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Factors affecting the protective effect provided by sand fly saliva immunization on the outcome of *Leishmania* infection

Faktory ovlivňující protektivní efekt daný imunizací slinami flebotomů ve vztahu k vývoji leishmaniové infekce

DIPLOMA THESIS

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Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Declaration:

Hereby I declare that I made this thesis independently, using the mentioned references. I have not submitted or presented any part of this thesis for any other degree or diploma.

In Prague: 9.8.2022 Bc. Kristýna Jelínková

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ABSTRAKT

Leishmanióza je parazitické onemocnění působené prvoky rodu *Leishmania*, jejichž vektorem jsou samice rodu *Phlebotomus* a k přenosu leishmánií dochází během sání krve. Je-li hostitel před kontaktem s leishmániemi vystaven slinám flebotomů, dochází k sensitizaci; v některých případech dochází k tzv. protektivnímu efektu, jenž ovlivní výslednou podobu onemocnění. Uvádí se, že tento efekt pramení z opožděné hypersenzitivní reakce na sliny flebotomů, která vrcholí přibližně 48 hodin po interakci se slinným antigenem a jejímž důsledkem je povzbuzení obranných mechanismů hostitele proti leishmániím. Cílem této diplomové práce bylo ověřit druhově specifické vlastnosti protektivního efektu, závislé mimo jiné právě na načasování infekce po poslední expozici slinám flebotomů. Předložené výsledky prokázaly, že zatímco u imunizovaných myší BALB/c, které byly infikovaných prvokem *Leishmania major* týden po vystavení slinám *Phlebotomus duboscqi*, se protektivní efekt vyvinul, tak při infekci provedené 48 hodin po expozici k tomu nedošlo. Protektivní efekt daný vystavení slinám flebotomů před infekcí v tomto případě dokonce úplně vymizel, což bylo prokázáno statisticky nesignifikantními rozdíly měřených parametrů v porovnání s kontrolní neimunizovanou skupinou. Vystavení sání flebotomů druhu *Sergentomyia schwetzi* navíc neovlivnilo výslednou podobu onemocnění při infekci s *Leishmania major* spolu s homogenátem slinných žláz druhu *Phlebotomus duboscqi*, tedy druhu fylogeneticky vzdálenému rodu *Sergentomyia*. Zkrácené načasování infekce tak nemělo vliv na specifitu protekce proti leishmániím vyvolanou imunitní reakcí na sliny flebotomů. V rámci experimentů byly měřeny markery poukazující na závažnost infekce, jakož i její imunologické aspekty. Závěry práce přinášejí nové poznatky pro výzkum dosud přehlížených faktorů ovlivňujících výslednou podobu onemocnění leishmaniózou, které se mohou uplatňovat i v endemických oblastech.

Klíčová slova: *Leishmania*, sliny flebotomů, *Phlebotomus duboscqi*, *Sergentomyia schwetzi*, protektivní efekt, hypersenzitivní reakce opožděného typu, pre-expozice

ABSTRACT ENGLISH VERSION

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*, transmitted by its vector – female sand flies during the process of blood feeding. Preexposure to sand fly saliva prior to *Leishmania* infection affects the host immune system, leading to possible establishment of protective effect on the disease outcome. This effect is based on the delayed type hypersensitivity reaction to sand fly saliva which has been shown to peak about 48 hours after antigen encounter, leading to the more effective host immune response towards the *Leishmania* parasites. In this project, we found this protective effect as species-specific, additionally dependent also on the timing of the infection post last immunization. Results of presented experiments showed that infection of BALB/c mice with *Leishmania major* performed after 48 hours post last *Phlebotomus duboscqi* sand fly saliva immunization, did not confer protection in comparison to infection performed at one-week after the last exposure. Moreover, the effect of the sand fly saliva pre-exposure was at this shorter timing of infection rather diminished, supported by insignificant differences in the measured parameters compared to the unpre-exposed infected control group. Additionally, immunization with sand fly *Sergentomyia schwetzi*, did not affect the disease outcome in case of infection with *Leishmania major* along with salivary gland homogenate of *Phlebotomus duboscqi*, a species phylogenetically distant to *Sergentomyia*. The shorter timing of infection did not affect the specificity of the protection against leishmaniasis provided by anti-sand fly immunity. Several parameters were measured and analysed, including markers describing the disease outcome as well as the immunological aspects. In summary, the results of this project provide an insight into the situations that might occurre in the endemic areas of leishmaniasis, adding a new, yet neglected, factor affecting the disease outcome.

Key words: *Leishmania*, sand fly saliva, *Phlebotomus duboscqi*, *Sergentomyia schwetzi*, protective effect, delayed type hypersensitivity, pre-exposure

CONTENT

ABBREVIATIONS

AMP	Adenosinemonophosphate
APC	Antigen-presenting cells
CL	Cutaneous leishmaniasis
CR	Complement receptor
DC	Dendritic cells
DCL	Diffuse cutaneous leishmaniasis
DMSO	Dimethyl sulfoxide
DTH	Delayed type hypersensitivity reaction
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EXP	preexposed and infected groupe
FBS	Fetal bovine serum
FMO	Fluorescent minus one
FSC	Forward scatter
gp 63	Glycoprotein 63
GPI	Glycosylphosphatidylinositol
iNOS	Inducible NO synthase
IFN	Interferon
IL	Interleukin
L.	*Leishmania*
LH	Langerhans cells
LPG	Lipophosphoglycans
Lu.	*Lutzomyia*
MAC	Membrane attack complex
MW	Molecular weight
MCL	Mucocutaneous leishmaniosis
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NEG	Negative control group
NK	Natural killer
NO	Nitrid oxide
NOS	Nitric oxide synthase
OD	Optical density
P.	*Phlebotomus*
PBMC	Peripheral body mononuclear cells
PBS	Phosphate buffered saline
p.i.	Post infection
p.l.i.	Post last immunization

POS	Positive control groupe
qPCR	quantitative polymerase chain reaction
ROS	Reactive oxygen species
RT	Room temperature
S.	*Sergentomyia*
SD	Standard deviation
SS	Single stained
SSC	Side scatter
SGH	Salivary gland homogenate
TCM	Central memory T cells
TEM	Effector memory T cells
TGF	Transforming growth protein
TNFα	Tumor necrosis factor alpha
TLR	Toll-like receptor
VL	Visceral leishmaniasis
WHO	World Health Organization
YRPs	Yellow related proteins

1 INTRODUCTION

Leishmaniasis is a neglected tropical disease, caused by protozoan parasite of genus *Leishmania*, causing thousands of deaths annually. The parasites are transmitted to the host by its vector female phlebotomine sand flies, during the process of blood feeding. Depending on the *Leishmania* species transmitted to the host, symptoms of the infection may vary, from cutaneous lesions (cutaneous leishmaniasis) to life-threatening visceral form (visceral leishmaniasis). Additionally, the outcome of the disease is affected by many other factors, such as pharmacologically active molecules present in the sand fly saliva inoculated to the bite site along with the parasite or immune response of the host. It has been experimentally shown that pre-exposure to sand fly saliva of uninfected sand flies prior to infection might elicit so called protective effect against the disease. According to previous studies, this effect is based on the development of delayed type hypersensitivity (DTH) in the host as a reaction to the repeated exposure to sand fly saliva, which skew the host immune response towards anti-*Leishmania* immunity. Delayed type hypersensitivity culminates 48 hours after the immunization, thus the *Leishmania* infection during that time might be affected by such immune response.

This diploma project is focused on that effect, firstly investigating the influence of the timing of the infection post last immunization on the disease outcome, and secondly also its antigenic specifity when the immunization and the infection differ in sand fly species used. By that, possible naturally occurring interactions of the vector and host are represented, reflecting the chances to encounter sand fly bites from more than one species as well as different timing of infection. To our knowledge, none of the published studies deal with such factor as the timing of the infection post last immunization, nor with this timing in studying the species-specificity of the protective effect within combination of *Sergentomyia schwetzi* pre-exposure and phylogenetically distant sand fly saliva of *Phlebotomus duboscqi* presence during the infection.

Aims of the project are:

1) The effect of timing between the last exposure to sand fly saliva and *Leishmania* infection, focusing on the shorter timespan in comparison to widely used model, when the host is infected not earlier than 1 week after the last exposure to sand fly saliva.

2) If this timing (shorter time span between the last exposure to sand flies and *Leishmania* infection) affect the species specificity of the protective effect.

The experiments were performed on the animal model of *Leishmania* infection using BALB/c mice and *Leishmania major*, and further employing two sand fly species – *Phlebotomus duboscqi* as the proven vector of *L. major* and *Sergentomyia schwetzi* as the nonvector species. The experiments were evaluated based on several parameters. The level of anti-sand fly saliva immunity was described using anti-sand fly saliva IgG antibodies. The outcome of *Leishmania* infection was evaluated based on the lesion size, parasite load in ear pinna and corresponding draining lymph node, and the levels of anti-*Leishmania* IgG antibodies. The cellular immune response behind the infection was characterized in the lymph node tissue draining the lesion site (local immunity) and in the spleen (systemic immune response), focusing on selected T cell populations.

2 LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is a parasitic disease caused by the protozoan parasite of the genus *Leishmania* (Kinetoplastea: Trypanosomatida), distributed mainly in tropical and subtropical regions. According to the World Health Organisation (WHO), 0.7 to 1 million new cases occur annually, causing approximately 65,000 deaths each year. Although leishmaniasis affects especially people from poor regions, without proper access to the health care system, more than 350 million people live at risk in endemic areas (WHO 2022, Schol *et al.* 2020).

The transmission of parasites occurs during the blood feeding of infected females of phlebotomine sand flies (Diptera: Nematocera: Phlebotominae) on the host. For mammalian hosts, the genus *Lutzomyia* in the New World and the genus *Phlebotomus* in the Old World are of primary medical importance as vectors of *Leishmania* parasites (Reithinger *et al.* 2007, Lestinova *et al.* 2017). The transmission cycle between the host and its vector includes several stages of the parasite, varying in its morphology and metabolism. The host phase is initiated by flagellated metacyclic promastigotes injected into the skin environment during blood feeding, followed by their engulfment primarily by immune phagocytic cells such as macrophages and neutrophils. Within macrophages, promastigote differentiates into immobile short-flagellated amastigotes, which replicate and infect other cells, causing the dissemination of the disease. The amastigote stage is also infective for the vector of *Leishmania* – sand flies, which become infected from the blood during the process of blood feeding. Ingested amastigotes transform themselves into promastigotes forms inside the gut of the vector, multiply, and undergo further development including several morphological changes. Finally, promastigotes migrate to the upper parts of sand fly midgut, from where they are ingested to the host together with sand fly saliva (Fig. 1) (Killick-Kendrick, 1990, Bates and Rogers, 2004, Reithinger *et al.* 2007, Kaye and Scott, 2011, Torres-Guerrero *et al.* 2017).

Depending on the transmitted species of *Leishmania*, the clinical manifestation of leishmaniasis can include a different range of symptoms, from localized self-healing skin lesions, known as cutaneous leishmaniasis (CL), to life-threatening visceral form of the disease affecting internal organs such as livers and spleen, known as visceral leishmaniasis (VL) (McGwire and Satoskar, 2014, Torres-Guerrero *et al.* 2017). Other forms known as mucocutaneous (MCL) or diffuse cutaneous (DCL) leishmaniasis are less prevalent, caused only by few species of *Leishmania,* including *Leishmania braziliensis* and *Leishmania mexicana* (Hepburn, 2000, Santos *et al.* 2014, McGwire and Satoskar, 2014). The outcome of the disease is dependent not only on the *Leishmania* species, but also on the host's immune system and genetic background, including also factors such as exposure to sand flies and thus their salivary antigens and immunomodulators (Remadi *et al.* 2016, Lestinova *et al.* 2017, Dos Santos Meira and Gedamu, 2019). To date, there are no vaccines available for humans against any type of leishmaniasis (Palatnik de Sousa and Nico, 2020, WHO 2022).

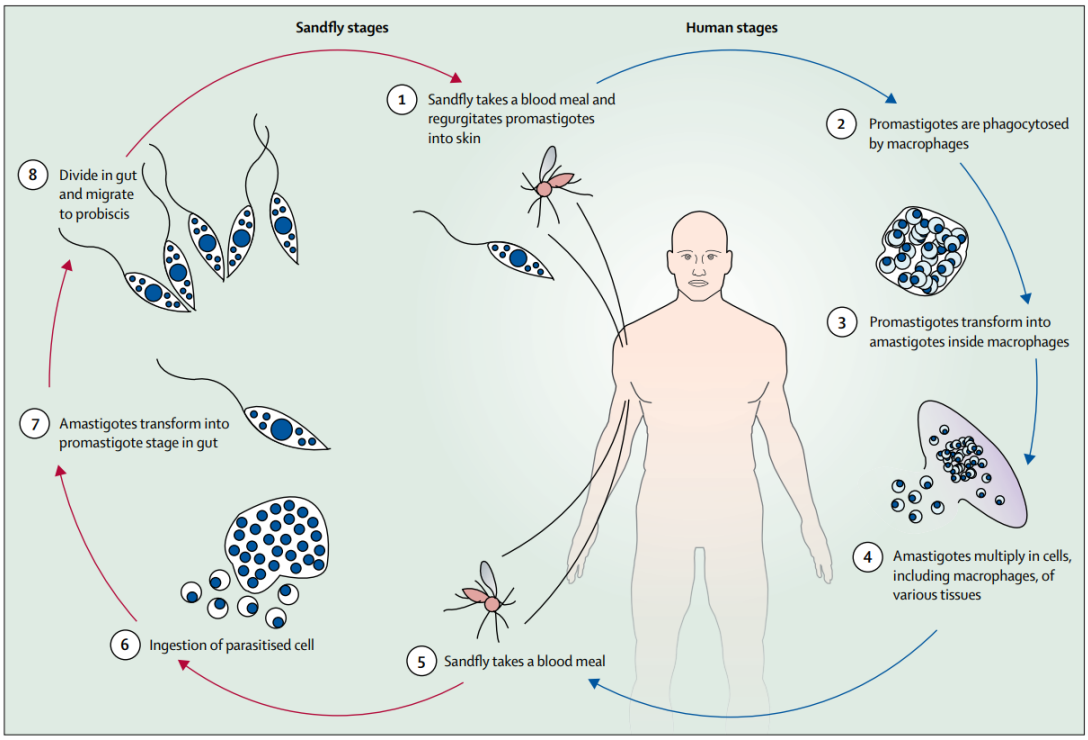


Fig. 1: General *Leishmania* life cycle including its mammalian host and vector phase (Burza *et al.* 2018).

2.1.1 *Leishmania major* and its vector sand flies

The most common form of the disease is the cutaneous leishmaniasis, caused by several *Leishmania* species, including *Leishmania major*, the model organism of this diploma project (Kaye and Scott, 2011, Masmoudi *et al.* 2013). *Leishmania. major* is distributed in African and Eastern Mediterranean regions, where it is preserved in a zoonotic transmission cycle within the animal reservoirs such rodents (Beach *et al.* 1984, Rassi *et al.* 2008, Tomás-Pérez *et al.* 2014, Soleimani *et al.* 2022). Transmission to people occurs in endemic areas by bites of previously infected vectors. The incubation period of the parasite is usually about 1 to 4 weeks, followed by the formation of typical 'wet' lesions, which resolve from 2 months to a few years from the first symptoms (Jeronimo, 1987, Masmoudi *et al.* 2013)

Sand flies are major vectors of *Leishmania* parasites. The relationship between sand flies and *Leishmania* is species specific, including different distribution in the Old vs. New World (Killick-Kendric, 1990, Pigott *et al.* 2014). This project is focused on the combination P*hlebotomus duboscqi* and *Sergentomyia schwetzi* as vectors and the parasite *L. major*, thus following paragraphs are primarily focused on some general characteristics of these species.

Phlebotomus duboscqi is an Old World sand fly species, distributed mainly in Central Asia and Subsaharian Africa, belonging to the proven vectors of *L. major* (Beach *et al.* 1984, Akhoundi *et al.* 2016). *Phlebotomus duboscqi* feeds both on animals and humans, creating ideal conditions for *Leishmania* transmission from its animal reservoirs to the human hosts (Beach *et al.* 1984, Mukhopadhyay and Ghosh, 1999).

Sergentomyia schwetzi, is phylogenetically distinct species from *Phlebotomus* and *Lutzomyia*, and its preferential hosts are reptiles (Lewis, 1971, Mutinga and Omogo, 1986). *Sergentomyia schwetzi* is sympatrically distributed with *P. duboscqi*, however, its role in the *Leishmania* transmission to the human

hosts have not yet been conclusively confirmed. Nevertheless, its ability to feed on both mammals and man has been already demonstrated, together with its high prevalence in endemic areas of CL and VL (Anjili *et al.* 2011, Senghor *et al.* 2016, Maia and Depaquit, 2016).

