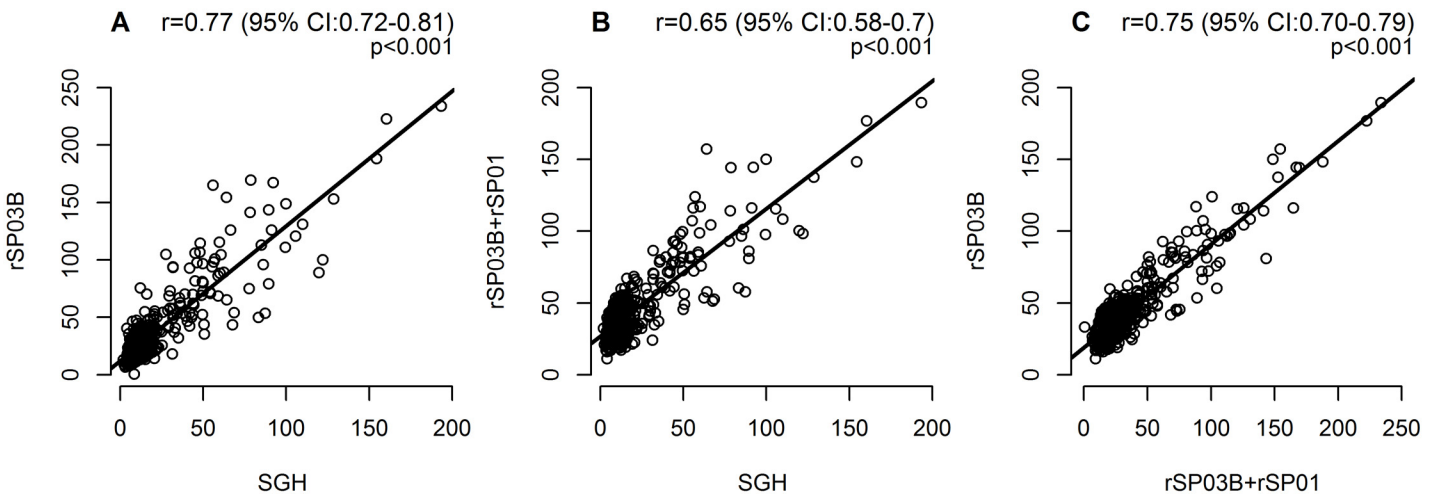

Fig 3. Correlations between IgG antibodies recognizing SGH and recombinant salivary proteins in dogs naturally exposed to sand flies. Correlation between SGH and rSP03B protein (A), between SGH and combination of rSP03B+rSP01proteins (B) and between rSP03B protein and combination of rSP03B+rSP01proteins (C) was performed using Spearman rank correlation. OD values were multiplied by 100. r = correlation index, CI = confidence interval.

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Multivariable relationship between salivary antibodies, season, and *L. infantum* infection

Multilevel models confirmed the strong association between antibodies against SGH and recombinant proteins and sampling month, and between SGH and active *L. infantum* infection.

Tables 2 and 3 present parameters estimates for models including only sampling date (model a) and both sampling date and *L. infantum* infection status (model b) for SGH and rSP03B, respectively (for combination of rSP03B+rSP01 data are showed in S1 Table). Estimates for sampling months alone reflect a significant increase in log OD values by September in comparison to July of the first year, when the study started, then they started to decrease in October, and raised again in March and especially in July of the second year, with the highest log OD estimate detected in the following month of September ($p<0.05$) (Tables 2 and 3, model a). Including dog as a random effect significantly improved the model and the variance estimate indicated that for SGH it was 33% [(10.06/(10.06+20.35))*100] of the variation in log OD dog related. For rSP03B it was 38% [(17.33/(17.33+28.67))*100]. This can be appreciated in Fig 4A and 4B representing the sampling month-specific ODs for the 56 study dogs. Finally, model b shows the sharp increase in log OD between March and July of the second year (Tables 2 and 3) and highlights the strong association between the amount of antibodies against whole saliva and dogs with active *L. infantum* infection ($p<0.05$) (Table 2). The association between active infection and anti-rSP03B antibodies (Table 3) and combination of rSP03B+rSP01did not reach statistical significance. The model also shows that the greatest variation in log ODs between dogs was observed in July of the second year (Tables 2 and 3). There was no evidence of correlation between repeated measures in any of the above models.

Discussion

This paper describes for the first time the dynamics and diagnostic potential of antibodies recognizing *P. perniciosus* salivary recombinant proteins in dogs following natural exposure to the sand flies over two years.

Table 2. Estimates of the multilevel linear regression model of the relationship between log transformed SGH OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).

Variable	Levels	Estimate	SE	P value
a) sampling month only				
Intercept		4.33	0.74	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	August (first year)	1.56	0.87	0.073
	September (first year)	2.85	0.86	0.001
	October (first year)	2.49	0.99	0.012
	December (first year)	1.60	0.86	0.064
	January (second year)	1.55	0.85	0.069
	March (second year)	2.36	0.85	0.006
	July (second year)	9.21	0.85	<0.001
	August (second year)	7.84	0.85	<0.001
	September (second year)	9.62	0.86	<0.001
Random effects	**Variance**			
Dog	10.06			
Residual	20.35			
b) sampling month and *Leishmania* infection status	Levels	Estimate	SE	P value
Intercept		4.33	0.11	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	March (second year)	1.71	0.56	0.003
	July (second year)	8.46	1.2	<0.001
Leishmania status	Negative	0.00		
	Exposed	1.39	0.90	0.13
	Subpatent	1.28	2.96	0.67
	Active	5.11	2.38	0.03
Random effects	**Variance**			
Dog	0.11			
March (second year)	10.42			
July (second year)	71.52			
Residual	0.51			

SE = standard error

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The study area is considered traditionally endemic for *L. infantum* [35]. The rate of dogs with active infection was increasing over the two-year period, and this pattern was in accordance with previous studies carried out in the area [5, 8]. Our results indicate that there is a significant positive association between anti-*P. perniciosus* saliva antibodies and active *L. infantum* infection. This finding could reflect that these dogs had a greater sand fly challenge compared to other dogs and therefore developed a stronger humoral response against salivary proteins, thus had bigger chance to become infected. Alternatively, because active *L. infantum* infection in dogs is characterized by a mixed Th1/Th2 response associated with marked antibody production [reviewed in [36]], it is possible that our *Leishmania* infected dogs were more sensitive to develop a humoral antibody response against sand fly salivary gland proteins. So far, there is only one report describing an association between CanL and positivity for anti-sand fly saliva antibodies. The study, performed in a *L. infantum* focus in Apulia region of

Table 3. Estimates of the multilevel linear regression model of the relationship between log transformed rSP03B OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).