Therefore, exposure to bites of both *P. duboscqi* and *S. schwetzi* under natural conditions is presumable. Considering the fact, that sand fly saliva plays a major role during *Leishmania* transmission to the host (described in chapter 2.1.2), part of the presented project was also focused on the relationship between the role of saliva of *S. schwetzi – P. duboscqi* during *L. major* transmission.

2.1.2 Effect of sand fly saliva on the host

As already briefly mentioned in Chapter 2.1, the establishment of *Leishmania* parasites in the host is a complex process, influenced by its vector, phlebotomine sand flies. The transmission of infectious metacyclic promastigotes to the host skin occurs during the blood-feeding process of female sand flies. Insertion of proboscis parts into the skin environment results in laceration of cells in the skin and creation of an extravascular pool of blood from which sand flies ingest blood (Bates *et al.* 2007, Abdelahim *et al.* 2014, Cecílio *et al.* 2022). This disruption of the skin environment immediately triggers the host response to prevent blood loss and pathogen entry. However, sand flies developed an evolutionary advantage in counteracting this mammalian defence to take the blood meal (Serafim *et al.* 2021). Their saliva is composed of several pharmacologically active molecules, which can alter host protective mechanisms and therefore contribute to successful blood feeding (Rohousova and Volf, 2006, Gomes and Oliveira, 2012, Abdelahim *et al.* 2014). These molecules affect the process of haemostasis and immune response, providing enough time and a safe environment for vector feeding (Gomes and Oliveira, 2012, Lestinova *et al.* 2017).

2.1.3 Role of sand fly saliva during blood feeding

The host response to *Leishmania* parasites is influenced not only by the genetic and immunological background of the host (Kurey *et al.* 2009, Oliviera *et al.* 2013), but also by the exposure to sand fly saliva. Repeated exposure to phlebotomine saliva elicits host immune reaction based on the interaction with sand fly salivary molecules that skews the skin microenvironment towards anti-*Leishmania* effector mechanisms (Belkaid *et al.* 1998, Kamhawi, 2000, Rohousova *et al.* 2011). However, this protection is highly dependent on the frequency of the exposure and the timing of the infection since the last immunization with sand fly saliva (Rohousova *et al.* 2011). On the other hand, the exacerbation of the disease can manifest itself in the saliva-naive host, where saliva serves as a factor promoting the infection in the so called ´enhancing effect´ (Theodos *et al.* 1991, Norsworthy *et al.* 2004, Lestinova *et al.* 2017).

Sand fly saliva possesses a variety of pharmacologically active molecules, which affect the host's haemostasis and the immune system during the blood feeding process. Haemostasis is a set of reactions that leads to prevention of blood loss from the vascular system in the event of disturbance. The process consists of blood coagulation, vasoconstriction, and platelet aggregation (Kamhawi, 2000, Ribeiro and Francischetti, 2002). The principle of inhibiting the host haemostatic system remains the same among different species of sand fly, although the components of the sand fly saliva may vary (Ribeiro *et al.* 2010, Abdelahim *et al.* 2014). Blood coagulation is made up of a series of enzymatic reactions, leading to

insoluble fibrin production, creating a clot through interaction with blood vessels and platelets (Blanco and Blanco, 2017, Malcolm, 2013). Platelet aggregation is primarily triggered by thrombin, which is the last enzyme in the blood coagulation cascade (Hou *et al.* 2015, Al-Almer, 2022). Inhibition of blood coagulation is characterized by the interaction of salivary proteins such as apyrases or *Lutzomyia* factor Xa inhibitor (Lufaxin) with molecules included in the host reaction of blood coagulation and platelet aggregation (Charlab *et al.* 1999, Valenzuela *et al.* 2001, Collin *et al.* 2012, Abdeladhim *et al.* 2014, Serafim *et al.* 2021). Vasoconstriction is triggered in the initial phase of haemostasis around the wound area, which decreases blood flow and contributes to the prevention of blood loss (Periayah *et al.* 2017). To ensure sufficient blood flow to the site of bite, the saliva of sand fly also contains strong vasodilators, such as maxadilan in *Lutzomyia* (Lerner *et al.* 1991) or adenosin and 5´AMP in *Phlebotomus* (Ribeiro *et al.* 1999).

Other molecules such as biogenic amines, including, most importantly, histamine and serotonin, also contribute to the haemostatic process. The role of histamine includes enhancing platelet aggregation, together with the increasing vascular permeability and vasodilatation (Masini *et al.* 1998, O´Mahony *et al.* 2011) Serotonin is on the other hand implicated mainly in the process of vasocontriction (Schoenichen *et al.* 2019). Yellow related proteins from saliva of sand flies have been shown to bind to these biogenic amines and therefore inhibit their function (Sumova *et al.* 2019, Spitzova *et al.* 2020).

Sand fly saliva do not counteract only with the haemostatic system of the host, but also the immune system. The effect has been shown to be species-specific, affecting both humoral and cellular immune response in repeatedly bitten hosts (Gomes and Oliveira, 2012, Lestinova *et al.* 2017). Only very few studies are available that are focused on the immunomodulatory effect of saliva of species presented in this project – *P. duboscqi* and *S. schwetzi*. Both sand fly species have been shown to elicit production of anti-saliva IgG antibodies, in the laboratory setting as well as in endemic area (Rohousova *et al.* 2011, Polanska *et al.* 2014). Effect on the cellular immune response was so far shown only for *P. duboscqi* saliva, having the chemotactic effect on mouse monocytes in vitro (Anjilli *et al.* 1995).

The immune response to sand fly saliva is of great importance in terms of the transmission and establishment of the *Leishmania* parasite in the host, which is closely related to the status of host pre-exposure. Immunomodulatory effect of sand fly saliva in the context of *L. major* infection is discussed in following chapters.

2.1.4 Protective effect of sand fly saliva

Repeated exposure to bites of uninfected sand flies leads to a protective effect on subsequent infection with *Leishmania* parasites (Belkaid *et al.* 1998, Kamhawi *et al.* 2000). This effect is manifested for instance by smaller and less serious lesion appearance, decrease in number of parasites, and establishment of self-healing phenotype of the disease (Belkaid *et al.* 1998, Kamhawi *et al.* 2000, Rohousova *et al.* 2011, Teixeira *et al.* 2014, Lestinová *et al.* 2017). In laboratory settings, a protective effect has been observed in the scenario in which the host model is immunized and infected with sand fly saliva from the same species. The combinations of sand fly species and *Leishmania* strains used in these experiments are summarized in the Tab. 1, including *Phlebotomus* as well as *Lutzomyia* species highlighting the fact that the protective effect is not exclusively specific only to Old World or New World sand fly species. Experiments were carried out with well-established laboratory mice host models that included mice strains susceptible to infection, BALB/c (Kamhawi *et al.* 2000, Carregaro *et al.* 2013) and *Leishmania* resistant strain C57BL/6

(Kamhawi *et al.* 2000). Immunization was performed using salivary gland homogenates (SGH) from dissected sand flies (Belkaid *et al.* 1998, Carregaro *et al.* 2013, Gomes *et al.* 2016), plasmid encoding salivary proteins (Gomes *et al.* 2008) or by repeated exposure to sand fly bites themselves (Kamhawi *et al.* 2000, Rohousova *et al.* 2011), as listed in Tab. 1.

Tab. 1: Summary of studies investigating the protective effect of sand fly saliva. + denotes enhancement of the presented parameter, - denotes for decrease of the presented parameter

Sand fly in immunization	**Mode of immunization**	***Leishmania* infection**	**Host model organism**	**Outcomes**	**References**
P. papatasi	Exposure to bites of uninfected sand flies	*L. major*	BALB/c + C57BL/6 mice	+ protection + DTH reaction + cellular infiltrate to the bite site - lesion size - parasite load	Kamhawi *et al.* 2000
P. duboscqi	Exposure to bites of uninfected sand flies	*L. major*	BALB/c mice	+ protection -parasite load anti-*L. major* IgG	Rohousova *et al.* 2011
Lu. longipalpis	Immunization with a DNA plasmid coding for salivary proteins of *Lu. longipalpis*	*L. infantum*	Syrian golden hamsters	+ protection + DTH reaction + proinflammatory response (↑ IFNγ, ↑ NO)	Gomes *et al.* 2008
Lu. longipalpis	Immunization with SGH of *Lu. longipalpis*	*L. major*	C57BL/6 mice	+ protection -lesion size	Xu *et al.* 2011
Lu. longipalpis	Immunization with SGH of *Lu. longipalpis*	*L. braziliensis*	BALB/c mice	+ protection -lesion size + proinflammatory response (↑IFNγ, ↓IL-10) + ↑CD4+, ↑CD8+ T cells	Carregaro *et al.* 2013
Lu. whitmani	Immunization with SGH of *Lu. whitmani*	*L. braziliensis*	BALB/c mice	+ protection + DTH reaction + protective immune response (↑IFNγ, ↑IL-4, ↑IL-10	Gomes *et al.* 2016

Sand flies may occur sympatrically and a probability of exposure to saliva from more than one species is assumed (Fonseca *et al.* 2021, Isräel *et al.* 2022). Considering this possibility, several experiments have been performed to investigate the cross-reaction of the host immune system with saliva from sand fly of different species. So far, the ability to induce protective effect even between different species has been

observed only for a few closely related species, namely *P. papatasi* for immunization and *P. duboscqi* for infection with *L. major* in a mouse model (Lestinova *et al.* 2015) and *Lu. longipalpis* for immunization and *Lu. intermedia* for infection with *L. braziliensis et al.* in a hamster model (Tavares *et al.* 2011). The combinations of more phylogenetically distant species such as *Lu. longipalpis* for immunization and *P. papatasi* or *P. sergenti* for infection with *L. amazonensis* did not mediate the protective effect, and the disease outcome did not differ from nonimmunized controls (Thiakaki *et al.* 2005). The authors hypothesize that the the protective effect was not established probably due to the different molecular characteristics of saliva from the Old and New World species, resulting in an immune response dissimilar to the environment required for protection (Thiakaki *et al.* 2005, Lestinova *et al.* 2017).

2.1.5 Immunological background of the protective effect

Host protection elicited by repeated exposure to sand fly saliva is believed to be mediated by a delayed type hypersensitivity reaction (DTH) (Belkaid *et al.* 1998, Kamhawi, 2000, Kamhawi *et al.* 2000). Characterization of its features is based on the general characteristic of DTH, also known as type IV hypersensitivity, which occurs later than 24 hours after antigen exposure in a sensitized host. This type of reaction is mediated by T cells and high immune cellular influx to the site of the exposure, usually manifested as a granulomatous dermatitis-like reaction (Marwa and Kondamuni, 2022). According to the literature, the DTH response is divided into 5 subtypes, based on the immune cells involved. Type IVa includes Th1 cells, which further activate macrophages to produce cytokines such as IFNγ and TNFα, type IVb involve Th2 cell subsets with production of IL-4, IL-5, IL-13, and induce eosinophilic inflammation connected with elevated levels of IgE, type IVc is mediated by CD8+ T cells and their cytotoxic activity that induces the death of keratinocytes. Last but not least, type IVd is characterised by T cell response connected with the tissue damage by neutrophilic inflammation (Pichler, 2003, Dispenza, 2019).

Studies focused on DTH reaction to sand fly bite do not take this more in-depth differentiation into consideration and the monitored immune cells populations and humoral features are not united through the available literature. However, the observations mainly involve detections of cellular influx of $CD4^+$ T lymphocytes and macrophages, eosinophils, neutrophils, and dendritic cells at the bite site, together with an increase in pro-inflammatory cytokine levels such as IFNγ and IL-12 that peak 48 hours after bite (Belkaid *et al.* 1998, Kamhawi, 2000, Kamhawi *et al.* 2000). Such change in the skin environment alters not only the physiological and immunological response of the host but also the behaviour of sand flies, which preferably take blood at the site of ongoing DTH reaction, due to the increased blood flow (Belkaid *et al.* 1998).

DTH reaction to sand fly bites has been observed not only in experimental animals, but directly also in humans. The experimental exposure of volunteers to *P. duboscqi* bites from endemic areas in Mali resulted in the development of a DTH reaction in more than 60 % of the participants in that testing group. This reaction peaked at 48 hours after exposure and was shown to be age-dependent, decreasing around the 50^{th} year of the participants, due to the loss of the sensitivity to sand fly bites. The main infiltrate at the bite site consisted of $CD3^+$ T cells together with macrophages and higher levels of IFNγ with decreased levels of TNF, TGFβ, IL-13 and IL-5, which refers to the Th1 response. Immunological changes after the bites of *P. duboscqi* were systemic, where peripheral blood mononuclear cells (PBMC) obtained from the majority

of DTH positive individuals showed mixed Th1 and Th2 responses, after stimulation with *P. duboscqi* SGH in vitro. However, approximately 25 % of individuals with systemic response had their systemic reaction polarized to the Th1 or Th2 response. These findings lead to the conclusion that part of the population is more resistant to CL, whereas some proportion of people is more susceptible to the infection, regardless of the exposure to sand fly bites (Oliveira *et al.* 2013).

DTH reaction to sand fly bites was proved also for individuals repeatedly exposed to saliva of *Lu. longipalpis*. *In vitro* stimulation of PBMC cells from these volunteers produced higher levels of IFNγ even one year after the last exposure. However, these cells also exhibited increased levels of $CD4^+CD25^+$ and $CD8^+CD25^+$ cells. Nevertheless, the authors assume that due to the increased IFNγ production, these cells possessed rather effector cell function than regulatory potential (Vinhas *et al.* 2007). Another study focused on the development of DTH reaction in humans was done by Belkaid *et al.* (1998) in combination with *P. papatasi* exposure. The humans and laboratory mice in that experiment developed the DTH reaction in response to repeated exposure to *P. papatasi* sand fly bites, which in this case was characterized by increased blood flow, together with a typical nodular lesion.

The priming of the immune system by repeated exposure to sand fly saliva results in a different reaction toward the *Leishmania* parasite during the infection. In mice pre-exposed to bites of the Old World species *P. duboscqi, L. major* parasite infection increased the expression of proinflammatory cytokines (IFNγ) and chemokines (CCL9, CCL19 and CCl25), leading to a high cellular flow indicating DTH reaction, while having a suppressive effect in unexposed mice infected by *L. major*. Naïve mice also developed a response characterized by an increase in pro-inflammatory cytokine levels, but this response was delayed from the pre-exposed group, occurring one-week versus 48 hours, respectively (Teixeira *et al.* 2014). Consistent with these results, mice pre-exposed to vector saliva from *P. papatasi* and infected with *L. major* also showed the influx of immune cells characteristic of the DTH reaction (neutrophiles, macrophages, $CD4^+$ T cells) to the bite site with elevated concentrations of IFNγ and IL-12, indicating the pro-inflammatory Th1 response (Belkaid *et al.* 1998, Kamhawi *et al.* 2000). Single exposure of naïve mice to SGH of *P. papatasi* also induced rapid production of cytokines connected to Th2 (IL-4, IL-5) response in epidermal cells, which was abolished by repeated exposure to vector saliva (Belkaid *et al.* 1998).

The presence of DTH cellular infiltrate can differ in the context of the immunization with sand fly saliva from different species, corresponding to the variable saliva molecules among species (Gomes *et al.* 2016). However, as mentioned in the previous paragraphs, due to the inconsistence of studied immunological parameters among available studies, no comparative study exists. Single exposure of mice to SGH of New World species *Lu. longipalpis* lead to massive influx of immune cells, with the majority of neutrophils and macrophages, but this recruitment was drastically reduced when the mice were pre-exposed repeatedly to SGH, regardless of the presence of *L. braziliensis* in the inoculum (Carregaro *et al.* 2013).

2.1.6 Salivary proteins as promising vaccine candidates

Further research using only particular proteins from vector saliva to elicit protective immune response has been successfully applied on laboratory host models including mice (Valenzuela *et al.* 2001, Oliveira *et al.* 2008), hamsters (Gomes *et al.* 2008) and nonhuman primates (Oliveira *et al.* 2015). Salivary proteins that serve as potential vaccine candidates are selected based on their ability to induce a DTH response in the host, which is believed to be the immunological mechanism behind the protective effect (Valenzuela *et al.*

2001, Gomes *et al.* 2008). However, only few molecules from sand fly saliva can induce this effect, and yet the capacity of DTH-inducing molecule does not ensure reaction resulting in the protection (Belkaid *et al.* 1998, Oliveira *et al.* 2008).

For the New World species of the genus *Lutzomyia,* the maxadilan molecule as a potential vaccine candidate has been tested mainly due to its ability to induce the Th1 response (Morris *et al.* 2001) and LJM19 from the family of yellow related proteins (YRPs), which has shown its protective capacity in the hamster model by inducing the DTH reaction after multiple immunization (Tavares *et al.* 2011). Another study showed the immunomodulatory effect of the YRPs family protein LJM 11, resulting in the protective effect on *L. major* infection (Sima *et al.* 2016), which was proved on the BALB/c model (Xu *et al.* 2011).

For the Old-World species of the genus *Phlebotomus*, the protein PpSP15 has been shown to induce a strong DTH reaction in the host itself, and therefore protect from the *Leishmania* parasite (Valenzuela *et al.* 2001). Different proteins from sand fly saliva have different outcomes on the disease. When experimental mice models were immunized with other salivary protein inducing DTH, PpSP44 from saliva of *P. papatasi*, the effect resulted in an enhanced effect on subsequent infection with *L. major*, which contrasts with observations of immunization with whole saliva or other single DTH inducing protein such as PpSP15 (Oliveira *et al.* 2008).

DTH inducing proteins from sand fly saliva of *P. ariasi* such as ParSP25 and ParSP15 elicited rather mixed or Th2 response, in contrast to other protein like ParSP03, which immunization resulted in response similar to other 'protective' proteins like PpSP15 from *P.papatasi* (Valenzuela *et al.* 2001) and LJM19 from *Lu. longipalpis* (Tavares *et al.* 2011). DTH reaction associated with immunization with salivary proteins from *P. ariasi* was characterized mostly by macrophages and $CD3^+$ cells, with only low amount of neutrophiles and mast cells. This finding is slightly different from DTH reaction to *P. papatasi* bites and to PpSP15 protein, where neutrophiles and eosinophils were dominant cell types recruited to the bite site (Oliveira *et al.* 2006)

2.2 Innate immune response to *Leishmania major*

Leishmania parasites are causative agents of different clinical forms of Leishmaniasis, based on the *Leishmania* species and host genetic and immunological background. The various outcome of the disease is connected to the species-specific diverse interaction of parasites with the mammalian immune system (Kaye and Scott, 2011). For that reason, following literature overview is focused mainly on the immune interaction of the model species used in the experimental part of this project – *L. major*, within the mammalian – mice or human hosts.

2.2.1 *Leishmania* virulent factors – surface molecules

As soon as infectious metacyclic promastigotes of *Leishmania* are injected into the host skin, complement factors interact with molecules expressed on the surface of *Leishmania*, triggering the assembly of the membrane attack complex (MAC), leading to the cell lysis. To escape this deadly mechanism, *Leishmania* parasites possess several features to prevent MAC formation, including the expression of two major *Leishmania* surface molecules - lipophosphoglycans (LPG) and glycoprotein 63 (gp63). These molecules have been detected as major virulence factors for *Leishmania* stages present in mammalian host (Bouvier

et al. 1985, Späth *et al.* 2000) and serve as essential components in early *Leishmania* survival and establishment inside the host. Moreover, LPG together with gp63 are key factors in immunomodulation and protection of parasites from host immune cells (Brittingham and Mosser, 1996, Kulkarni *et al.* 2006).

LPG is creating high coat covering the *Leishmania* parasites, which length, density, molecular and chemical composition is dependent on the life stage of the parasite – amastigote or promastigote form (Sacks *et al.* 1990, Becker *et al.* 2003). The prolonged LPG in the stage of infectious metacyclic promastigotes protects the parasite cell membrane when exposed to the complement factors in the serum. Formation of MAC is prevented by spontaneous release of the assembled complement complex C5b-9, which loses its activity (Puentes *et al.* 1991, Brittingham and Mosser, 1996). This molecule is not present consistently in parasite life cycle and vary also in species-specific modifications (Descoteaux and Turco, 1992).

Immunomodulatory effects of LPG also depend on the *Leishmania* species, however, the effect on the host immune system is mainly towards parasite protective environment. Once present in the host tissue, LPG is immediately recognized by complement factor C3b, resulting in the opsonization. This process helps with recognition of *Leishmania* parasites through the complement receptors CR1 and CR3 presented on phagocytic cells like macrophages, which facilitate the intake of parasites into the cell – a process, necessary for *Leishmania* survival and the disease establishment (da Silva *et al.* 1989, Mosser and Endelson, 1985, Brittingham and Mosser, 1996). In case when *Leishmania* LPG is recognized through the Toll-like receptors, this interaction can trigger increased production of proinflammatory cytokines such as IFNγ, which play the role in host protection (Schleicher *et al.* 2007). However, *L. major* was proved to downregulate the TLR-9 expression through the TLR-2 interaction, which inhibits the host protective mechanisms (Srivastava *et al.* 2013).

LPG does not lose its immunomodulatory capabilities by being phagocytized and still can be implicated during phagolysosome maturation, suppression of the oxidative burst and NO production in macrophages (Frankenburg *et al.* 1990, de Assis *et al.* 2012), and is involved in downregulation of expression of specific toll like receptors (Becker *et al.* 2003, Srivastava *et al.* 2013).

Taken together, LPG plays a key role during the first phase of interaction between the *Leishmania* parasite and the host defence mechanism and is important in immunomodulation even after being phagocytized by immune cells.

LPG is not the only essential molecule presented on the surface of *Leishmania*. Another frequently expressed molecule, also being considered as major virulent factor, is zinc metalloprotease gp63, known as leishmanolysin. It is another key factor molecule contributing to the establishment of *Leishmania* infection in the host, when mice infected with promastigotes lacking gp63 presented delayed and reduced infection in both the susceptible (BALB/c) and the resistant (C57BL/6) mice strains (Chan *et al.* 2021). Focusing on the interaction of *Leishmania* parasite with the mammalian host, gp63 is presented both on promastigotes and amastigotes (Yao *et al.* 2003) and can be found in three main forms – as GPI anchored surface molecule, released from the *Leishmania* coat as a soluble form freely into the surrounding environment or localized inside the cells (Bouvier *et al.* 1985, McGwire and Satoskar, 2002, Kulkarni *et al.* 2006). The intracellular localization is based on the ability of freely released gp63 to gain access to the macrophages through lipid-raft dependent manner, while its proteolytical activity remains preserved and active. Once in the

macrophages, gp63 can interact with macrophage protein tyrosine phosphatases, the crucial molecule in macrophage signalling pathways affecting NO production and its proinflammatory effector functions (Forget *et al.* 2006, Gomez *et al.* 2009, Shio *et al.* 2012).

One of the main roles of gp63 occurs already during the first effector response of the host by complement system, where gp63 can cleave the active form of factor C3b into its inactive form iC3b (Brittingham and Mosser, 1996) and by that protect the parasite from formation of the lytic pore on its membrane. Moreover, the iC3b molecules can bind to CR3 complement receptor, which facilitate the uptake of promastigotes by macrophages (Brittingham and Mosser, 1996, Brittingham *et al.* 1999).

2.2.2 Neutrophils and their role in *Leishmania major* infection

Neutrophils, together with monocytes, are the first immune cells recruited to the bite site of a sand fly. For the long time, neutrophils have been considered to be the predominant cell type during the early phase of infection, carrying also the majority of *Leishmania* promastigotes (Peters *et al.* 2008, Ribeiro-Gomes *et al.* 2012). In contrast with these findings, recent study by Chaves *et al.* 2020 identified dermal tissue resident macrophages (TRMs) as main cell type infected by *L. major* promastigotes during the first 24 hours of the infection. However, neutrophils are still one of the main cell populations involved during the first hours and days post-infection. The accumulation of neutrophils is elicited both by bites of infectious as well as non-infectious sand flies appearing as early as 30 minutes after the bite. The initial neutrophil recruitment is driven mainly by the proboscis insertion and host tissue disruption, followed by *Leishmania* parasites itself (Peters *et al.* 2008, Ribeiro-Gomes *et al.* 2012, Chaves *et al.* 2020).

Research conducted by Ribeiro-Gomes *et al.* 2012 described neutrophils as the primary infected cells during the early phase 1-12 hours of infection which are being later progressively substituted by the infiltrating macrophages. To ensure and facilitate the process of continuous influx of immune cells, *Leishmania* parasites are endowed with several mechanisms to achieve that. It includes triggering of endothelial cells into the chemokine production such as CXCL8 (IL8) – neutrophil chemoattractant (Brennan, 2007) and CCL5 – the chemotactic factor for several immune cells, including monocytes, eosinophils, basophils, NK cells and immature T cells (Dembic, 2015).

Migration of neutrophils to the bite site, however, carry on for longer period, with several waves which differ according to the presence of *Leishmania* parasites. This endothelial activation is promoted regardless to the *Leishmania* species and it generally contributes to *Leishmania* cell invasion and survival (D´Alessandro *et al.* 2021). Later influx of neutrophils is maintained mainly by the *Leishmania* parasite themselves in the infected mice. Recruited monocytes have started to possess phenotype features reflecting their differentiation into macrophages and monocyte derived DCs since the end of the first week of infection. These finding correlate with the fact, that macrophages, main *L. major* host cells, are recruited to the bite site later in the infection – appearing from day 4 post-infection (Ribeiro-Gomes *et al.* 2012).

Macrophages are essential cells for *Leishmania* long-term establishment and their absence during the first hours after the infectious inoculation is being hindrance for *Leishmania* promastigotes effective cell infection (Serafim *et al.* 2021). Extracellular environment of the host skin is lethal for the freely released infectious metacyclic promastigotes (Guimaraes-Costa *et al.* 2009). However, *Leishmania* parasites were able to develop an escape strategy to overcome this delay of macrophage influx by finding the temporary shelter also inside other immune cells including neutrophils. The uptake of *Leishmania* promastigotes by

neutrophils also induce the production of several cytokines and chemoattractants like IL-8 (enhance the recruitment of neutrophiles to the site of infection (Bickel, 1993)), TNFα (important in leucocyte migration and DCs and macrophages activation and differentiation (Idriss and Naismith, 2000), IL-1b and IL-6 (proinflammatory cytokines (Zhang and An, 2007) together with the chemokine MIP1b – the chemoattractant of nature killer cells and monocytes (Menten *et al.* 2002, Oualha *et al.* 2019).

Moreover, infected neutrophils enable the 'silent' entry of *Leishmania* into the macrophages when the apoptotic cell cover the presence of the parasite (van Zandbergen *et al.* 2004). Therefore, neutrophils are known as temporary host for *Leishmania*, enabling the parasites to escape from the effector immune mechanisms prior their main host cell appearance and they are also contributing to their uptake by macrophages during phagocytosis of apoptotic neutrophils (Peters *et al.* 2008, Ritter *et al.* 2009). The process of silent *Leishmania* uptake into the macrophages has been known as the 'Trojan horse' strategy (van Zandbergen *et al.* 2004). During this process, *Leishmania* benefit from the immune response distinct from the one elicited by direct promastigote uptake, during which levels of anti-inflammatory cytokine TGFβ are elevated, together with inhibition of macrophage effector killing functions (van Zandbergen *et al.* 2004. Ritter *et al.* 2009). There is one more strategy similar to Trojan horse silent entry, where instead of staying on inside the apoptotic neutrophils, *Leishmania* promastigotes are released in their transitional stage to the vicinity of surrounding macrophages. These stages are suggested to have better adaptations for uptake and survival inside macrophages (Peters *et al.* 2008). The apoptotic neutrophil in that case still serves as silencers of macrophage killing functions, skewing the cell response towards parasite protective mechanisms. Ritter *et al.* 2009 has named this as 'Trojan rabbit' strategy.

Neutrophils preferentially take up promastigotes which, however, lack the ability to transform into amastigotes stage inside these cells and reproduce. So far only amastigotes of *L. mexicana* have been observed to be able to replicate inside neutrophils, however, this happens only in the case, when neutrophil ingest amastigote in the later phase of infection. Ingested amastigotes are able to escape neutrophile eliminative machinery by formation and residing inside the acidic phagolysosome-like compartments called parasitophorus vacuoles, commonly known from macrophages (Mollinedo *et al.* 2010, Regli *et al.* 2017, Hurrel *et al.* 2017).

Leishmania promastigotes released into the skin tissue are rapidly phagocytized by incoming neutrophils, which causes their migration arrest. The rapid influx of these cells together with the reduction of their motility after parasite uptake are crucial factors for the development of cutaneous *Leishmania*sis, when mice lacking neutrophils showed significantly reduced numbers of parasites (Peters *et al.* 2008). Although neutrophils are main contributors for *Leishmania* survival, they are also short living cells, their life span is only few hours after their release into peripheral blood (Hidalgo *et al.* 2019). The early death of the *Leishmania* temporary host cell would not be much beneficial for the promastigote survival inside the host tissue before the macrophage arrival, therefore *Leishmania* developed another immune escape mechanism – the ability to postpone the neutrophil apoptosis itself. *Leishmania* parasites can decrease the caspase 3 activity, delaying the apoptotic process(Aga *et al.* 2002). This provides enough time to *Leishmania* for macrophage encounter, when apoptotic neutrophils can be later directly ingested by incoming macrophages (Aga *et al.* 2002, van Zandbergen *et al.* 2004).

Uptake of apoptotic neutrophils can be performed also by other immune cells including neutrophils itself. The neutrophil capacity to engulf the apoptotic neutrophils has been reported to be enhanced in the

presence of *L. major* parasites, when apoptotic cells act in similar way as in Trojan horse strategy with macrophages. Apoptotic neutrophils contribute to the survival of *Leishmania* inside neutrophils by inhibiting the effector neutrophil antimicrobicidal functions and decreasing the activity of signalling pathways leading to reactive oxygen species (ROS) production, observed in *L. major* infected neutrophils. Infected neutrophils have enhanced capacity to engulf apoptotic cells, which is connected to increased expression of components of complement receptors CR3 and CR1 on *Leishmania*-infected neutrophils, the mediators of phagocytosis by phagocytic cells (Salei *et al.* 2017).

Leishmania possess even more mechanisms to escape neutrophil killing microbicidal functions. Another main contributor for their survival is their ability to interfere with the process of granule fusion with *Leishmania* containing phagosome, which prevent the destruction of parasite by microbicidal granule content and acidification (Mollinedo *et al.* 2010). Further interaction with the function of NADPH oxidase results in the suppression of ROS production (Regli *et al.* 2017).

2.2.3 Macrophages and their role in *Leishmania major* infection

Macrophages can play dual role during the *Leishmania* infection. On one side, they possess effector killing mechanisms to eliminate *Leishmania* parasites, on the other hand, *Leishmania* uses macrophages as the main host cells, enabling them to replicate and survive in the host (Tomiotto-Pellisier *et al.* 2018, Costa da Silva *et al*. 2022).

The entry of *Leishmania* parasites to the macrophages occurs in different ways, which involve direct phagocytosis of promastigotes and amastigotes through several different receptors recognizing their surface molecules (Kelleher *et al.* 1992, Brittingham and Mosser, 1996) as well as opsonized parasites by complement components iC3b (Blackwell *et al.* 1985), or phagocytosis of apoptotic neutrophils containing *Leishmania* promastigotes, known as Trojan horse strategy as mentioned already above – see chapter 2.2.2 (van Zandbergen *et al.* 2004, Ribeiro-Gomes *et al.* 2012).

Promastigotes and amastigotes of *L. major* are recognized by different macrophage receptors, which reflects the stage of the infection and inflammation (Guy and Belosevic, 1993). Amastigotes are recognized mainly through Fc receptors, when parasite-specific antibodies are already presented at that later time point and facilitate the parasite uptake (Guy and Belosevic, 1993). Promastigotes are on the other side taken up mainly via complement receptors such as CR1 and CR3, mannose receptors, fibronectin receptors and Fcγ receptors as well (Blackwell *et al.* 1985, Brittingham and Mosser, 1996, Woelbing *et al.* 2006, de Menezes *et al.* 2016).

Leishmania parasites has developed specific mechanisms, how to escape the intracellular killing machineries and survive within the host cells. *Leishmania* promastigotes are internalized within phagolysosome-like compartments, of the macrophage plasmamembrane origin. These phagosomes then undergo remodelling and fusion with endocytic organelles, resulting in creation of parasitophorus vacuole, further modified as a lysosomal compartment (Antoine *et al.* 1998).

The internalized phlagellated promastigotes start to differentiate inside macrophages into the smaller and non-motile form – amastigotes, which can further replicate. This transformation is pH dependent, requiring the acidic environment for induction of amastigote related protein expression (Zilberstein *et al.* 1991). Vacuoles containing amastigotes possess lysosomal features, where acidic

environment is good for their optimal metabolism, which is in oppose to ideally neutral pH optimum for promastigotes (Moradin and Descoteaux, 2012). Amastigotes of *L. major* segregates into small vacuoles during the replication, which is typical for Old World *Leishmania* species (Berman *et al.* 1981, Castro *et al.* 2006).

Both amastigotes and promastigotes can inhibit maturation of parasitophorus vacuole and by that avoid the destruction by antimicrobicidal activity of the cell (Podinovskaia and Descoteaux, 2015, Le Moal and Loiseau, 2016). Amastigote form serves as a keeper of the infection. This form can reside in macrophages for several days and is responsible for clinical manifestation of the disease. Moreover, the uptake of apoptotic or dead neutrophils triggers TGF production by macrophages, which exacerbate the replication of *L. major* inside (Ribeiro-Gomes *et al.* 2004).