Variable	Levels	Estimate	SE	P value
a) sampling month only				
Intercept		8.18	0.91	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	August (first year)	4.15	1.03	<0.001
	September (first year)	5.87	1.02	<0.001
	October (first year)	3.61	1.18	0.002
	December (first year)	2.14	1.02	0.037
	January (second year)	1.53	1.01	0.131
	March (second year)	2.88	1.01	0.005
	July (second year)	10.81	1.01	<0.001
	August (second year)	8.50	1.01	<0.001
	September (second year)	9.67	1.02	<0.001
Random effects	**Variance**			
Dog	17.33			
Residual	28.67			
b) sampling date and *Leishmania* infection status	Levels	Estimate	SE	P value
Intercept		8.18	0.32	<0.001
Fixed effects				
Sampling month	July (first year)	0.00		
	March (second year)	2.26	0.74	0.003
	July (second year)	10.13	1.49	<0.001
Leishmania status	Negative	0.00		
	Exposed	1.47	1.11	0.188
	Subpatent	-1.11	3.59	0.758
	Active	4.68	3.14	0.139
Random effects	**Variance**			
Dog	2.39			
March (second year)	15.34			
July (second year)	104.54			
Residual	3.31			

SE = standard error

doi:10.1371/journal.pntd.0003855.t003

Italy, showed that *Leishmania* positive and negative dogs did not differ in IgG and IgG1 production against the whole *P. perniciosus* salivary lysates but the *Leishmania* positive ones had significantly decreased levels of IgG2 antibodies [12]. However, we did not find any association between antibodies against rSP03B protein and active CanL infection. Although, the use of rSP03B protein as a marker of exposure to sand flies is promising, use of recombinant proteins as risk markers for infection need more investigation.

In the present study *P. perniciosus* salivary recombinant proteins, rSP03B (yellow- related protein) and its combination with rSP01 (apyrase) were used. For rSP03B, the repeatability of ELISA test was even higher than for SGH, proving this recombinant protein to be better antigen for large scale studies. The achieved high correlation score between SGH and rSP03B was in agreement with results from previous studies [20, 21]. However, the combination of two recombinant proteins (rSP03B and rSP01) did not show better performance than a single

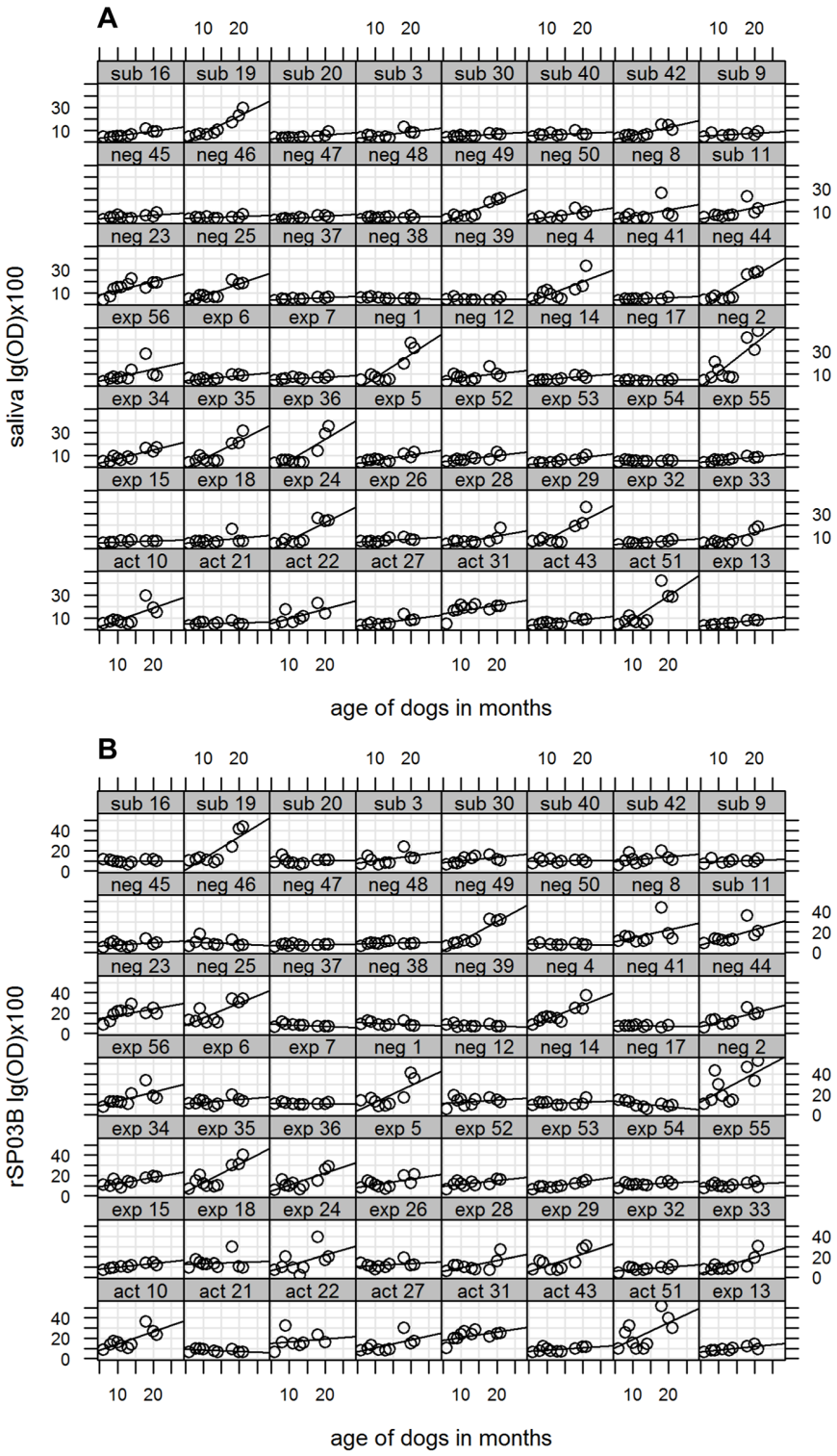

Fig 4. Dynamics of IgG antibodies recognizing SGH (A) and rSP03B protein (B) in individual dogs from CanL endemic locality. *Leishmania* infection status was assigned for 53 dogs according their infection status at the end of the study in March third year and for 3 dogs according their infection status in July second year (dog number 7, 8, 49). OD values were log transformed and multiplied by 100. OD = optical density, neg = negative, exp = exposed, sub = subpatent, act = active.