Phagocytosis or internalization via several receptors by professional phagocytes such as macrophages triggers the activation of NADPH oxidase, which leads to the production of reactive superoxide ions, effective in killing of intracellular pathogens (Underhill and Ozinsky, 2002). The expression of inducible NO synthase (iNOS) is triggered also by the presence of proinflammatory cytokines such as IFNγ (Ding *et al.* 1988). Its activity correlates with the *Leishmania* disease progression and loads of parasites in the tissues. Levels of iNOS have been found to correlate with the levels of TGFβ, when TGFβ downregulates the iNOS expression (Stenger *et al.* 1994). Amastigotes taken up by macrophages were also shown to supress the superoxide and NO production of macrophages, which are normally induced by phagocytising, thus inhibiting their leishmanicidal activities (Assereuy *et al.* 1994, Kima, 2007).

Macrophages can be divided into 2 basic phenotypes, namely M1 and M2, which are characterized by different metabolism and effector functions. M1 and M2 macrophages are directly connected to T cell immune response, which is polarized by their cytokines into Th1 or Th2 – M1 stimulating Th1 and M2 stimulating Th2 response (Mills, 2012). The main feature of these different types of macrophages is their metabolism of arginine, which can be metabolize via nitric oxide synthase (NOS) to NO and citrulline (typical for M1 macrophages) or via arginase to ornithine and urea (M2) (Rath *et al.* 2014). These two phenotypes are also characterized by their effector functions, when NO inhibits *Leishmania* replication and ornithine promotes it (Mills, 2012). *Leishmania* major has been reported to preferentially invade M2-like dermal macrophages through the mannose-receptor, which are able to locally maintain M2 functionality by even very low levels of IL-4 and IL-10, despite the high levels of IFNγ and TNFα, which are produced by other cells to control the infection (Lee *et al.* 2018).

In general, macrophages play an important role in the elimination of parasite through the production of nitric oxide and they also contribute to recruitment of pro-inflammatory cells and priming of T cell response. *Leishmania* parasites however developed several mechanisms, how to overcome macrophage effector functions and change them into parasite-hospitable environment (Costa da Silva *et al.* 2022).

2.3 Adaptive cellular immune response to *Leishmania*

2.3.1 CD4+ T cells polarization in response to *Leishmania* infection

Major role in fighting and elimination of *Leishmania* in the host is attributed to macrophages and their capacity to produce NO and ROS which are effector killing molecules against this parasite (Tomiotto-Pellissier *et al.* 2018). However, this type of immune response is closely related to the adaptive immune response, where especially $CD4^+$ Th1 response is crucial to support protection against *Leishmania* parasites. Once *Leishmania* are phagocytized by innate immune cells, this process triggers the production of cytokines determining further immune response. Protective mechanisms are related to Th1 polarization characterized by higher levels of proinflammatory cytokines like IFNγ, TNFα, IL-6 and IL-12, whereas nonprotective Th2 response includes anti-inflammatory cytokines such as IL-13, IL-4, TGFβ and IL-10, associated with *Leishmania* progression (Muraille *et al.* 2014, Rossi and Fasel, 2018 dos Santos Meira *et al.* 2019).

The polarization of macrophages into M1 or M2 type is mainly dependent on the antigenic stimulation, and it is crucial in Th1 or Th2 establishment respectively (Mills *et al.* 2000, Muraille *et al.* 2014, Tomiotto-Pellissier *et al.* 2018). However, their contribution to the T cell priming is rather indirect, by production of cytokines. Major roles are playing by other antigen-presenting cells (APC) such as dendritic cells (DC). In the early phases of the *Leishmania* infection, T cells are underrepresented at the bite site, thus macrophages infected by *Leishmania* parasites have lower chance to encounter T cells. The APC presenting cells play important role in the infection, by creating the link to T cells and further priming the immune response (Will *et al.* 1992, Woelbing *et al.* 2006). Dendritic cells (DCs) are the main type of APCs, which preferentially uptake amastigotes opsonized by anti-parasite specific IgG through the Fcγ receptors, in contrast to macrophages that mediatethe uptake of *Leishmania* mainly through the complement receptors (Mosser and Endelson, 1985, Woelbing *et al.* 2006, Liu and Uzonna. 2012). Study by Woelbing *et al.* 2006 showed that macrophages together with DCs are sequentially involved in the infection, when phagocytosis of *L. major* during the early phase occurred predominantly by macrophages via complement receptors and in later phases by DCs via Fcγ. By that, DCs become especially important in establishment of the disease. Uptake of *L. major* by DCs through Fcγ triggers high IL-12 production by DCs, which is important in priming the Th1 response (Woelbing *et al.* 2006).

Langerhans cells (LHs) are major antigen presenting cells in the skin. They are members of DC cells family (Clayton *et al.* 2017). Their capabilities involve phagocytosis of *Leishmania* parasites, however, their main function is not to eliminate parasite but rather to present its antigens to T cells and prime T cell response. They were proved to migrate from epidermis to dermis to uptake *L. major* antigens, followed by further migration and then to the draining lymph nodes where they present *Leishmania* antigen to naïve $CD4^+$ and $CD8^+$ cells (Will *et al.* 1992, Moll *et al.* 1993, Woelbing *et al.* 2006).

Langerhans cells are not the only type of DCs involved in *Leishmania* infection. Other DCs subset like monocyte-derived DCs from the blood have been also shown to possibly phagocyte parasites and present them in the draining lymph nodes. In the lymph nodes, they produce high levels of proinflammatory IL-12 and by that stimulate the Th1 response (León *et al.* 2007). This DCs cell type was found to be more important in the later phases of the infection in comparison to LHs, which are active mainly during the early phases of the infection (Feijó *et al.* 2016).

After the internalization of pathogen, DCs present antigens on their MHC II molecules to the T cells (Hennecke and Willey, 2001). The exact role of the DCs in Leishmaniasis depends on the murine strain and DCs subpopulations used in the experiments. Langerhans cells of susceptible BALB/c mice upregulated anti-inflammatory IL-4 secretion and downregulated IL-12 production in response to *L. major* infection, which resulted in the inhibition of Th1 response. These results contrast to observations with the resistant C57BL/6 strain (Moll, 2002). *Leishmania major* LPG was also proved to possess the ability to downregulate the migration of LHs from skin, which can play a role in induction of effective immune response against the parasite (Ponte-Sucre *et al.* 2001).

The Th1 or Th2 response during *Leishmania* infection is mainly based on the balance between Th1 and Th2 cytokines. The main cytokine contributing to Th1 protective immune response during *Leishmania* infection is IL-12, associated with the clearance of *L. major* parasites from cutaneous tissues of BALB/c mice in the early phase of infection. The production of IL-12 is closely related also to IFNγ production, contributing to the Th1 profile and proinflammatory response as well (Sypek *et al.* 1993, Constantinescu *et al.* 1998). IL-4 and IFNγ has been shown to work reciprocally; the progressive *Leishmania* infection is associated rather with Th2 response, characterized also by elevated IL-4 in the tissues, whereas protective immune response is associated with Th1 response, characterized by high levels of IFNγ (Heinzel *et al.* 1989).

$CD4^+$ T cells are important in terms of polarization of immune response towards *Leishmania* parasites protective (Th1) or susceptible (Th2) immune response subtypes. Another T cell subset – Th17 - has been recently put in focus in terms of its contribution to the immune response in *Leishmania* infection. Studies have shown that *L. major* infection strongly stimulates the production of IL-17, which relates to the more severe disease outcome (Anderson *et al.* 2009, Lopez Kostka *et al.* 2009). The effect of Th17 response is, however, species specific. IL-17 has been related to neutrophil recruitment and disease progression in case of *L. major*. This effect was strong in susceptible BALB/c mice in contrast to resistant strain C57BL/6 (Lopez Kostka *et al.* 2009). Th17 response has been also shown to last during the infection and the presence of neutrophils triggered by higher levels of IL-17 supressed the IL-10 mediated infection control, which further contributed to the disease progress (Gonçalves-de-Albuquerque *et al.* 2017).

2.3.2 CD8+ T cells in *Leishmania* infection

$CD8^+$ T cells have been also found to highly contribute to *Leishmania* infection, especially in case of low dose infections. Nevertheless, their role in the infection is species-specific and dose and localization dependent, leading to both protective and exacerbating effect (Campos *et al.* 2017).

Study by Uzonna *et al.* 2004 showed that low dose infection in resistant C57BL/6 mice primed $CD4^+$ T cell into Th2, however, the IFNγ producing $CD8^+$ T cells were also activated, which promoted switch to Th1 response, that control the infection. Thus $CD8^+$ T cells were found to be implicated in control of the low dose infection, whereas high dose infection did not require $CD8^+$ T cells for the healing phenotype and promote strong $CD4^+$ T cells proliferation and cytokine production (Belkaid *et al.* 2002, Uzonna *et al.* 2004, Okwor *et al.* 2014).

However, $CD8^+$ T cells did not affect the control of secondary *L. major* infection in mice healed from primary low/high dose infections. These results suggest that $CD8^+$ play role rather in primary infection

as important producers of IFNγ that help to activate macrophages into the M1 phenotype. (Belkaid *et al.* 2002, Okwor *et al.* 2014).

2.3.3 Chronic *Leishmania* infection

The study on resistant C57BL/6 mice showed, that persistence of *L. major* parasites in the skin of healed mice is conferred by peripheral $CD4^+CD25^+$ regulatory T cells that accumulate in the dermis, where they supress the effector mechanisms of $CD4^+$ cells to eliminate the parasite. These $CD4^+CD25^+$ T cells are probably naturally occurring T regulatory cells producing IL-10 cytokine that inhibits the effector cell response in response to *L. major*, thus contributing directly to parasite persistence (Belkaid *et al.* 2002).

IL-10 is an anti-inflammatory cytokine, conferring protection of the host in long term infections by preventing immunopathological reactions (Couper *et al.* 2008). During the infection of *L. major*, it, however, plays a dual role, by also disabling the complete clearance of parasite from the infectious site. The deficiency in this cytokine resulted in sterile immunity. Interestingly, IFNγ levels remained high even in chronic phase of infection, which leads to conclusion, that IL-10 is responsible for the host irresponsiveness to IFNγ and effector killing mechanism. The chronic site of infection is then maintained only by DCs and macrophages which kept their responsiveness to IFNγ and IL-10 (Belkaid *et al.* 2001).

2.3.4 Memory cells in *Leishmania* infection

Although no effective vaccine for cutaneous *Leishmania*sis is currently available for humans, some protection against reinfection has been observed in patients with healed infection (Mandel *et al.* 2017, WHO 2022).

Protection against reinfection to *Leishmania* is maintained by several populations of memory T cells. The CD4+ effector memory T cells characterized by tissue homing receptors - $CCR7^{low}CD62L^{low}$ were shown to be dependent on the presence of parasite to possess protection. CCR7 together with CD62L are priming the lymphocyte migration into the lymph nodes (Sallusto and Lanzavecchia. 1999, Kaech *et al.* 2003, Zaph *et al.* 2004).

Leishmania specific effector memory T cell population ($CD4^+CCR7^{neg}CD62L^{low}IL\text{-}7R^{low}$) and *Leishmania* specific central memory T cells ($CD4^+CCR7^{high}CD62L^{high}IL\text{-}7R^{high}$) development was observed already during the first 2 weeks post infection with *L. major*, corresponding with the Th1 $CD4^+$ T cells development in C57BL/6 mice. These early-emerging cells produced only IL-2 and needed further stimulation by antigen to trigger generation of Th1 IFNγ producing cells. These cells however arrested their proliferation soon and the authors assume, that this phenomenon is due to the lack of antigen presentation, caused by *Leishmania* suppressing effect on antigen presenting cells (Colpitts and Scott, 2010).

Central memory T cells are mainly located in the lymph nodes, from where they can migrate to the tissues (Sallusto and Lanzavecchia. 1999, Kaech *et al.* 2003). Another population of memory cells has been documented residing in the skin after the infection, and it was characterized as tissue resident memory $CD4^+$ T cell population (Glennie *et al.* 2015). Its presence has been observed even in the absence of circulating *L. major* specific $CD4^+$ T cells subsets (including effector $CD4+CD62Ll^{low}IL\text{-}7R^{low}$, effector memory $CD4^+CCR7^{neg}CD62L^{low}IL\text{-}7R^{low}$ or central memory $CD4^+CCR7^{high}CD62L^{high}IL\text{-}7R^{high}$ T cells) and it persisted even in long term point of view (for up to one year) as observed in the study by Glennie *et al.* 2015. The tissue memory T cells produce IFNγ and are able to promote the recruitment of *Leishmania*

specific memory cells, in case of new infection. Their presence is required for the protection since mice immunized with *Leishmania* specific central memory T cells was not able to achieve efficient protection. However, the tissue resident cells are also unable to mediate protection alone and they require the circulating *L. major* specific $CD4^+$ T cells to provide the protection (Glennie *et al.* 2015). Thus, it can be seen, that the protection requirements are fully efficient only in the presence of parasite (Glennie *et al.* 2015, Collpits and Scott 2010).

3 MATERIALS AND METHODS

Solutions and its reagents used for the experiments are summarized in the following table:

Solution:	Reagents:
Anaesthesia	2% ketamine (Narketan; Vétoquinol); 0,5% xylazine (Rometar; Bioveta), physiological solution, Ketamine 62.5 mg/kg and xylazine 25 mg/kg
Salivary gland homogenate (SGH)	Salivary glands homogenate of sand flies in physiological solution, 1 salivary gland per 1 µl of physiological solution
Physiological solution	0.85% NaCl
Leishmania cultivation and infection	
Cultivation medium for L. major (LV561)	M199 medium supplemented with 10% fetal bovine serum (Gibco), M199 (Sigma), 1% amikacin (Brystol-Myers Squibb), 1% BME vitamins (Sigma), 0.5% sterile urine
Diluting solution	1% formaldehyde, 0.85% NaCl
Infective inoculum	*L. major* promastigotes in physiological solution + SGH of *P. duboscqi*
Tissue sample collection	
Phosphate buffered saline (PBS)	140mM NaCl, 3mM KCl, 4 mM $Na_2HPO_4.12H_2O$, 1.5 mM KH_2PO_4, distilled H_2O, pH = 7.4
1% FBS/PBS	1% fetal bovine serum diluted in PBS
Zinc fixative	BD Biosciences, cat. no. 552658, diluted 1:10 with dH_2O
Erythrocyte lysis buffer	0,15M NH_4Cl, 10mM $KHCO_3$, EDTA (EDTA-2Na) 0,1mM, dH_2O; pH = 7,2-7,4; filtered through 0.2 um filter (SigmaAldrich)
Fixative solution	4% formaldehyde (SigmaAldrich), 1% PBS
Detection of antibody response	
Coating buffer	20mM Na2CO3-NaHCO3, pH = 9
Phosphate buffered saline (PBS)	140mM NaCl, 3mM KCl, 4 mM $Na_2HPO_4.12H_2O$, 1.5 mM KH_2PO_4, distilled H_2O, pH = 7.4
Washing buffer (PBS-TWEEN)	0.05 % Tween (Serva) in PBS; pH = 7.5
Substrate buffer (McIwein phosphate-citrate)	0,11 M $NaH_2PO_4 \cdot 12H_2O$ (9 ml; Penta); 0,5 M citric acid (1 ml; Lachner); pH 5,5 10 mg OPD (o-fenylendiamin, ThermoFisher scientific), 20 µl 30% H2O2 added right before use
Blocking buffer	6% milk (Blotting-grade blocker; BIO RAD; 170-6404) in PBS-TWEEN
Diluting buffer for sera	2% milk (Blotting-grade blocker; BIO RAD; 170-6404) in PBS-TWEEN
Immunoblot	
Tris buffer	20 mM Tris (Trizma base, Sigma), 150 mM NaCl (LACHNER), 500 ml dH_2O
Tris-TWEEN	Tris buffer with 0.05% Tween 20 (Tris-Tw)

3.1 *Leishmania major* – cultivation and preparation of the inoculum

For the purpose of this project, a common laboratory model of cutaneous leishmaniasis in BALB/c - *Leishmania major* LV561 (MHOM/IL/67/LRC-L137 JERICHO II) was used. Our laboratory possesses a collection of several *Leishmania* strains, which promastigotes are kept in liquid nitrogen in the freezing ampules CryoTubeTM Vials (NUNC). *Leishmania* are cryopreserved by addition of dimethyl sulfoxide (DMSO; Duchefa Biochemie) as the cryoprotectant in total amount of 5-10 % of the sample volume.

Leishmania promastigotes were defrosted at room temperature (RT), aseptically distributed equally into 2 cultivation flat tubes with 1 ml of cultivation medium and kept at 23°C. Passaging of culture was done each 5-7 days; 2-3 drops of the culture were put into the new flat tubes with 1.5 ml of cultivation medium. Passages (p) used to infect mice were from p3 to p5.