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SP03B. Repeated experiments suggest that this could be due to instability of rSP01 protein and its higher susceptibility to repeated thawing and freezing. Previously, better results for recombinant proteins combination were obtained for detection of anti-*Lu. longipalpis* antibodies when two recombinant yellow-related proteins were combined together [18].

Kinetics of anti-SGH and anti-rSP03B IgG antibodies developed with similar pattern and were clearly seasonal: rising during summer months when sand fly density is the highest and decreasing during winter months when sand flies are not active. This positive correlation between antibodies against salivary proteins and seasonal abundance of blood feeding insects has been previously reported in mosquitoes [37, 38]. The smaller increase of antibodies against *P. perniciosus* salivary proteins during the first transmission season was probably due to the fact that dogs were exposed to the vector for the first time and were moved to an endemic locality in the middle of the transmission season (July). In central and southern peninsular Italy, the sand fly season usually lasts from late May to late October, with two density peaks [25, 39]. The significant increase of IgG antibodies for SGH and rSP03B protein in March compared to January in the second year (Fig 2) remains unexplained, since it cannot be attributed to such an early activity of sand flies. Even in the southernmost region of Italy (Sicily) the earliest collection of *P. perniciosus* was recently reported to be in April [40]. The marked increase in antibody levels in the second transmission season is much probably caused by re-exposure of dogs to sand flies following antigenic priming in the previous season. Similar antibody responses to sand fly saliva were observed in mice re-exposed to *P. papatasi* [17] and in humans re-exposed to *P. argentipes* [11]. Moreover, the vector population density could vary between years due to the different climatic conditions [39] and therefore, the dogs could have been exposed to higher sand fly challenge during the second transmission season. However, as shown by the multilevel models, there was significant variation in the amount of anti-saliva antibodies between dogs, particularly during July of the second year, the reason of which remains unclear. It could be associated with innate differences in their antibody responses to sand fly salivary antigens or in attractiveness to sand fly bites, or with other unknown factors that need to be investigated. Interestingly, it was recently reported that Beagle dogs may exhibit markedly different attractiveness to *P. perniciosus* under experimental exposure [41].

In conclusion, the dynamics of antibody response against *P. perniciosus* salivary proteins clearly showed seasonal changes due to the expected sand fly abundance. Our study confirmed that recombinant yellow-related protein rSP03B of *P. perniciosus* is a valid alternative to whole sand fly saliva as marker of sand fly exposure. Serology tests based on this recombinant protein could be a practical and economically-sound tool for investigations dog exposure to sand flies in endemic settings of zoonotic visceral leishmaniasis.

Supporting Information

S1 Table. Estimates of the multilevel linear regression model of the relationship between log transformed rSP03B+rSP01 OD values (multiplied by 100) and sampling time (model a), and *Leishmania* status and sampling time (model b).
(DOCX)

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Author Contributions

Conceived and designed the experiments: PV IR LG MG. Performed the experiments: GO AS LG MG EF TK TL PS MV. Analyzed the data: TK EB. Contributed reagents/materials/analysis tools: PV LG MG. Wrote the paper: TK PV LG MG EB.

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The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

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The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

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Short title: Recombinant protein as exposure marker

Abstract

Frequency of sand fly-host contacts can be measured by host antibody levels against sand fly salivary proteins. Recombinant salivary proteins are suggested as valid replacement for salivary gland homogenate (SGH), however, it is necessary to prove that such antigens are recognized by antibodies against various populations of the same species. *Phlebotomus perniciosus* (Diptera: Psychodidae) is the main vector of *Leishmania infantum* (Trypanosomatida: Trypanosomatidae) in south-western Europe, being widespread from Portugal through Italy. In this study, sera were sampled from naturally exposed dogs from distant regions, Campania (south Italy), Umbria

(central Italy) and Metropolitan Lisbon region (Portugal), where *P. perniciosus* is the unique or principal vector species. Sera were screened for anti-*P. perniciosus* antibodies using SGH and 43 kDa yellow-related recombinant protein (rSP03B). Robust correlation between antibodies recognizing SGH and rSP03B was detected in all regions, suggesting substantial antigenic cross-reactivity among different *P. perniciosus* populations. No significant differences in this relationship were detected between regions. Moreover, we showed that rSP03B and the native yellow-related protein share similar antigenic epitopes, as canine IgG binding to the native protein was inhibited by pre-incubation with recombinant form. We propose rSP03B protein as universal marker of sand fly exposure throughout geographical distribution of *P. perniciosus*.

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Key words: *Leishmania infantum*, antibody response, dog, markers of exposure, Mediterranean region, salivary proteins, sand flies

Leishmaniasis is a widely distributed disease, caused by *Leishmania* protozoans and transmitted by phlebotomine sand fly vectors. During blood-feeding, sand flies inoculate saliva into the host. Bitten hosts then develop species-specific antibody response against salivary antigens that reflects the intensity of sand fly exposure and thus provides a useful marker of exposure to generate epidemiological data (Vlkova *et al.*, 2011; Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015).

Large-scale serological studies using total sand fly salivary gland homogenate (SGH) are currently impractical due to the difficulty of dissecting large numbers of sand flies to obtain sufficient amount of SGH. Another potential complication is variability in protein composition of the sand fly saliva that was found to fluctuate depending on physiological factors such as sand fly age and diet (Volf *et al.*, 2000; Prates *et al.*, 2008). Studies on Old World sand fly species also revealed a certain degree of intra- and inter-population variability in protein and mRNA levels e.g. (Rohousova *et al.*, 2012; Ramalho-Ortigão *et al.*, 2015). Therefore, salivary recombinant proteins were suggested to replace the whole salivary gland protein cocktail, and some have

already been validated in the field e.g. (Martín-Martín *et al.*, 2014; Kostalova *et al.*, 2015). The use of specific recombinant salivary antigen circumvents the necessity for laborious maintenance of sand fly colonies, and potentially refined to minimise antigenic cross-reactivity with taxonomically close sand fly relatives. A useful recombinant salivary protein would demonstrate comparable antigenicity to SGH and share similar antigenic epitopes with the native proteins, and demonstrate similar antigenic patterns throughout the geographical distribution of a particular sand fly vector.