3.1.1 *Leishmania major* inoculum preparation

To infect mice, the inoculum contained 10^4 *L. major* promastigotes (p3-p5) and salivary gland homogenate (SGH) of *Phlebotomus duboscqi* (the equivalent of 0.5 gland) in a physiological solution. The inoculum was prepared under sterile conditions from stationary phase *L. major* promastigotes (LV561). The culture of *L. major* was transferred from the flat tubes to 1.5 ml vials and spin for 5 minutes, 3500g (Eppendorf MiniSpin). After centrifugation, supernatant was discharged into disinfection and sediment was resuspended in 1 ml of sterile physiological solution. This process was repeated twice, and the washed culture was kept on ice.

In case of *Leishmania* corpuscular antigen preparation, used in ELISA (see Chapter 3.9), all morphological forms of *Leishmania* parasites were counted in Bürker´s chamber and solution put into -80°C prior use.

Leishmania parasites were counted in Bürker´s chamber by counting only metacyclic promastigotes (long-flagellated slender cells). For this purpose, 10 ul of the *Leishmania* culture was diluted in 990 µl of 1% formaldehyde/0.85% NaCl solution to kill and immobilize the parasites. The inoculum was then prepared in a concentration of 2,2x10^6 metacyclic promastigotes/ml, which corresponds with required concentration of 10^4 *Leishmania* in 4.5 µl of 1 infection dose. Each of the infection dose was supplemented with prepared SGH of *P. duboscqi* in an equivalent to 0.5 gland in volume of 1 µl, thus the total volume was 5.5 µl per 1 infection dose. The preparation of SGH of *P. duboscqi* is described below (chapter 3.2.1.).

3.2 Sand flies *Sergentomyia schwetzi* and *Phlebotomus duboscqi* – maintenance and preparation of the salivary gland homogenate

Sergenotmyia schwetzi colony originates in Etiopia and *Phlebotomus duboscqi* colony comes from Senegal. Both colonies are reared and breed under standard conditions in the insectary as described in Volf and Volfová (2011). The eggs, larvae and adults are kept at 26 °C, humidity up to 50 %, and 14/10 hours of light/dark cycle.

The adults are kept in nets made from monofil, stretched on the metal cages to achieve cubic shape of the net with size 40 x 40 x 40 cm. The cage is covered with plastic bag and the inserted wet cotton wool ensure sufficient humidity. Both genders of sand flies are allowed to feed ad libitum on 50% sugar solution

in distilled water soaked in the cotton wool, which is placed inside the nets and regularly changed three times a week.

Adult females require proteins diet for the egg development, which is provided by the access to the mammalian blood. Mice are put under anaesthesia for blood feeding of at least 5 days old females once a week. Blood feeding takes place in dark room with stable temperature around 24°C for about 60 minutes. After 4 days, blood fed females are placed to the plastic boxes coated inside with moisturized plaster of Paris and covered with monofil. These boxes are placed inside the bigger plastic box filled with moisturized sterilized sand to keep the humidity. Within 2-6 days, blood fed females lay the eggs and consequently die. They are removed from the boxes to prevent egg contamination from fungi. Hatched larvae are fed by grinded fermented rabbit faeces. The development continues through four larvae stages (L1-L4) to pupae stage and to adults, which are released to new cages 3 times a week. The whole life cycle of sand flies reared in our lab last about 5 to 8 weeks, depending on the species.

3.2.1 *Sergentomyia schwetzi* and *Phlebotomus duboscqi* salivary gland homogenate preparation

Phlebotomus duboscqi and *S. schwetzi* sand flies at least 5 days old were dissected for salivary glands, which were collected to small vials with physiological solution in concentration 1 salivary gland/1 µl of physiological solution. Dissected glands were stored at -20°C, until use. For the purposes of ELISA test and infections, vials with dissected glands were defrosted and immersed into liquid nitrogen and lukewarm water three times. By that, salivary gland homogenate was achieved.

3.3 Experimental animals

Laboratory mice used in our experiments were from an inbred BALB/c strain in the total amount of 35 females for experiment no. 1 and 18 females for experiment no. 2. Mice were obtained from Anlab, s.r.o. (Company ID 45796301) at the age of 7 weeks and placed in the approved animal facility at the Department of Parasitology, Faculty of Science, Charles University (approval no. 37428/2019-MZE-18134). In the animal facility, animals were placed in plastic containers type 4 (58 x 37 x 20 cm), maximum 6 individuals per box. Boxes were placed in the room with monitored temperature (20-24 °C) and humidity (40 – 50%). The boxes were equipped with bedding from non-dusty dried wooden fibres and the enrichment was provided by paper houses and tubes. Mice had access to water and standard maintenance dietST-1 (Velaz) ad libitum.

Manipulation with animals was performed in calm environment with maximum effort to prevent stress of the animal, under the supervision of certified person (Dr. Iva Kolářová, certificate no. CZ 02439). All experiments involving mice were approved by the ethical committee of Faculty of Science, Charles University and by the Ministry of education youth and sports (approval no.: MSMT-32011/2020-3).

3.4 Experimental design

3.4.1 EXPERIMENT no. 1 – The effect of timing between the last exposure to sand fly saliva and *Leishmania* infection

This project was focused on the course of infection by *L. major* in BALB/c mice, performed 2 days versus 7 days after the last immunization (= preexposure) by bites of *P. duboscqi*. Uninfected *P. duboscqi* females were used for immunization three times in one-week intervals and the infection was performed into both ear pinnae 2 days or 7 days post last immunization (p.l.i) as illustrated in Fig. 2. Mice were divided into following 4 groups: pre-exposed and infected 2 days p.l.i. (group D2), pre-exposed and infected 7 days p.l.i. (group D7), nonimmunized and infected mice served as a positive control group (POS) and nonimmunized and uninfected (naïve) mice served as a negative control group (NEG). Two independent experiments were performed (1A and 1B) including mice from all experimental groups in each replication according to the Tab. 2. Mice were euthanized 5 weeks post infection (p.i.), and tissue samples were collected and analysed as follows.

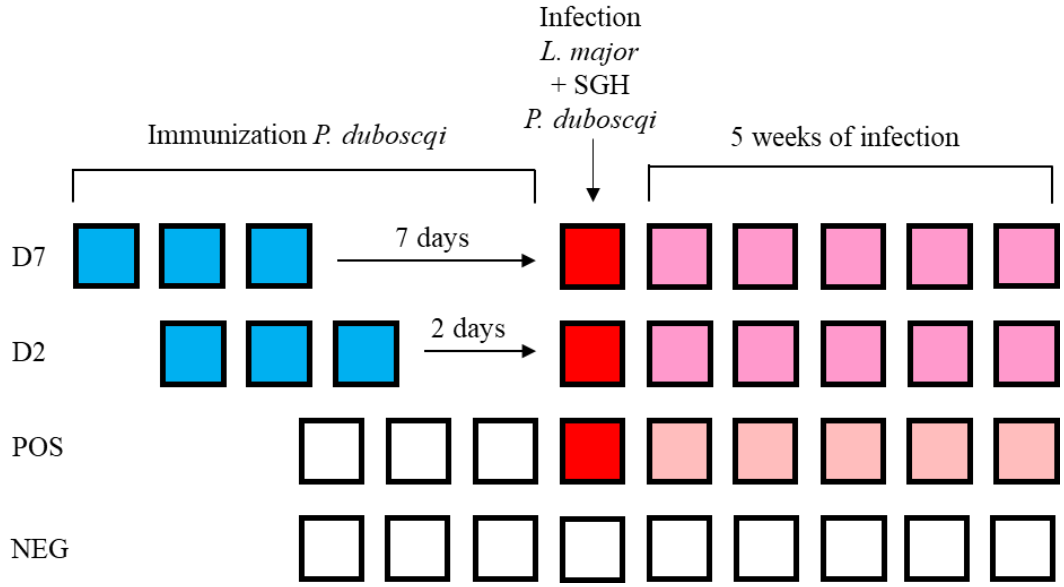


Fig. 2: Time schedule of Experiment no. 1. Each blue square represents 1 exposer to *P. duboscqi* bites, red squares indicate infection with inoculum containing *L. major* and SGH of *P. duboscqi* (week 0 of infection), pink squares represent weeks of infection and white squares indicate no intervention. Each square denotes one week.

Tab. 2: Numbers of mice per each replication of experiment no. 1. Three mice from the POS group of replication B were excluded from the statistical analysis since the infection did not result in the disease development (more details are provided in chapter 4.2).

Group	Replication 1A	Replication 1B	Total
D2	5	5	10
D7	5	5	10
POS	3	5	8
NEG	2	5	7

3.4.1.1 Immunization of mice with *Phlebotomus duboscqi*

Mice from groups D2 and D7 were individually exposed to 30 females of *P. duboscqi* per mice three times in 1-week intervals, whereas POS and NEG groups were left unimmunized. Sand flies were at least 5 days old, unfed, kept under standard conditions as described in chapter 3.2. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution (62.5.mg/kg and 25 mg/kg, respectively), and their eyes were protected from drying out by the eye gel (RecuGel) together with wet tissue covering also the nose and mouth parts (Fig. 3). Mice were covered by cotton fabric in a way that sand flies were allowed to feed blood preferentially on the ears. During the immunization, mice were kept individually in small nets with 30 – 40 sand fly females per net, under standard conditions (humidity 50% and temperature 25-26°C) and were checked regularly. Nets were covered by black fabric to simulate dusk conditions (Fig. 4), during which the sand flies are the most active in natural conditions. Sand flies were left to feed on mice for one hour. After each immunization, blood fed sand flies were counted, mice were checked for full recovery from anaesthesia, and were placed back to the boxes in animal facility.

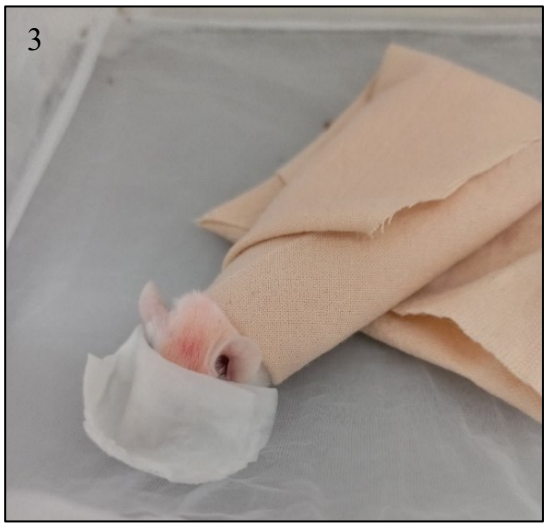


Fig. 3+4: Manipulation with mice during the immunization. **3** – anaesthetised mouse inside the net with the body and the eye/nose parts covered, **4** – ten cages covered under one black fabric.

3.4.2 EXPERIMENT no. 2 – The effect of *Sergentomyia schwetzi* saliva on the course of the infection by *Leishmania major* inoculated with salivary gland homogenate of *Phlebotomus duboscqi*

The aim of this part of the project was to test the specificity of protective effect of sand fly saliva against *Leishmania* infection on a mouse model. The immunization was performed on BALB/c mice by bites of unfed and uninfected *S. schwetzi* sand flies 3 times in one-week intervals. Infection with inoculum containing *L. major* promastigotes and salivary gland homogenate of *P. duboscqi* was performed 48 hours after the last immunization. Mice were infected into both ears (Fig. 5). Mice were divided into 3 groups: pre-exposed and infected (group EXP), nonimmunized infected positive control (group POS), and nonimmunized uninfected negative (naïve) control (group NEG). Mice were euthanized at week 9 p.i.; the tissue samples were collected and analysed as described further. For sufficient statistical analysis, three independent experiments were performed – 2A, 2B, 2C. Each repetition included mice from EXP, POS and NEG group, according to the Tab. 3. Covid restriction affected the immunization scheme of 2A group, which is illustrated in Fig. 5.

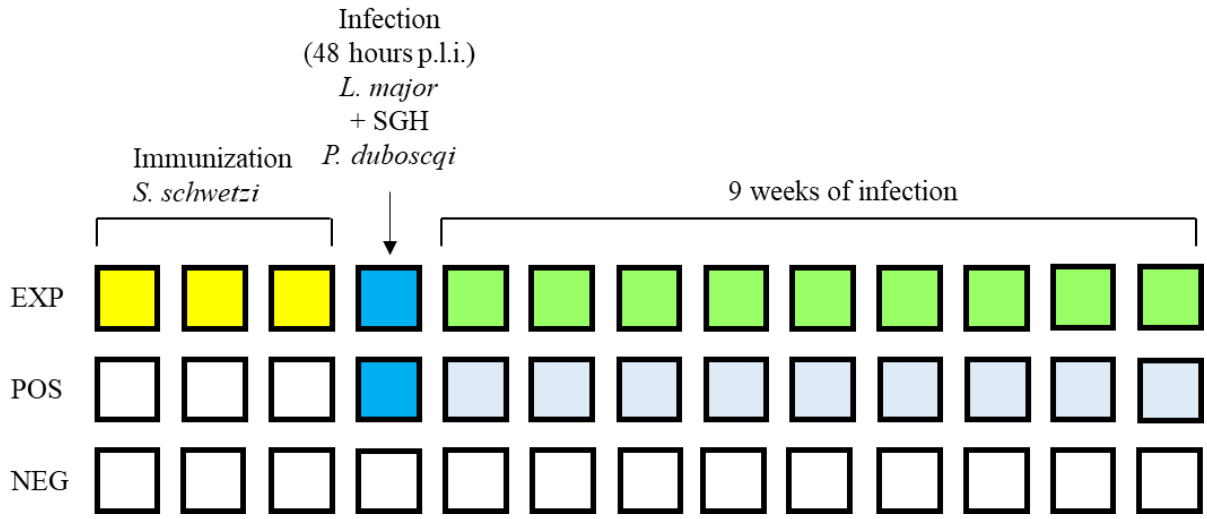


Fig. 5: Time schedule of Experiment no.2. Yellow colour represents immunization with *S. schwetzi*, blue colour infection with *L. major* and SGH of *P. duboscqi,* green colour is for infection of preexposed group, light blue is for infection without pre-exposition and white colour are for no interventions. Each box denotes one week, except the infection, which was performed 48 hours p.l.i.

Tab. 3: Numbers of mice per group for each replication of experiment no. 2

Group	Replication 2A	Replication 2B	Replication 2C	Total
EXP	3	3	2	8
POS	1	2	2	5
NEG	2	1	2	5

3.4.2.1 Immunization of mice with *Sergentomyia schwetzi*

Mice were divided into 3 groups, but only EXP group was immunized by bites of uninfected *S. schwetzi* females. The other two groups – POS and NEG were left naïve – without immunization. Mice were immunized 3 times in a one-week interval, 30-40 S. *schwetzi* females per mouse each time. Conditions were the same as previously described in the part Immunization of mice with *Phlebotomus duboscqi* (chapter 3.4.2) with following modifications: the first and the second immunization was done on the whole mouse body with covered head by the wet tissue, ensuring breathing but protecting from bites of sand flies. The third exposure (and the fourth one in case of group 2A) was performed only on mice ears, with the rest of the mouse body covered by cotton fabric for the protection. During the third exposure, only a small number of sand flies took blood (Tab. 4) and therefore immunization was repeated one week later.

The planned infection within the experiment 2A was postponed due to the covid restrictions, and therefore, 17 weeks later, additional sand fly immunization was performed prior to the infection as illustrated in Fig. 6.

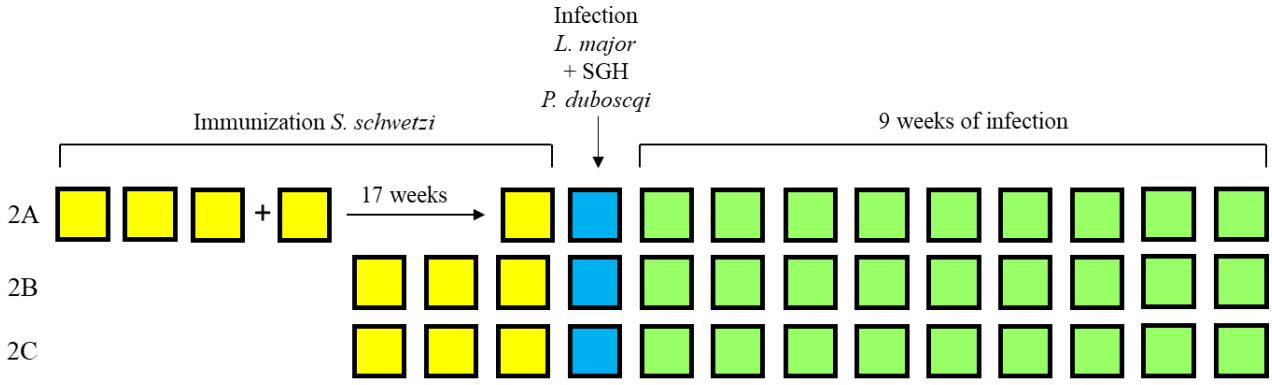


Fig. 6: Time schedule of EXP group divided according to the experiment repetition 2A/2B/2C; yellow colour represents immunization with *S. schwetzi*, blue colour infection with *L. major* and SGH of *P. duboscqi*, and white colour are for no interventions. Each square denotes one week.