In this study, we focused on *Phlebotomus perniciosus,* a sand fly species widely distributed in south-western Europe where it acts as a proven vector of *Leishmania infantum*, the causative agent of canine leishmaniasis (CanL), human visceral leishmaniasis and sporadic cutaneous leishmaniasis in Portugal, Spain, Malta, France and Italy (Maroli *et al.*, 2013). We tested (i) the levels of individual canine antigenic responses to *P. perniciosus* 43 kDa yellow-related recombinant protein (rSP03B) compared to *P. perniciosus* SGH; and (ii) the degree of similarity in these antigenic associations, across endemic canine populations, in Portuguese and Italian foci where *P. pernicious* is the unique or principal phlebotomine vector, in order to assess its universal use as a marker of natural sand fly exposure. Additionally, we studied the antigenic similarity of rSP03B to its native form.

Canine sera originated from three regions: (i) Campania (n=118), a traditional high risk area for CanL in southern continental Italy (Oliva *et al.*, 2006); (ii) Umbria (n=96), an inland area of central Italy recently recorded as medium-high risk area of CanL (Di Muccio *et al.*, 2012); and (iii) the Metropolitan Lisbon region (n=341), which is a well-known CanL endemic locality in west-coast Portugal (Cortes *et al.*, 2012). In all three areas, *P. perniciosus* is the unique or principal vector of CanL. Single sera samples from Campania and Umbria were purposely selected from archived samples collected in 2007-2013 to represent the period from July (i.e. at least two months after the beginning of the sand fly season) through to October (i.e. the end of the sand fly season).Single sera samples from Metropolitan Lisbon region were randomly collected from dogs at the beginning of sand fly season in May 2012.

Samples from Campania consisted of stored sera sent by veterinary clinics to Istituto Superiore di Sanità for routine serological diagnosis of suspected CanL in owned dogs. Sera from Umbria were collected from healthy dogs that were enrolled on a voluntary basis in the Perugia University CanL surveillance program. Blood sampling were performed in accordance with the

Italian guidelines for animal welfare, following owners' consent, and did not include additional or unnecessary invasive procedures. Collection of sera in Metropolitan Lisbon region was ethically approved by the board of the Institute of Hygiene and Tropical Medicine (IHMTUNL) (authorization no.8 2011- PI) complying with the Portuguese legislation for the protection of animals (Law 113/2013).

Detection of anti-*Leishmania* IgG in canine sera from Campania and Umbria was performed by an in-house IFAT assay using *L. infantum* promastigotes as antigen, as described in Gradoni & Gramiccia (2008). Samples showing an IFAT titre equal or above 1:40 were considered as indicative of exposure to *Leishmania*. Detection of IgG antibodies against *Leishmania* in canine sera from the Metropolitan Lisbon region was performed by enzyme-linked immunosorbent assay (ELISA) kit (Bordier Affinity Products SA, Switzerland) according to manufacturer guidelines (Maia *et al.*, 2010). The result was considered positive when the absorbance of the analyzed sample was higher than the absorbance of the weak positive control serum provided with the kit.

Long term established laboratory colony of *P. perniciosus* originating from Spain (Murcia) was reared under standard conditions as described in Volf & Volfova (2011). Salivary glands and rSP03B from *P. perniciosus* (Genbank accn. DQ 150622) were obtained for this study as previously described (Kostalova *et al.*, 2015) and used as antigens for testing the canine sera.

Antibodies against *P. perniciosus* SGH and rSP03B protein were measured by ELISA as described by Kostalova, *et al.*(2015). Each serum was tested in duplicate. Test absorbance values were reported as optical densities with subtracted blanks (the ELISA plate background mean absorbance value measured in control wells).

Western blot analysis was used to confirm the similarity of antigenic epitopes between the native yellow-related protein found in *P. perniciosus* SGH and the corresponding recombinant protein rSP03B. SDS-PAGE of SGH (equivalent of 4 μg total salivary proteins per lane) and rSP03B (1 μg per lane) was run on a 12% gel and blotted onto the nitrocellulose membrane using the iBLOT instrument (Invitrogen). Membrane with separated proteins was cut into strips and blocked in 5% milk diluted in Tris-buffered saline with 0.05% Tween 20 (Tris-Tw) overnight at 4°C. For the inhibition test, three Italian canine sera possessing high levels of anti-*P. perniciosus* IgG against SGH and rSP03B, were pooled. The positive serum pool was diluted 1:50 in Tris-Tw and split into halves. The first half was incubated for 2 hours on a shaker with rSP03B (10

µg/ml), the latter half was incubated without rSP03B. Negative control sera (canine sera from non-endemic locality) were diluted 1:50 in Tris-Tw and incubated without rSP03B on a shaker for 2 hours. In the next step, a part of the positive serum pool, either incubated with or without rSP03B protein, and part of the negative control sera, were incubated with strips of separated *P. perniciosus* SGH. The same procedure was repeated for strips containing rSP03B, except that sera were diluted 1:100 in Tris-Tw. After 1 hour, all strips were rinsed in Tris-Tw and subsequently incubated for 1 hour with peroxidase-conjugated anti-dog IgG (1:3000, Bethyl laboratories). The colour reaction was developed by substrate solution containing 3, 3'-diaminobenzidine (Sigma).

Statistical analyses were carried out using R software (http://cran.r-project.org/) and Stata v. 13. 1 software (Stata Corporation, College Station, Texas, USA). Correlations were analysed using Spearman rank correlation test and medians compared between groups using a Wilcoxon rank sum test. In Figure 1 the OD values were logarithmised (natural logarithm) for better readability. Statistical analyses of the relationships between SGH and rSP03B OD values between the canine populations were statistically tested by fitting general linearised (GLM) Poisson models with a ln link function, having shown that the right skewed frequency distributions did not follow a negative binomial distribution (Deviance goodness-of-fit chi2>56.2; P=1, D.F=549, for each antibody). The full GLM Poisson models included interaction terms to test differences between the regions, both in terms of baseline anti-rSP03B value (intercept where anti-SGH equals 0) and the relationship between antibodies against SGH and rSP03B (slopes). Statistical significance was considered when the P-value was below 0.05.

We tested the use of *P. perniciosus* rSP03B as an epidemiological tool for investigations of canine exposure to sand fly bites in geographically distinct localities where *P. perniciosus* is the prevalent phlebotomine vector. The recombinant protein rSP03B used in this study, was obtained from the salivary glands of *P. perniciosus* laboratory-reared colony originated from Murcia in Spain and was used as antigen for serology of dogs living in Campania and Umbria regions in Italy and in Metropolitan Lisbon region in Portugal.

Levels of canine IgG antibodies reacting with SGH and rSP03B were measured by ELISA. We observed positive but variable correlations between antibody responses to SGH and rSP03B antigens in all three localities (Campania: r=0.73, 95% CI: 0.62-0.82, P<0.001; Umbria: r=0.56, 95% CI: 0.38-0.71, P<0.001; Metropolitan Lisbon region: r=0.81, 95% CI: 0.76-0.84,

P<0.001) (Figure 1). Table 1 summarises the OD values for each region indicating that the OD frequency distributions were over-dispersed. To query possible differences in the relationships between SGH and rSP03B antibody responses between geographical regions, we tested the equality of the population specific regression slopes by fitting a Poisson model. No significant differences were detected (population*antigen interaction terms: z>-0.85, P>0.365). Relative to the Metropolitan Lisbon region, both Campania and Umbria populations tended to produce higher baseline antibody responses against rSP03B, though this failed to reach significance at the 5% level (Campania: z=1.66, P=0.097; Umbria: z=1.95, P=0.051). One plausible explanation for the putative difference in baseline rSP03B antibody levels between populations is that the populations differ in their condition or past history of infections affecting their general immunological responses to certain antigens, and/or the sand fly biting pressure in these populations differed. Age is a frequent covariate of cumulative exposure used to model cross-sectional age-prevalence data of *Leishmania* infection (Courtenay *et al.*, 1994), where the average older dog is expected to experience more sand fly seasons. Canine age was not collected systematically in this study hence we could not adjust the analyses by this likely confounder. Despite this short-coming, the results nonetheless indicate substantial salivary antigen cross-reactivity amongst *P. perniciosus* populations from Campania, Umbria and the Metropolitan Lisbon region. The strong antigenic cross-reactivity between populations of the same sand fly species was similarly observed between two geographically distant colonies of *Phlebotomus orientalis* in Ethiopia (Vlkova *et al.*, 2014), and among colonies of *Phlebotomus sergenti* originating from Israel and Turkey (Rohousova *et al.*, 2012).

The similarity of antigenic epitopes between native yellow-related proteins in Spanish *P. perniciosus* SGH and rSP03B was demonstrated by an inhibition test (Figure 2). For this analysis, sera of dogs from Campania and Umbria with high levels of specific antibodies were selected and pooled. The inhibition test showed that all IgG antibodies specific for the native yellow-related protein bind to the recombinant form during pre-incubation of the sera, which resulted in complete disappearance of the corresponding band on western blot (Figure 2). Thus, we demonstrated that rSP03B shares antigenic epitopes with the native yellow-related proteins contained within *P. perniciosus* saliva and we assume that it identifies proportion of bitten dogs in a similar way as using SGH.

Italy and Portugal are generally assumed to be under CanL endemic transmission (Oliva *et al.*, 2006; Cortes *et al.*, 2012; Di Muccio *et al.*, 2012). In this study, CanL seropositivity ranged from 5% to 30%, with the lowest prevalence in Umbria and the highest in Campania (Table 2). The use of antibodies against sand fly salivary proteins as risk markers of *L. infantum* infection was already proposed not only for SGH (Vlkova *et al.*, 2011) but also for salivary recombinant proteins, among which rSP03B proved to be a powerful marker (Kostalova *et al.*, 2015). Therefore, we analysed the relationship between anti-*P. perniciosus* antibodies and *Leishmania* serological status. Using rSP03B antigen, significantly higher levels of specific IgG in *Leishmania* seropositive dogs (median [interquartile range]=0.346 [0.257-0.536]) than in *Leishmania* seronegative dogs (median [interquartile range]=0.32 [0.229-0.422]) was found only in Metropolitan Lisbon region (Wilcoxon rank sum test, W=5391.5, P=0.025). In Campania, the difference in antibodies against rSP03B between *Leishmania* seropositive (median [interquartile range]=0.457[0.357-0.55]) and *Leishmania* seronegative (median [interquartile range]=0.379[0.303-0.499]) dogs were marginally significant (Wilcoxon rank sum test ,W=1123.5, P=0.053). Previous studies of the relationship between anti-*P. perniciosus* antibodies and seropositivity to *L. infantum* show variable correlations. In Kostalova *et al.* (2015), a positive association was observed between levels of canine IgG antibodies against sand fly saliva and active CanL infection in dogs sampled longitudinally over two years. In contrast, the study by Vlkova *et al.* (2011) described a negative correlation between levels of specific IgG2 and the risk of *Leishmania* infection. Comparisons between studies are difficult following observations that anti-saliva antibodies wax and wane with sand fly exposure and seasonality (Kostalova *et al.*, 2015), whereas, at least in dogs actively infected, anti-*Leishmania* antibodies tend to persist after an initial increase whereas in exposed resistant animals they tend to fluctuate or convert to negative (Oliva *et al.*, 2006). In the current study, the canine samples were collected at variable periods relative to the sand fly season, hence we could not control for this potential confounder in our analyses. As studies tend to be cross-sectional, and use different approaches to determine the *Leishmania* infection status, makes cross-study comparisons difficult. Certainly, longitudinal studies demonstrate the potential usefulness of sand fly saliva antigenic responses in dogs as a marker for *Leishmania* infection (Kostalova *et al.*, 2015; Quinnell, 2016, personal communication). The possibility of using sand fly salivary recombinant proteins in a similar way in cross-sectional surveys still needs to be validated.

In conclusion, this study showed that *P. perniciosus* rSP03B, the 43 kDa yellow-related recombinant protein, possesses the same antigenic epitopes as its native form in salivary glands, and that it binds similarly in canine sera from Italy and Portugal foci. Therefore it could serve as a universal marker of sand fly exposurein dogs across the *P. perniciosus* geographical distribution.