Tab. 4: Average number of blood fed *S. schwetzi* females per mouse of EXP group during immunization, divided according to the experiment repetition; the data are expressed as mean ± SD. N.A. means not applicable.

	1. immunization	**2. immunization**	**3. immunization**	**4. immunization**	**5. immunization**
2A	26 ± 3	15 ± 8	8 ± 5	28 ± 7	17 ± 5
2B	27 ± 2	28 ± 2	33 ± 2	N.A.	N.A.
2C	28 ± 2	28 ± 2	28 ± 2	N.A.	N.A.

3.5 Serum samples

3.5.1 Experiment no. 1

Serum from mice was examined for the presence of antibodies against *L. major* and against *P. duboscqi* saliva using ELISA test. For those purposes, blood was obtained at two time points – after the immunization meaning before infection within two days or one-week p.l.i. (preinfection/W0) and at the end of the experiment, at week 5 post infection (W5). The preinfection samples were taken from mice tails by cutting the tip of tail with sharp scissors and collecting the blood drops into heparinized capillaries (Keraglass). Capillaries were sealed at one end and centrifuged for 5 minutes at 3500 g (Thermo scientific hereus pico 17) and serum was collected to new vials and stored at -20°C for later analysis. The postinfection sample was obtained during the tissue collections at the end of the experiment at week 5 p.i. Mice were deeply anaesthetised, and the blood was collected by the disruption *vena subclavia*, which is described in detail in chapter 3.7.1.

3.5.2 Experiment no. 2

In this experiment, serum was collected at three time points: before first immunization = preimmunization, before infection = preinfection (W0) and at the end of the experiment, at week 9 of the infection = postinfection (W9). The preimmunization and preinfection blood samples were collected from the mouse tails, the postinfection blood sample was collected from *vena subclavia* as described in detail in chapter 3.7.2.

3.6 Infections of mice with *Leishmania major*

3.6.1 Experiment no. 1:

Mice from group D2 were infected 2 days after the last immunization by *P. duboscqi*, while mice from D7 group were infected 7 days after the last immunization. Half of the mice from POS group were infected with the D2 group, the rest was infected with D7 group. NEG group was left uninfected. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution and then infected with inoculum containing 10^4 *L. major* promastigotes and 0.5 µl SGH of *P. duboscqi* by needle (Chirana U40 29Gx1/2) intradermally into both ears. All groups of mice were infected in a volume of 5.5 µl of previously prepared inoculum and the infection was centred to the middle of the outer part of ear pinna, directed to the auricle. Mice from all groups were euthanized at week 5 of the infection, while collecting tissue samples for further analysis as described below.

3.6.2 Experiment no. 2:

Mice from groups EXP and POS were infected in the same way as mice from experiment no. 1. Mice from the control NEG group were intradermally inoculated with the same volume – 5.5 µl of physiological solution. The lesion development was monitored weekly by together with lesion area measurement (two perpendicular diameters) by digital calliper. Mice from all groups were euthanized 9 weeks post infection while collecting tissue samples for further analysis as described below.

3.7 Tissue samples collection

3.7.1 Experiment no. 1

Mice were euthanized at week 5 p.i., D2 and D7 groups were processed separately (5 days apart from each other) corresponding with their different infection time points, thus ensuring the same duration of the infection. Collection of tissue samples and its processing is summarized in Tab. 5. Mice from POS and NEG groups were divided into two groups and processed with D2 and D7 groups, to have representatives from each group in both experiment end points. All mice were put into deep anaesthesia by ketamine/xylazine solution and blood was obtained from *vena subclavia* into 1.5 ml vials and kept at 4°C for subsequent processing in centrifuge to obtain the serum sample. After blood collection, mice were euthanized by cervical dislocation. Both ear pinnae were cut off with scissors, documented, left one was fixed in 5 ml of zinc fixative solution and kept at room temperature (RT) - the subsequent histological and immunohistochemical analysis was performed in collaboration with doc. Andrea Bardůnek Valigurová and Bc. Karolína Poláková at Masaryk University, as described in the appendix no. 1 (page 92). The contralateral right ear was kept in pre-cold empty vial at -20°C for subsequent *Leishmania* quantification by qPCR.

Lymph nodes draining the ears were dissected and collected – left one was put into 1% FBS/PBS solution to measure lymphocytes populations by flow cytometry and the right one was kept in pre-cold empty vial at -20°C for *Leishmania* quantification by qPCR analysis, together with corresponding right ear pinna.

Tab. 5: Obtained tissue samples, their storage conditions, and intended analysis in Experiment no. 1:

Mice tissue:	Analysis:	Storage medium:	Temperature:
Left ear pinna	Neutrophils and macrophages (immunohistochemistry)	Zinc fixative	RT
Right ear pinna	*Leishmania* quantification (qPCR)	None	-20°C
Left lymph node	T lymphocytes (flow cytometry)	1% FBS/PBS	on ice
Right lymph node	*Leishmania* quantification (qPCR)	None	-20°C
Serum	Anti-sand fly saliva/*Leishmania* antibodies (ELISA)	None	-20 °C

3.7.1.1 T cell analysis by flow cytometry

Left lymph nodes from each mouse were analysed for selected T cell populations by flow cytometry: T cells ($CD3^+$), T helper cells ($CD3^+CD4^+CD8^-$), and cytotoxic T cells ($CD3^+CD4^-CD8^+$). Antibodies used for the analysis are listed in Tab. 6.

Tab. 6: Antibodies with corresponding fluorophores used for the flow cytometry analysis:

Antibody	Fluorophore	Concentration	Company	Catalogue number	Isotype
CD3	APC- Cy7	1:100	Biolegend	100222	Rat IgG2b, κ
CD4	FITC	1:10	Biolegend	100406	Rat IgG2b, κ
CD8b	APC	1:100	Biolegend	126614	Rat IgG2b, κ
CD16/32	-	1:100	Invitrogen	14-0161-82	

To obtain single cell suspension, lymph nodes were homogenized in the 1.5 ml vials using the proportionaly sized sterile plastic microhomogenizators and subsequently filtered through the micro-strainer with size of pores 70 um (Greiner bio-one) into 50 ml tubes. Strainers were washed with 1% FBS/PBS up to 25 ml. All samples were then centrifuged at 350g, 4°C (Heraus Megafuge 8R) for 10 minutes and supernatant was discharged to disinfection. The sediment of cells was resuspended in 25 ml of 1% FBS/PBS and samples were centrifuged again at 350g, 4°C (Heraus Megafuge 8R) for 5 minutes. The supernatant was again discharged into disinfection and the sediment was resuspended in 500 µl of 1% FBS/PBS. During the whole process, samples were kept on ice and the processing was continuous to prevent dying of obtained cells. Cells were counted by Countess™ Automated Cell Counter (Invitrogen) by taking 10 µl from each sample and mixing it with methylene blue in the ratio 1:1 from which 10 µl were used for the measurement on provided slides.

3.7.1.2 Staining of cells with specific T cell markers

Isolated cells from the lymph node tissues were stained with fluorescent markers listed in Tab. 6. Antibodies for flow cytometry were selected with the help of FluoroFinder program[1] to minimalize the spectral overlap of fluorophores. To prevent non-specific binding of Fc parts of antibody markers, 50 µl from each sample was first incubated with blocking CD16/32 antibody in final concentration 1:100 at 4°C for 15 minutes.

Meantime, conjugated antibodies were diluted in 1% FBS/PBS according to the manufacturer recommendation (Tab. 6) and added to blocked samples to final volume of 100 ul. One sample from NEG group served as a blank control (no labelled antibodies were added). Fluorescent minus one (FMO) and single stained (SS) controls were also prepared according to the Tab. 7. Samples were incubated in dark at 4°C for 45 minutes. Hoechst (Invitrogen, 33258) was added to samples 10 minutes before measuring in concentration 1 ug/ml diluted in 1% FBS/PBS except for blank samples and SS controls to mark dead cells and samples were kept in dark at 4°C.

[1] https://fluorofinder.com/

Measuring was done on CytoFLEX (405/488/561/638, Beckman Coulter) in Flow Cytometry Core Facility maintained by Jozef Janda, DVM, PhD. Each sample was shortly vortexed and measured in test tubes. Compensation matrix was counted during the first analysis using the SS controls and applied to all data within Experiment no. 1. Samples were measured with 50 000 events as the limiting factor. Two samples from the NEG group (1B) were excluded from the subsequent analysis due to low number of cells in the vials.

Obtained data were analysed by FlowJo (version 10.4.2). The compensation matrix was recalculated according to the data from single stained controls. Cell populations were distinguished based on the FMO and SS control analysis. Obtained data were counted as the ratio to all measured events (in %).

Tab. 7: FMO and SS controls for flow cytometry in experiment no. 1:

FMO controls	**Antibody mixture**	**Single stained controls**	**Antibodies**
FMO $CD3^-$	CD4, CD8b, CD197, CD62L	SS $CD3^+$	CD3
FMO $CD4^-$	CD3, CD8b, CD197, CD62L	SS $CD4^+$	CD4
FMO $CD8b^-$	CD3, CD4, CD197, CD62L	SS $CD8b^+$	CD8

3.7.1.3 Gating strategy

Immune cell populations were detected according to the gating strategy at Fig. 7. Firstly: lymphocytes were sorted out according to the size (FSC) and granularity (SSC) together with exclusion of dead cells and non-single cells from the further analysis. More precise selection of T cell subpopulation was achieved by distinguishing according to surface markers.

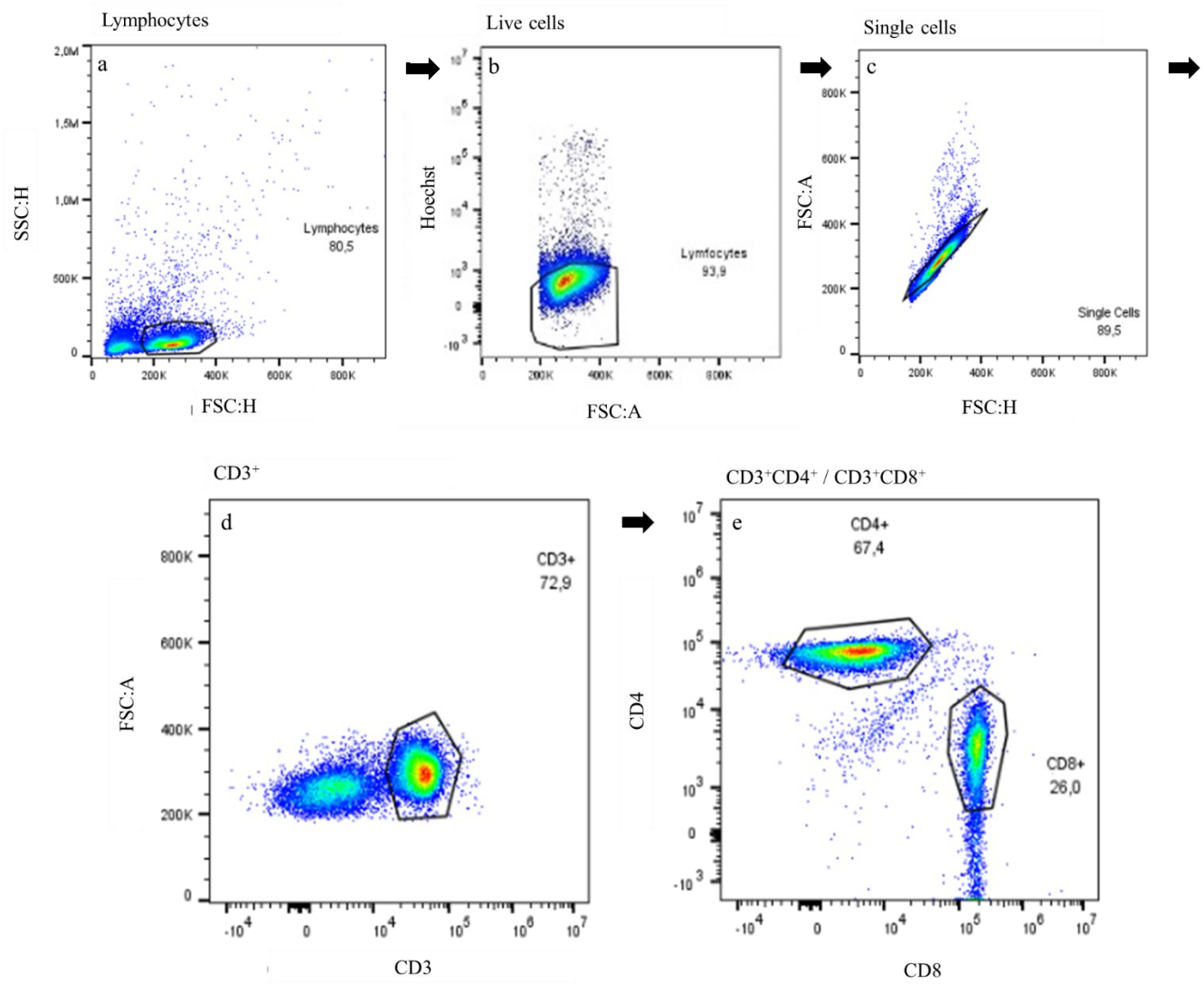


Fig. 7: Gating strategy of T cells populations on a representative sample of the lymph node single cell suspension: a) lymphocytes gated according to their size (FSC) and granularity (SSC); b) exclusion of dead cells using the Hoechst dye; c) single cells gating - exclusion of cell clusters d) detection of $CD3^+$ lymphocytes; e) detection of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subpopulations; gating was used for all the samples from one measurement in the same way.

T cell populations were characterized based on surface markers combinations summarized in Tab. 8:

Tab. 8: T cells populations according to the markers

T cell subsets	Surface markers
Lymphocytes	FSC/SSC
T cells	CD3+
Helper T cells	$CD3^+CD4^+$
Cytotoxic T cells	$CD3^+CD8^+$

3.7.2 Experiment no. 2

Mice from all groups were deeply anesthetized by intraperitoneal injection of anaesthesia (ketamine/xylazine) and blood sample was obtained from *vena subclavia* to vials for following serum separation. After blood collection, mice were euthanized by cervical dislocation and both ear pinnae were cut off with scissors and documented. Left ear was then fixed with 5 ml of 4% formaldehyde fixative solution and kept at 4°C for next processing for cryosections. Right ear was placed into pre-cold empty vials and kept at -20°C for later analysis by qPCR.

Lymph nodes draining the ears were dissected and collected – left one was put into 1% FBS/PBS solution for direct flow cytometry and right one was kept in empty vial at -20°C for later qPCR analysis. The spleen was collected under sterile conditions, placed into 800 µl of 1% FBS/PBS and proceeded for the flow cytometry as described in the chapter 3.8.6. Processing of collected tissues is summarized in Tab. 9.

Tab. 9: Obtained tissue samples, storage conditions, and intended analysis in Experiment no. 2:

Mice tissue:	**Analysis:**	**Storage medium:**	**Temperature:**
Left ear	Neutrophils and macrophages (immunohistochemistry)	4 % formaldehyde	RT
Right ear	*Leishmania* quantification (qPCR)	None	-20°C
Left lymph node	T lymphocytes (flow cytometry)	1% FBS/PBS	On ice
Right lymph node	*Leishmania* quantification (qPCR)	None	-20°C
Spleen	T lymphocytes (flow cytometry)	1% FBS/PBS	On ice
Blood - serum	Anti-sand fly saliva/*Leishmania* antibodies (ELISA)	None	Serum in -20 °C

3.7.2.1 Cryopreservation of the ear tissues

Left mouse ear was processed for intended immunohistochemistry analysis. As a fixative solution was used 4% formaldehyde, in which mice ears were placed right after the dissection. Ears were incubated on 360° rotating shaker at 4°C in volume of 5 ml overnight. Fixed ears were washed 3 times for 15 minutes with 5 ml of PBS the next day followed with 3-step saturation of the tissue with sucrose solution with rising concentration. Saturation took place at 4°C on rotating shaker when ear samples were first put for 2 hours in 5 ml of 10% sucrose solution, then in 20 % sucrose solution for additionally 6 hours, and finally, in the highest concentration of sucrose solution – 30% over the night till the next day (approximately 18 hours).

Ears were left in 30% sucrose solution at RT for additional 1 hour. The completed saturation with sucrose was indicated by drowning of the ear pinna to the bottom of vials (visible mainly in the samples with infected ears). The sucrose solution was then replaced with a few drops of tissue freezing medium, so the surface of the tissue was completely covered. Plastic moulds (teats cut from Pasteur pipettes) were filled with tissue freezing medium (Leica Biosystems) and ears were transferred there. Each ear was placed

into one mould, incubated at -20°C for 3 hours and then moved to -80°C and stored there until planned use. The immunohistochemical analysis of these cryopreserved samples is part of the follow-up diploma project.

3.7.2.2 T cells analysis by flow cytometry

Cells analysed by flow cytometry were obtained from spleen and lymph node tissues. Antibodies with corresponding fluorophores were used according to the Tab. 10. Based on the preliminary experiment, CD183 and CCR7 antibodies performed the best in dilution of 1:200 for CCR7 and at 1:150 for CD183. Therefore, these dilutions were used in further experiments. Other antibodies were used based on the manufacturer recommendations.

Tab. 10: Panels for detection of memory and Th cells populations. *denotes the concentrations based on the preliminary experiments. Other concentrations were used based on the manufacturer recommendations.

Antibody	Fluorophore	Concentration	Producer	Catalogue number	Isotype
CD3	APC- Cy7	1:100	Biolegend	100222	Rat IgG2b, κ
CD4	FITC	1:10	Biolegend	100406	Rat IgG2b, κ
CD8b	APC	1:100	Biolegend	126614	Rat IgG2b, κ
CCR7	PE	1:200*	Biolegend	120106	Rat IgG2b, κ
CD62l	PerCP-Cy5.5	1:100	Biolegend	104432	Rat IgG2b, κ
CD169	PerCP-Cy5.5	1:100	Biolegend	129809	Rat IgG2b, κ
CD183	PE	1:150*	Biolegend	126505	Rat IgG2b, κ
CD16/32	-	1:100	Invitrogen	14-0161-82	

Tissues were processed similarly to experiment no. 1, with following modification. Spleen and lymph nodes were homogenized in small vials using the plastic microhomogenizators. Acquired homogenates were filtered through sterile micro-strainer with size of pores 70 um (Greiner bio-one) into 50 ml tubes, and strainers were washed with 1% FBS/PBS up to 25 ml. All samples were then centrifuged at 350g, 4°C (Heraus Megafuge 8R) for 10 minutes and supernatant was discharged to disinfection. Sediment was resuspended in 25 ml of 1% FBS/PBS and centrifuged again at 350g, 4°C (Heraus Megafuge 8R) for 5 minutes. Supernatant was discharged into disinfection and 1 ml of erythrocyte lysis buffer was added to each spleen sample, carefully resuspended, and let incubated for 5 minutes with occasional gentle mixing. Successful disruption of erythrocyte was indicated by reddish supernatant visible after 5 minutes of incubation. Prior to following centrifugation, the erythrocyte lysis buffer in samples was diluted by 15 ml of 1% FBS/PBS, which slowed down the reaction and prevented disruption of white blood cells. Samples were centrifuged at 350g, 4°C (Heraus Megafuge 8R) for 5 minutes. Supernatant was discharged into disinfection and spleen homogenate was resuspended in 2 ml of 1% FBS/PBS.

Samples from lymph nodes were not incubated with erythrocyte lysis buffer and the second washing with 1% FBS/PBS was directly followed by discharging the supernatant and resuspendation in final volume of 500 µl of 1% FBS/PBS. All samples were kept on ice during the whole process. Part of the homogenate from spleen and lymph nodes samples was taken as blank controls.

3.7.2.3 Staining of cells with specific T cell markers

Lymph nodes and spleen cells were washed and prepared for staining with fluorescent markers as described above. Two panels of marker´s combinations were prepared to examine memory T cells and Th1/Th2/Th17 subsets of T cells. Chosen markers and corresponding fluorescent labels are listed in Tab. 10. Blocking antibody CD 16/32 was added to each sample in final concentration 1:100 to prevent non-specific binding of Fc parts of antibody markers and incubated at 4°C for 15 minutes.

Meantime, mixture of antibodies for both panels – Th1/Th2/Th17 and memory T cells - was prepared. Specific antibodies were diluted in 1% FBS/PBS according to Tab. 10 in final volume of 100 µl per sample.

Fluorescent minus one (FMO) and single stained (SS) controls were prepared according to the Tab. 11. All the markers were added with exception of Hoechst (Invitrogen, 33258) that was added to the samples 10 minutes before measuring in concentration 1ug/ml. Samples with antibodies were incubated in dark at 4°C for 45 minutes.

Hoechst dye to distinguish dead cells was added 10 minutes before measurement and samples were kept in the dark in 4°C. The measurement was done on CytoFLEX (405/488/561/638, Beckman Coulter). Each sample was shortly vortexed and measured in test tubes. Compensation matrix was counted during the first analysis using the SS controls and applied to all replicates (2A, 2B, 2C). Samples were measured with 50 000 events as the limiting factor.

Obtained data were analysed by FlowJo (version 10.4.2). The compensation matrix was recalculated according to the data from single stained controls. Cells populations were distinguished based on the FMO and SS control analysis and samples were analysed with respect of these parameters. Obtained data were counted as the ratio to all measured events (in %). Since some samples had low number of cells, the data were first transformed into the ratio to all events before subjected to the statistical analysis.

Tab. 11: FMO and SS controls for flow cytometry in experiment no. 2:

FMO controls	Antibody mixture	Single stained controls	Antibodies
Memory T cells			
FMO CD3-	CD4, CD8b, CD197, CD62L	SS CD3+	CD3
FMO CD4-	CD3, CD8b, CD197, CD62L	SS CD4+	CD4
FMO CD8b-	CD3, CD4, CD197, CD62L	SS CD8b+	CD8
FMO CD197-	CD3, CD4, CD8b, CD62L	SS CD197+	CD197
FMO CD62L-	CD3, CD4, CD8b, CD197	SS CD62L+	CD62L
Th cell populations			
FMO CD196	CD3, CD4, CD8b, CD183	SS CD196+	CD196
FMO CD183	CD3, CD4, CD8, CD196	SS CD183+	CD183

3.7.2.4 Gating strategy

Within this experiment, we measured memory T cells and Th1/Th2/Th17 subpopulations based on the markers summarized in Tab. 12. The analysis revealed that the dilution of antibodies against CD197 (memory T cell marker) and CD196 (T cell subpopulations marker) need further optimalization and therefore these markers were excluded from the analysis. Flow cytometry was performed with cells from

spleen and draining lymph node from each mouse as described earlier. Gating strategy is illustrated in fig. 8.

Tab. 12: Analysed T cells populations; only populations used in result analysis are noted

T cell populations	Surface markers
Lymphocytes	FSC/SSC
T cells	CD3+
Helper T cells	$CD3^+CD4^+$
Cytotoxic T cells	$CD3^+CD8^+$
Th1 cells	$CD3^+CD4^+CD183^+$
Naïve T cells + central memory T cells	$CD3^+CD4^+CD62L^+$

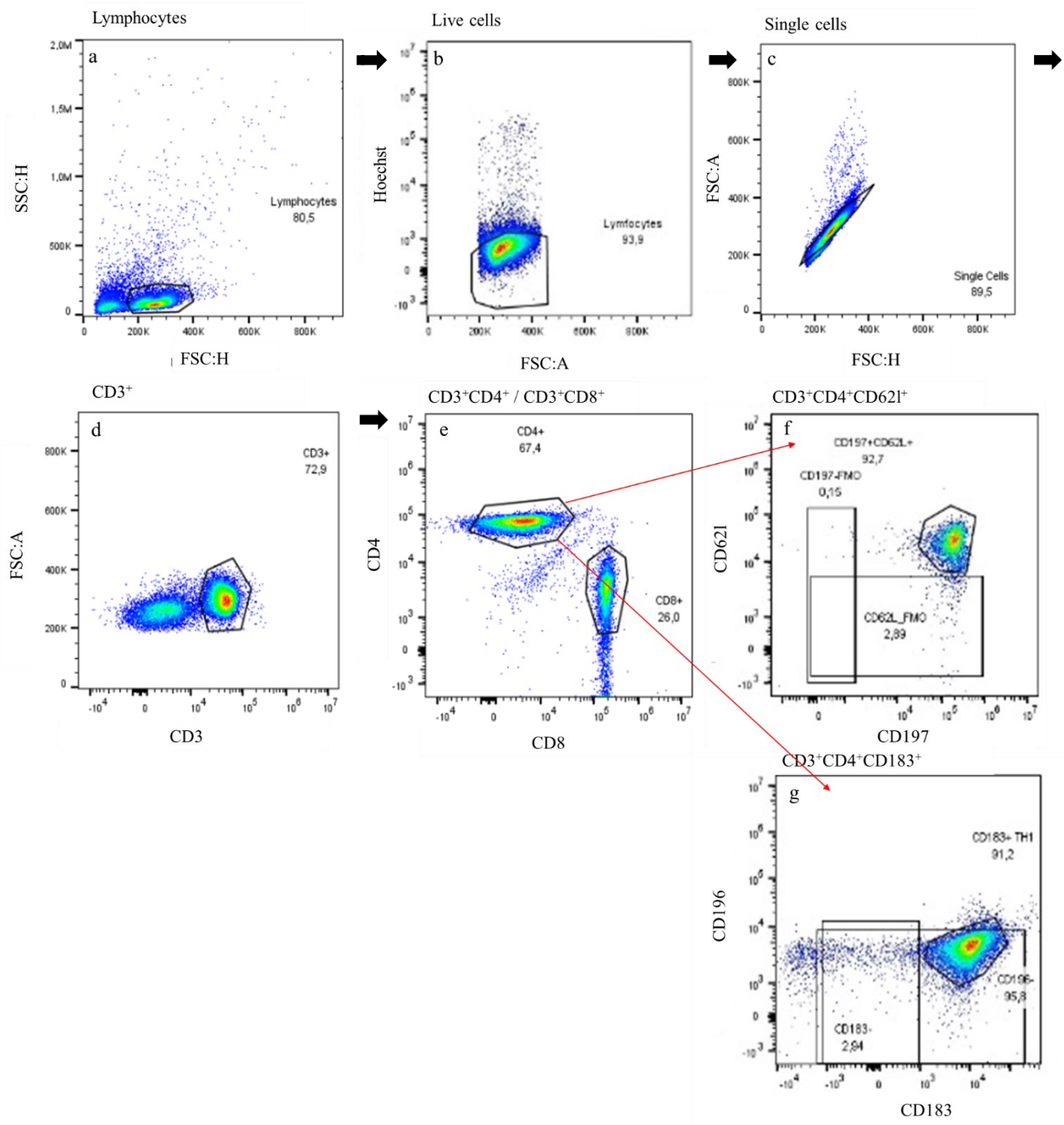


Fig. 8: Gating strategy of memory T cells and T cells populations: a) lymphocytes gated out according to their size (FSC) and granularity (SSC); b) exclusion of dead cells by DNA dye Hoechst; c) single cells gating - exclusion of cell clusters d) detection of CD3+ lymphocytes; e) division of CD3+ lymphocytes into CD3+CD4+ and CD3+CD8+ T cell subpopulations; f) gating of memory CD62l markers from CD4+ population; g) gating of CD4+CD183+ Th1 markers from CD4+ population; gating was used for all the samples from one measurement in the same way.

3.8 *Leishmania* quantification

For the purposes of *Leishmania* quantification, DNA was isolated from the tissue samples (one ear pinna and its draining lymph node from each mouse) using High Pure PCR Template Preparation Kit (Roche, cat. No. 11796828001) according to the manufactured protocol with minor modifications.

Briefly, tissue samples were stored in empty vial at -20°C. Before DNA isolation the ears and lymph nodes were homogenized to ensure proper isolation. Ears were cut into small pieces, put into homogenization tube with homogenization ball and 200 µl of lysing buffer and processed by tissue homogenizator (QIAGEN, TissueLyser LT) for 5 minutes, 50s. Each sample was then shortly centrifuged (Eppendorf 5424, 13 000g) and 50 µl of homogenates (1/4 of the sample) from each sample were taken to a new vial. Lysis buffer in volume 200 µl and 40 µl of Proteinase K were added to each sample, including tubes with lymph nodes, which were not homogenized prior to lysis buffer addition. Samples were shortly vortexed and incubated 1 hour at 55°C. Complete digestion of tissue was ensured by using clean plastic microhomogenisators for lymph nodes samples. After incubation, 200 µl of binding buffer was added to each sample, vortexed and incubated for 10 minutes at 70°C. Incubation was followed by adding 100 µl of isopropanol (Lachner) to each sample and mixed well. Insoluble tissue particles were disposed from the soluble part of the DNA.

The soluble part of the digested homogenate was centrifuged through High Pure Filter for 1 minute at 8000g (Eppendorf 5424). The filtrate was discarded, and the filter was filled with 500 µl of Inhibitor Removal buffer and centrifuged with new collection tube. This process was repeated twice with 500 µl of Wash buffer and finished with centrifugation on full speed for 10 seconds, which removed the residual washing buffer. Elution of DNA from filter tube was done by 200 ul/sample of prewarmed elution buffer and centrifuged again for 1 minute at 8000 g. Obtained DNA was stored in 1.5 ml vials at -20°C for later analysis by qPCR.

3.8.1 Calibration curve of *Leishmania major* DNA for qPCR

To quantify *Leishmania* parasites in tissue samples, serial dilution of *L. major* DNA equivalent to 10^1 to 10^7 *Leishmania* promastigotes were used for the calibration curve. Cultivated *L. major* (LV561) prepared as described in chapter 3.1.1 was diluted in physiological solution to concentration 10^8 *Leishmania* parasites/ml in physiological solution (0.85% NaCl). This suspension was then diluted in decimal series, achieving 10^1 – 10^7 as number of parasites in 100 µl of solution. Before the DNA isolation, samples were mixed with ear tissue of non-infected mouse. DNA was obtained using the Isolation of Nucleic Acids from Mammalian Tissue Sample Lysis and DNA Binding Protocol (High Pure PCR Template Preparation Kit; Roche, 11796828001) as described above.For the analysis of results from qPCR, values from NEG group were used as a cut off in both experiments.

3.8.2 Quantitative polymerase chain reaction (qPCR)

The quantification was performed based on the 18S DNA. Isolated DNA samples were defrosted, shortly vortexed and centrifuged 10 seconds at maximum speed (Centrifuge 5424R) before use. The master-mix contained fluorescent dye SYBR Green (SsOAdvancedTM Universal SYBR®, Bio-Rad, 172-5270), PCR H_2O (Takara), and reverse and forward primers (Invitrogen) as presented in Tab. 13.

Tab. 13: qPCR reaction reagents

Reagents:	Volume per 1 sample [µl]:
SYBR Green	10
PCR H2O	9
Forward primer 18S rDNA 5´-AGATTATGGAGCTGTGCGACAA-3´	0,5 ≙ 5 pmol
Reverse primer 18S rDNA 5´-TAGTTCGTCTTGGTGCGGTC-3´	0,5 ≙ 5 pmol
Sample DNA	1

Samples were analysed in duplicates in Thermocycler Biorad iQ5. The qPCR was run under the following conditions: 95°C for 3 minutes, 35 cycles at 95°C for 20 seconds each, annealing at 53°C for 30 seconds, extension at 72°C for 40 seconds and the end was at 72°C.

3.9 Detection of antibody response

The Enzyme-linked Immunosorbent assay (ELISA) was used for the purposes of detection antigen-specific antibodies from the serum of experimental mice.

3.9.1 Experiment no. 1

Salivary gland homogenate of *P. duboscqi* (preparation in chapter 3.2.1) and corpuscular antigen of *L. major* LV561 (preparation in chapter 3.1.1.) were used as antigens to coat the 96-well plate (Fischer Scientific, 478042). For coating, *P. duboscqi* SGH was diluted in coating buffer to final concentration 40 ng per well (100 ul) and the corpuscular antigen of *L. major* was diluted into final concentration of 10^6 promastigotes per well (100 ul).

Plates were incubated overnight at 4°C. Following day, plates were washed twice with PBS-TWEEN (100 µl per well/washing) and the non-specific binding was blocked by adding 100 µl of 6% milk in PBS-TWEEN per well, incubating for 1 hour at 37°C. Subsequently, plates were washed three times with PBS-TWEEN (100 µl per well/washing). Sera from experimental mice were diluted in 2% milk in PBS-TWEEN, 1:400 for anti – *L. major* antibodies detection and 1:200 for anti-*P. duboscqi* SGH antibodies detection, in a final volume 100 µl per well. Incubation was done at 37 °C for 1.5 hour with plates containing *L. major* antigens and for 1 hour with plates containing *P. duboscqi* antigens. Unbounded antibodies were washed out five times with PBS-TWEEN (100 µl per well/washing) and the secondary antibody IgG (Goat antimouse IgG, BIORAD, cat. no. 20391) was diluted 1:1000 with PBS-TWEEN and added into plates (100µl per well). Incubation was done for 45 minutes with both *L. major* and *P. duboscqi* antigen-coated plates at 37°C. Plates were then washed 6 times with PBS-TWEEN (100 µl per well/washing) followed by adding of 100 µl of substrate buffer to each well. Plates were incubated in dark (the substrate solution is light-sensitive) for 6 minutes. The reaction was stopped by adding 100 µl of 10% H_2SO_4 to each well. The absorbance (OD) was measured at 492 nm using the spectrophotometr Infinite M 200 (Tecan).