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284

285 **Table 1.** Summary statistics of OD values recorded by ELISA using *Phlebotomus perniciosus*
286 salivary antigens

Antigen	Region	N	Median (IQR)	Min-Max
SGH	Campania*****	118	0.131 (0.073-0.241)	0.011-0.1.899
	Umbria******	96	0.218 (0.133-0.409)	0.005-1.652
	Lisbon*******	341	0.221(0.165-0.311)	0.081-1.39
rSP03B	Campania*****	118	0.407 (0.311-0.516)	0.091-1.761
	Umbria******	96	0.495 (0.386-0.649)	0.026-1.925
	Lisbon*******	341	0.323 (0.234-0.436)	0.092-1.766

287 N=number of dogs
288 IQR=interquartile range
289 *****Southern Italy
290 ******Central Italy
291 *******Metropolitan Lisbon region (Portugal)
292

293 **Table 2.** Proportion of *Leishmania* seropositive and seronegative dogs from different regions

		anti-*L. infantum* IgG positive / total animals sampled (%)		
Diagnostic method	Serological status*****	Campania	Umbria	Lisbon
IFAT	Positive	35/118 (30%)	5/96 (5%)	-
	Negative	83/118 (70%)	91/96 (95%)	-
ELISA	Positive	-	-	46/341 (13%)
	Negative	-	-	295/341 (87%)

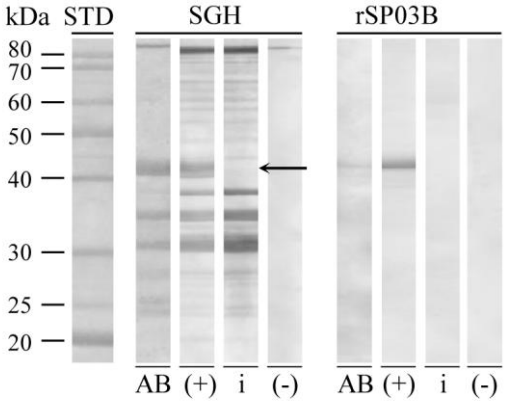
294 *****As determined by the IFAT titre or the ELISA cut-off

295

Figure legends

Fig. 1. Correlation between antibodies recognizing SGH and rSP03B in naturally bitten dogs from different localities of *P. perniciosus* occurrence. Correlation between SGH and rSP03B in dogs from Campania (A), Umbria (B) and Metropolitan Lisbon region (C) was performed by Spearman rank correlation. r=correlation index, CI=confidence interval.

Fig. 2. Western blot analysis of SGH and rSP03B and inhibition test. A mixture of canine sera positive to *P. perniciosus* SGH was pre-incubated with rSP03B and then tested in Western blot against SGH. The arrow indicates the emplacement of rSP03B. STD=standard, AB=strip stained by amidoblack, i=inhibition strip, (+)=positive control strip, (-)=negative control strip.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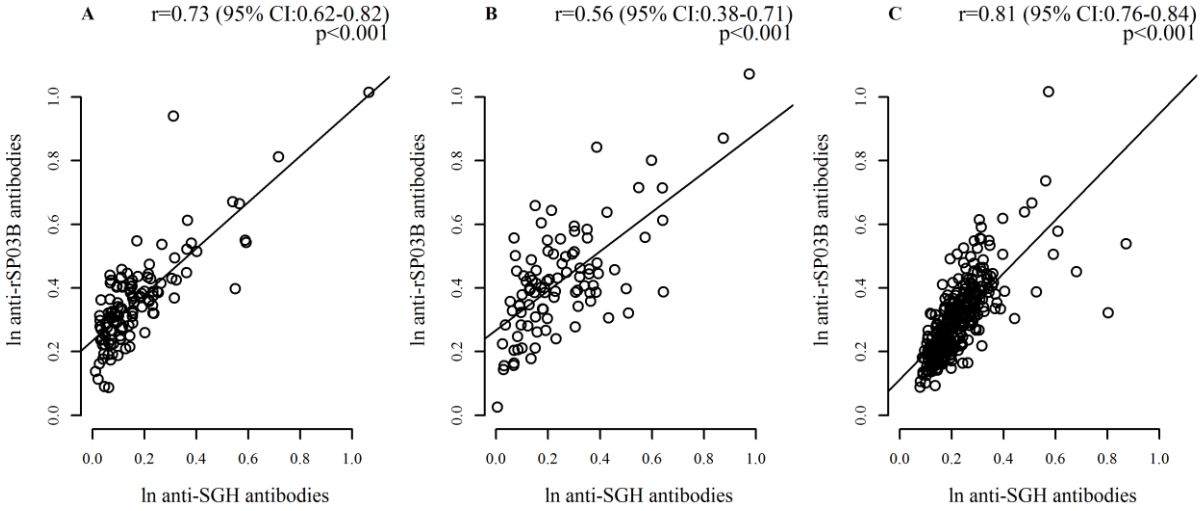
A
r=0.73 (95% CI:0.62-0.82)
p<0.001
B
r=0.56 (95% CI:0.38-0.71)
p<0.001
C
r=0.81 (95% CI:0.76-0.84)
p<0.001
ln anti-rSP03B antibodies
ln anti-SGH antibodies
0.0
0.2
0.4
0.6
0.8
1.0

SUMMARY AND DISCUSSION

This thesis summarises data included in four publications that describe results of three projects I participated on during my PhD study. The results, presented in a context of current knowledge, are briefly outlined in the following paragraphs.

While on one hand, a strict species-specificity of sand fly salivary proteins is appreciated mainly in serological studies conducted at locations with multispecies occurrence, on the other hand, this salivary proteins specificity constitutes an obvious restriction to their use in broad-spectrum anti-leishmania vaccines.

To date, saliva or salivary components from various sand flies were shown to confer protectivity against infection caused by *Leishmania* [reviewed in (Gomes and Oliveira, 2012; Abdeladhim et al., 2014)]. First salivary protein designated as a vaccine candidate was PpSP15[1] from *P. papatasi* providing protection against cutaneous leishmaniasis in mice (Valenzuela et al., 2001a; Oliveira et al., 2008). The homologue of this protein isolated from *P. duboscqi* (PdSP15) was able to protect rhesus macaques against *L. major* transmitted by infected sand fly bites (Oliveira et al., 2015). Large studies were performed also with salivary proteins from New World species *Lu. longipalpis* and *Lu. intermedia*. Rodents vaccinated with plasmids coding for LJM19, LJM11 or Linb-11 developed immunity that protected animals against *L. chagasi*, *L. major* or *L. braziliensis* infection, respectively (Gomes et al., 2008; Xu et al., 2011; de Moura et al., 2013). Furthermore, mice vaccinated with recombinant proteins of *Lu. longipalpis*, LJM11 or synthetic maxadilan, were protected against infection caused by *L. major* (Morris et al., 2001; Gomes et al., 2012).

A combination of a sand fly salivary protein with a *Leishmania* antigen is a new promising approach toward the anti-leishmania vaccine e.g. (Aguiar-Soares et al., 2014; Zahedifard et al., 2014). Zahedifard *et al.* tested the effectiveness of lizard protozoan parasite, *L. tarentolae*, combined with salivary antigen PpSP15 in eliciting protective immunity against *L. major* in BALB/c and C57BL/6 mice. Both strains showed stronger protective effect when compared with the single use of *L. tarentolae* or PpSP15 DNA, which demonstates enhanced efficacy of vaccine combining both factors (Zahedifard et al., 2014).

[1] GenBank accession numbers of sand fly salivary proteins/nucleotide sequences mentioned in Summary: PpSP15 = AF335487, PdSP15 = ABI15933, Linb-11 = KA660050, LJM19 = AY438271, LJM11 = AY445935/ AAS05318, rSP03B = DQ150622, rSP01 = DQ192490

It is necessary to highlight that all saliva-based vaccine candidates conferred protection against *Leishmania* sp. only when parasites were co-injected with the homologous vector saliva. As was shown by Thiakaki *et al*., immunization with *Lutzomyia longipalpis* saliva did not mediate cross-protection against *L. amazonensis* challenge together with saliva of *P. papatasi* or *P. sergenti* (Thiakaki et al., 2005). This is most probably due to antigenic species-specificity of vector saliva. The antigenic variability of sand fly salivary proteins was demonstrated not only between species but also between populations originating from distinct geographical areas e.g. (Warburg et al., 1994; Lanzaro et al., 1999; Volf et al., 2000; Rohousova et al., 2012b; Ramalho-Ortigão et al., 2015). This variability makes impossible the development of one universal saliva-based vaccine applicable to all *Leishmania*-vector combinations. Even one vaccine against *Leishmania* transmitted by the same vector species but at different areas of the vector distribution might be difficult to develop.

However, the intraspecific variability in saliva composition was refuted multiple times in several sand fly species (Elnaiem et al., 2005; Kato et al., 2006; Vlkova et al., 2014). The high degree of conservancy, not only among secreted salivary proteins but even among predicted T-cell epitopes, has been shown between Kenyan and Malian population of *P. duboscqi*, from the two places more than 2000 thousand kilometers apart (Kato et al., 2006). Similar conclusions were achieved for genetic polymorphism within *P. papatasi* SP15 isolated from Sudanese field population and from laboratory colonies collected in Egypt, Jordan, Israel and Saudi Arabia (Elnaiem et al., 2005). This suggests that host cross-protective cellular immune response may develop within those sand fly species thus making possible the concept of saliva-based vaccine for broader use.

It was suggested that interspecies differences in the SGH protein components could correspond with the phylogenetic position of individual species (Volf et al., 2000; Rohousova et al., 2012a; Vlkova et al., 2014). The vaccine based on more conserved salivary proteins could be cross-protective between closely-related vector species of the same subgenus and therefore applicable in more endemic foci. Several studies demonstrated similarity of salivary transcripts between *P. duboscqi* and *P. papatasi* (Kato et al., 2006; Abdeladhim et al., 2012), the two closely-related sand fly species belonging to the subgenus *Phlebotomus*, both serving as proven vectors of *L. major* (Killick-Kendrick, 1999) and occurring sympatrically in some areas [reviewed in (Maroli et al., 2013)].

In our study, we focused on these two sand fly species to test possible cross-antigenicity of their salivary proteins. Two groups of mice exposed to bites of *P. papatasi* and two control, non-immunized groups, were infected with *L. major* along with either *P.*

papatasi or *P. duboscqi* salivary gland homogenate (Lestinova et al., 2015). 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. Moreover, protection against *Leishmania* infection was also observed in mice exposed to *P. papatasi* sand flies but infected together with *P. duboscqi* SGH. The cross-protective effect was reflected 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 when compare with controls. Nevertheless, some differences in the immune response between the two exposed groups were noticed. Cytokine profile of peritoneal macrophages was affected only in the group challenged in the presence of homologous antigen. The absence of pro-inflammatory 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, triggered immunity was efficient enough to control the infection in mice infected together with heterologous antigen (Lestinova et al., 2015).

To sum up, we demonstrated for the first time the cross-protection caused by salivary antigens of two *Phlebotomus* species which suggests that 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* (Lestinova et al., 2015). Similar cross-protection was demonstrated also between two *Lutzomyia* species, *Lu. longipalpis* and *Lu. intermedia*, where immunogenic ability of *Lu. longipalpis* saliva or defined salivary protein LJM19 showed cross-protective potential against infection caused by *L. braziliensis* in the presence of *Lu. intermedia* SGS (Tavares et al., 2011).

The presence of anti-saliva antibodies in bitten hosts offers the potential to develop immunologically-based monitoring techniques for sand fly exposure in domestic animals and humans [reviewed in (Andrade and Teixeira, 2012; Gomes and Oliveira, 2012)]. Although similarity of sand fly salivary proteins among closely-related species is advantegous for saliva-based vaccine development, it is a disadvantage for their utilization in sand flies exposure testing; cross-reactivity of salivary proteins among sympatrically occuring species can lead to false positive results. For instance, salivary antigens do not cross-react between *Lutzomyia* and *Phlebotomus* species (Rohousova et al., 2005; Rohousova et al., 2015) but some weak cross-reactions were observed within the genus *Phlebotomus* (Volf and

Rohousova, 2001; Rohousova et al., 2005; Thiakaki et al., 2005) as well as within *Lutzomyia* species (de Moura et al., 2007; Gomes et al., 2007; Souza et al., 2010; Teixeira et al., 2010).

Anti-saliva antibody response can be employed to indicate an important blood source for sand flies or putative *Leishmania* hosts (Gomes et al., 2007; Martín-Martín et al., 2014). The main aim of our study was to screen domestic animals for anti-*P. orientalis* antibodies in northwest Ethiopia (Rohousova et al., 2015), an endemic area of *L. donovani* transmitted by *P. orientalis* (Gebresilassie et al., 2015). This *Leishmania* species is traditionally considered to have an anthroponotic transmission cycle; however, several authors suggest a zoonotic transmission cycle as well [reviewed in (Ready, 2014)]. Thus the results of antibody screening were also used to discuss possible role of domestic animals in the epidemiology of visceral leishmaniasis. In this collaborative project, I was responsible for verifying the specificity of *P. orientalis* SGH against anti-saliva antibodies elicited by sympatrically ocurring sand fly species (Gebre-Michael et al., 2004; Gebre-Michael et al., 2007; Lemma et al., 2014; Gebresilassie et al., 2015; Lemma et al., 2015). To test this, we evaluated SGH cross-reactivity with sera from mice repeatedly bitten by *P. papatasi*, *P. duboscqi* (subgenus *Phlebotomus*), *P. arabicus* (subgenus *Adlerius*) or *Sergentomyia schwetzi* (subgenus *Sergentomyia*). Antigens from *P. orientalis* (subgenus *Larroussius*) SGH was shown to react strongly only with homologous, anti-*P. orientalis* antibodies, while reactivity with heterologous anti-sand fly saliva antibodies were comparable to those from non-exposed mice (Rohousova et al., 2015).

In summary, we observed widespread exposure of domestic animals to *P. orientalis* bites indicating their involvement in the dynamics of visceral leishmaniasis caused by *L. donovani*, either as potential blood sources for sand flies or even as possible parasite hosts (Rohousova et al., 2015). However to prove their role in the transmission cycle of *L. donovani*, further evidence, such as host and transmission competence by xenodiagnostic tests, will be required (Rohousova et al., 2015).

The production of specific and highly antigenic salivary proteins in recombinant forms was shown to be of utmost importance for epidemiological studies estimating the level of sand fly bites [reviewed in (Andrade and Teixeira, 2012)]. The possible replacement of SGH by recombinant proteins in studies testing sera from endemic areas was already introduced for *Lu. longipalpis* (Souza et al., 2010; Teixeira et al., 2010; Soares et al., 2013), *P. papatasi* (Marzouki et al., 2012; Marzouki et al., 2015) or *P. perniciosus* (Martín-Martín et al., 2014). Although all of them confirm the advantages of salivary recombinant proteins utilization,

most of these experiments were performed on small sets of samples (Marzouki et al., 2012; Soares et al., 2013; Martín-Martín et al., 2014), their correlation with complete SGH was not convincing (Souza et al., 2010; Soares et al., 2013; Marzouki et al., 2015) and the results were never associated with the infection status of the hosts.

In our study, we have used for the first time sand fly salivary recombinant proteins for monitoring frequency of contact between dogs (reservoir hosts of *L. infantum* in Mediterranean) and *P. perniciosus* (proven vector of this *Leishmania* species), in a longitudinal field study (Kostalova et al., 2015). The antigenic capability of bacterially-expressed salivary proteins derived from *P. perniciosus*, rSP03B (yellow-related protein) and its combination with rSP01 (apyrase), was tested against 529 canine sera collected over two consecutive sand fly seasons in Italy, and was related to results obtained with SGH antigen. While relatively high correlation coeficient was observed between rSP03B and SGH (r=0.77), which is consistent with previously published data (Drahota et al., 2014; Martín-Martín et al., 2014), the combination of both recombinant proteins was less efficient (r=0.65). Further, the curve depicting sand fly occurence over biennial monitoring season developed with similar pattern comparing anti-SGH and anti-rSP03B IgG antibodies. Antibodies against both tested antigens raised during summer months and declined during winter months, which reflects well *P. perniciosus* seasonality in Italy (Rossi et al., 2008).

In summary, using recombinant form of *P. perniciosus* yellow-related protein as a marker of canine exposure in extensive epidemiological study, we confirmed rSP03B to be a valid alternative to salivary gland homogenate of *P. perniciosus* (Kostalova et al., 2015).

Intraspecific variability of sand fly salivary proteins has been demonstrated in several species e.g. (Warburg et al., 1994; Lanzaro et al., 1999; Volf et al., 2000; Rohousova et al., 2012b; Ramalho-Ortigão et al., 2015). The important question is if intraspecific salivary variances translate also to antigenic variability, which would limit broader utilization of host anti-saliva antibodies in seroepidemiological studies. SGH obtained from laboratory-reared sand fly colony as well as the recombinant protein based on a single colony might not be universally applicable as antigen in the whole area of sand fly distribution. However, careful selection of recombinant salivary proteins that can substitute the whole salivary gland protein cocktail in epidemiological studies may increase specificity of the reaction [reviewed in (Andrade and Teixeira, 2012)]. During the selection of salivary candidate for recombinant expression, researchers should give priority to those sharing antigenic epitopes intraspecifically but not interspecifically.

In our study, we tested the possible regionally-universal use of *P. perniciosus* 43 kDa yellow-related recombinant protein (rSP03B) obtained from laboratory-reared colony originated from Spain as a marker of sand fly exposure. We tested a total of 550 canine serum samples originating from distant localities of *P. pernicious* occurrence; from Campania and Umbria in Italy and from Metropolitan Lisbon region in Portugal (Kostalova et al., 2016). Anti-*P. perniciosus* IgG antibodies were screened by ELISA using SGH and rSP03B as antigens. Results showed robust correlation between antibodies recognizing SGH and rSP03B in all three localities tested which suggests substantial cross-reactivity between *P. perniciosus* populations (Kostalova et al., 2016). This is in accordance with previously described antigenic cross-reactivity between geographically distant colonies of *P. orientalis* (Vlkova et al., 2014) as well as between colonies of *P. sergenti* (Rohousova et al., 2012b). Further, we tested similarity of antigenic epitopes between native and recombinant forms of yellow-related protein. The binding of canine IgGs to the native protein was inhibited by pre-incubation with the recombinant form, which proves that bacterially-expressed rSP03B shares antigenic epitopes with the native yellow proteins of *P. perniciosus*.

In summary, we showed that rSP03B, the 43 kDa yellow-related recombinant protein reacts well with canine sera from Italy and Portugal and therefore it could serve as a universal marker of sand fly exposure for dogs from distant localities of *P. perniciosus* distribution.

In conclusion, the results included in this thesis bring new information about host´s immune response specificity to sand fly saliva and about possible replacement of salivary gland homogenate by recombinant proteins in ELISA tests to estimate sand fly exposure. In futher work, we would like to focus on predictions and identifications of putative B-cell salivary epitopes with subsequent designing of synthetic peptides serving as markers of sand fly exposure. Synthetic peptides were already used to assess human exposure to tsetse flies or moscquitoes, e.g. (Poinsignon et al., 2008; Poinsignon et al., 2009; Drame et al., 2010; Poinsignon et al., 2010; Dama et al., 2013; Londono-Renteria et al.,2015), but they were not yet applied on sand flies. Studies of antibodies reactivity to a single epitope could provide a better specificity and sensitivity when compared to whole saliva homogenate. Moreover, synthetic peptides have several advantages when compared with salivary recombinant proteins. While the production and storage of recombinant proteins are often problematic and require good facilities which limit their use in many contexts, peptides are more easily produced and can be stored lyophilized.

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