3.9.2 Experiment no. 2

Detection of anti-*L. major* and anti-sand fly saliva specific IgG antibodies was performed in a same way as in experiment no. 1, with additional test for anti-*S. schwetzi* specific IgG antibody response using the same concentration of SGH (40 ng/well).

3.10 Immunoblot

3.10.1 Experiment no.2

Immunoblot was used for the detection of putative cross-reactivity of anti-saliva specific IgG with antigens from SGH of other sand fly species. Serum samples used in the immunoblot were from hyperimmunized BALB/c mice used to keep the sand fly colony of indicated species. Mice were exposed to sand fly bites once a week, 14-times in case of *S. schwetzi* and at least 20-times in case of *P. duboscqi*. Fed females were not counted, but it is estimated that 50-100 females fed on mouse in each exposure. The sera were tested against SGH of *S. schwetzi* and *P. duboscqi*.

Sand fly salivary protein homogenates were separated by SDS-PAGE on a 0.75 mm 10% gel, under nonreducing conditions using the Mini-Protean III apparatus (BioRad). Five ug of salivary proteins in a volume of 39 ul was loaded in each lane (the equivalent of 26 glands of *S. schwetzi* and 14 glands of *P. duboscqi*) using a 5-well comb. Molecular weight was estimated based on the BenchMark unstained protein ladder (Invitrogen, cat. no. 10747-012) in a volume of 5 ul, run on the same gel as SGH of both sand fly species. Separated proteins were electro-transferred onto nitrocellulose membrane by iBlot device (Invitrogen) using the 7 min program. After transfer, the membrane was cut into strips and the free binding sites were blocked by 5% low fat dried milk (Blotting-grade blocker; BIO RAD; 170-6404) in Tris buffer with 0.05% Tween 20 (Tris-Tw) overnight at 4°C. The strips were then washed 3 times 5 min each with Tris-Tw and incubated with mouse sera diluted 1:100 in Tris-Tw for 1 hour at RT. Then, the strips were washed 3 times in Tris-Tw and incubated for 1 hour at RT with the peroxidase-conjugated Goat anti-mouse IgG (BIORAD, cat no. 20391) diluted 1:1000 in Tris-Tw. The strips were washed 2 times 5 minutes each in Tris-Tw, followed by the last washing in Tris for 5 min. The colour reaction was developed using substrate solution containing diaminobenzidine and H2O2, in dark, for 5 min at RT. To stop the reaction, the strips were placed in distilled water for 10 minutes at RT.

3.11 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (either 6.03 or 8.0.1 version). The difference was considered significant at $p < 0.05$.

In Experiment no. 1, three mice from the POS group 1B were excluded from the analysis due to the unsuccessful infection. Before the analysis, the data were transformed ($\ln(x+1)$) and subsequently analysed by nonparametric Mann-Whitney U Rank Sum test in case of comparison between groups (unpaired data) and by nonparametric Wilcoxon Signed Rank test in case of comparison within the group (paired data from serum samples). Correlation between measured parameters was analysed by nonparametric Spearman correlation test from the untransformed data. Correlation was determined as mild for $r < 0.6$ and strong for $r > 0.6$.

In Experiment no. 2, all mice were included into the analysis (2A, 2B, 2C), the data were transformed (ln(x+1)) and subjected to Multiple Comparison by Tukey Test in case of differences between groups (unpaired data) and to nonparametric Wilcoxon Signed Rank test in case of comparison within the group (paired data from serum samples). Additionally, 2A versus 2B+2C parameters were analysed for the EXP group using the Tukey test (this analysis was not possible to perform within group POS and NEG due to the small n). Spearman Correlation test was performed as described for Experiment no. 1 using the untransformed data from 2A, 2B, 2C.

4 RESULTS

4.1 EXPERIMENT No.1: The effect of timing between the last exposure to sand fly saliva and *Leishmania* infection

The aim of this part of the project was to investigate the outcome of the disease, in case when *Leishmania* parasites are inoculated into the environment of the skin altered by the last sand fly immunization at different time points after the last exposure. The laboratory immunization scheme in previous studies was followed by infection one-week after the last immunization, which was altered in our experiment, where the infection was done after the first 2 days, when the delayed type hypersensitivity reaction (DTH) should peak. As mentioned in the Introduction part (Chapter 2.1.4), DTH is the mechanism considered responsible for the formation of the protective effect when the ongoing immune response affects the survival of the parasite in the skin. The experiments were carried out on a *Leishmania* susceptible mouse strain – BALB/c.

Mice were divided into 4 groups: D2 (infection carried out 2 days postimmunization), D7 (mice infected 7 days after the last immunization), POS (mice infected without previous exposure to sand fly saliva) and NEG (unexposed – uninfected naïve mice). Mice were euthanized 5 weeks p.i. Analysis of disease outcome was performed by measuring the size of the lesion, quantification of *Leishmania* in the tissue of the ear and lymph nodes by qPCR, detection of antigen specific IgG antibodies by ELISA test, and cytometry analysis of the populations of T cells ($CD3^+$, $CD3^+$ $CD4^+$, $CD3^+CD8^+$).

4.1.1 *Leishmania* lesion size and its appearance

The size of the lesion was measured at week 5 after infection in all groups (D2, D7, POS, NEG). Three mice in the POS group did not develop any ear lesions at week 5 after infection and no *L. major* DNA was detected in both tissues by qPCR; thus, these mice were excluded from further statistical analysis.

Because of the covid restrictions, lesions size was not measured weekly as originally planned, but only at week 5 p.i. (W5). Lesion appearance at week 5 post infection is illustrated in Fig.9. Fig no.10 shows the lesion area for all experimental groups – D2, D7, and POS at week 5. Both pre-exposed (D2) and nonimmunized (POS) groups developed lesions significantly different from the NEG group ($p < 0.05$). Interestingly, the pre-exposed D7 group did not show significant difference in lesion size in comparison with NEG group ($p > 0.05$), however, as shown in the Fig. 10, some lesion development was measured. The overall trend in the graph is showing that mice pre-exposed to the sand fly saliva (D2 and D7) prior infection with *L. major* had lower lesion size, than nonimmunized control POS group. Differences between these infected groups were verified by statistical analysis, which revealed a significant difference only between D7 and POS group ($p = 0.029$). D2 group did not show any significant difference in lesion size from all other infected groups.

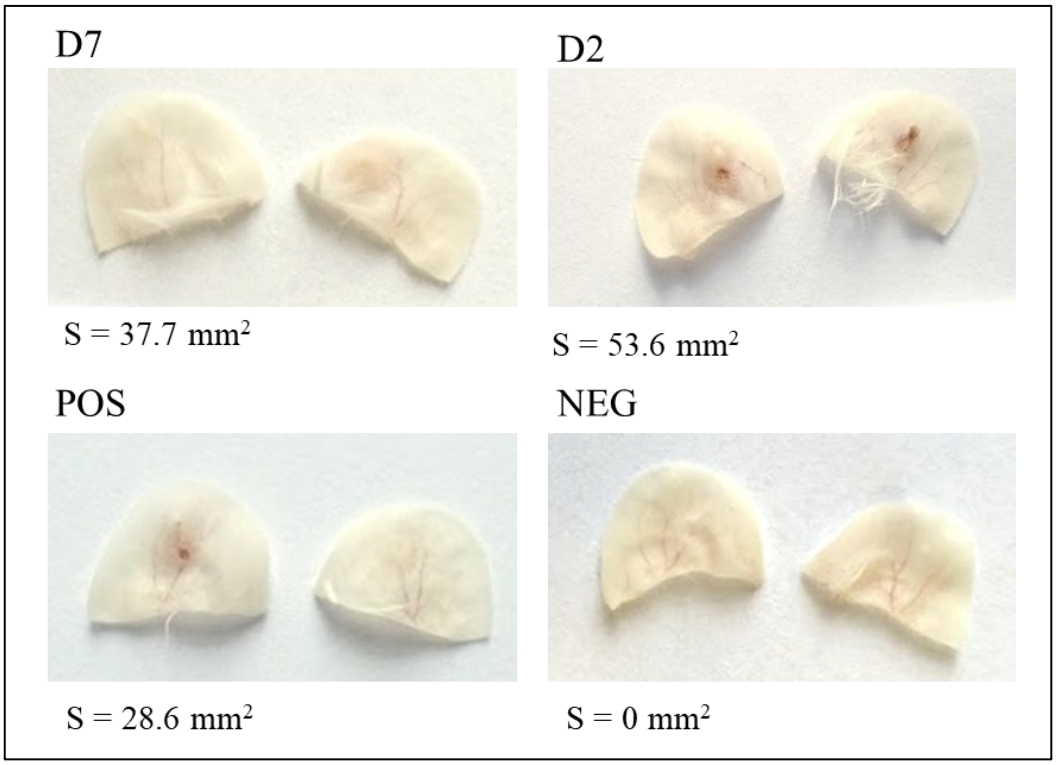


Fig. 9: Illustration of the lesion appearance at week 5 after the infection from representative mouse from each group. (D7) shows lesions of mice infected 7 days after the last immunization, (D2) represents lesions of mice infected 2 days after the last immunization, (POS) shows lesion of nonimmunized infected group (NEG) demonstrate ears of nonimmunized and noninfected negative control group. S denotes for the sum of lesion area of both ears.

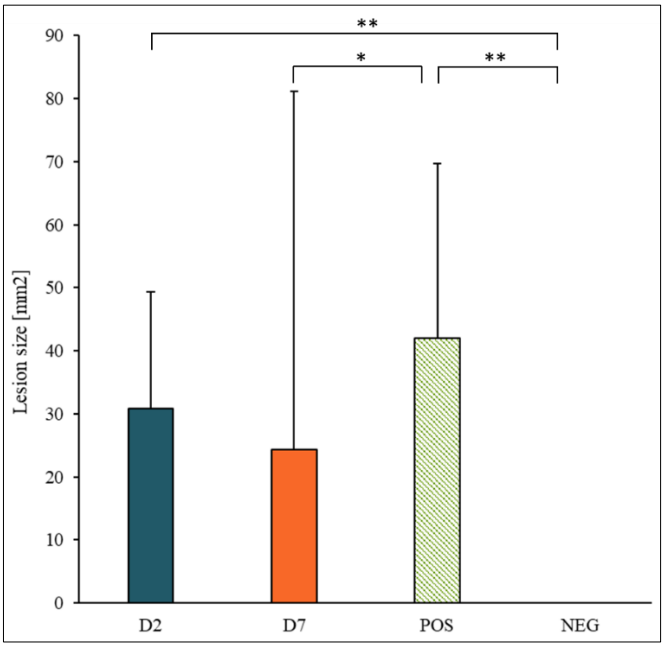


Fig. 10: *Leishmania* lesion size at week 5 p.i. The bars represent mean lesion size from the sum of both ears ± standard deviation (SD). * denotes significant difference at $p < 0.05$, ** $p < 0.01$

4.1.2 Quantification of *Leishmania major* in the ears and its draining lymph nodes

Leishmania infection was evaluated also by the amount of *L. major* in the infected tissue. Parasite load was measured in one ear pinna and the corresponding draining lymph node by qPCR using 18S ribosomal DNA gene. The results are summarized in Fig. 11A for the ear tissue and in Fig. 11B for the draining lymph node.

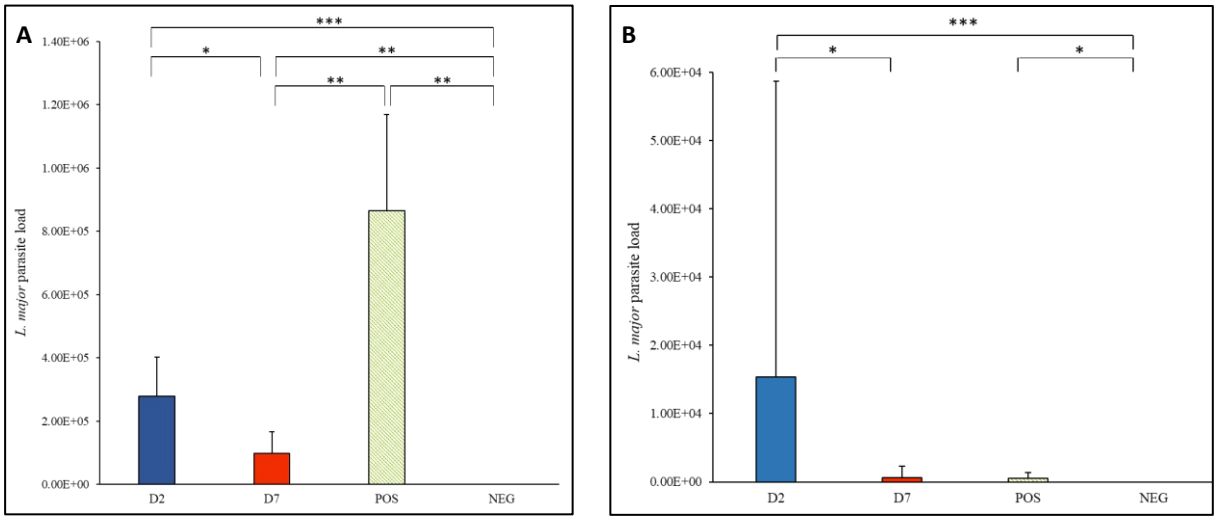


Fig. 11: Number of *L. major* detected by qPCR. Bar charts represent mean numbers of *L. major* per experimental groups per one ear (**11A**) and per one draining lymph node (**11B**) at week 5 p.i. ± SD. * denotes significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

As can be seen in the **Fig. 11A**, all infected groups – D2, D7 and POS did significantly differ in parasite load in the ear tissues from noninfected NEG control ($p < 0.05$). POS group had the highest number of *L. major* parasites compared to both preimmunized groups D2 and D7, reaching up to almost 10^6 parasites per the ear. This difference was proved to be significant from both naïve noninfected NEG control group ($p = 0.0001$) and from group D7 - infected one-week after the last immunization ($p = 0.008$). Additionally, D2 group showed also significantly higher parasite load from other experimental group infected 7 days after the last immunization – D7 ($p = 0.043$).

Draining lymph nodes (**Fig. 11B**) showed overall lower numbers of *L. major* in comparison with ears, when the highest amount of *L. major* was found in D2 group, coming up to mean of 2x10^4 of *L. major* per a lymph node. Similarly, to results from ear tissues, NEG group had significantly lower parasite load from POS ($p = 0.046$) and D2 group ($p < 0.0001$). D7 group did not significantly differ from NEG control, although similarly to data from ear tissues in previous paragraph, this group was significantly exceeded ($p = 0.027$) in *L. major* loads by the D2 group.

All three parameters – parasite load in the ear and lymph node tissues, and lesion size - were subjected to correlation analysis. The test showed mild correlation ($r = 0.605$, $p = 0.0002$) between lesion size and loads of *L. major* in the ear tissues as it is shown in the Fig. 12. Mild correlation was found also for lesion size and parasite load in the draining lymph node tissue ($r = 0.63$, $p < 0.001$) (Fig. 13). Mild correlation ($r = 0.6$, $p < 0.0001$) was found also between the parasite loads in the ear tissue and lymph node tissue.

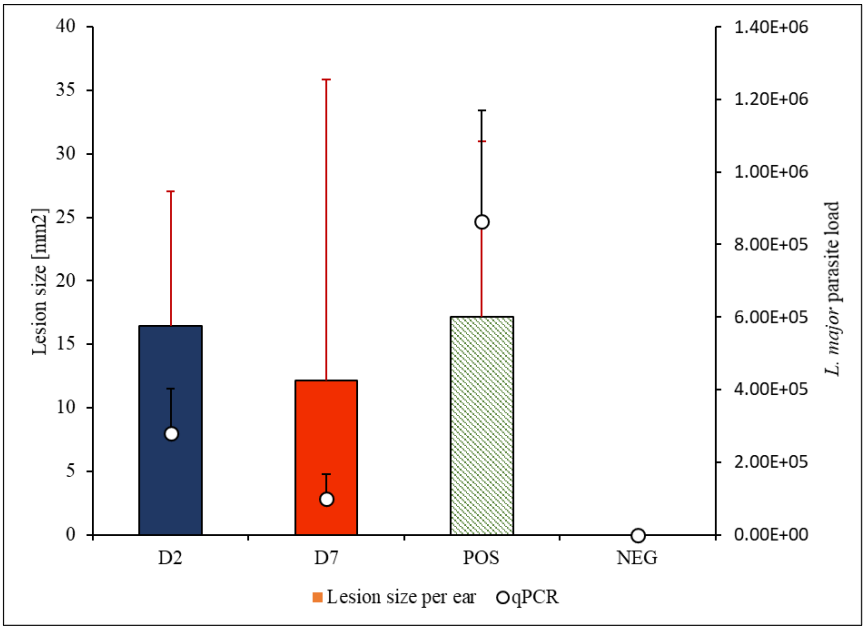


Fig. 12: The relationship between *L. major* load in the ear tissue and lesion size, both parameters from the same ear. Bar charts represents the mean lesion size ± SD, dots represent mean *L. major* load in the ear tissue ± SD.

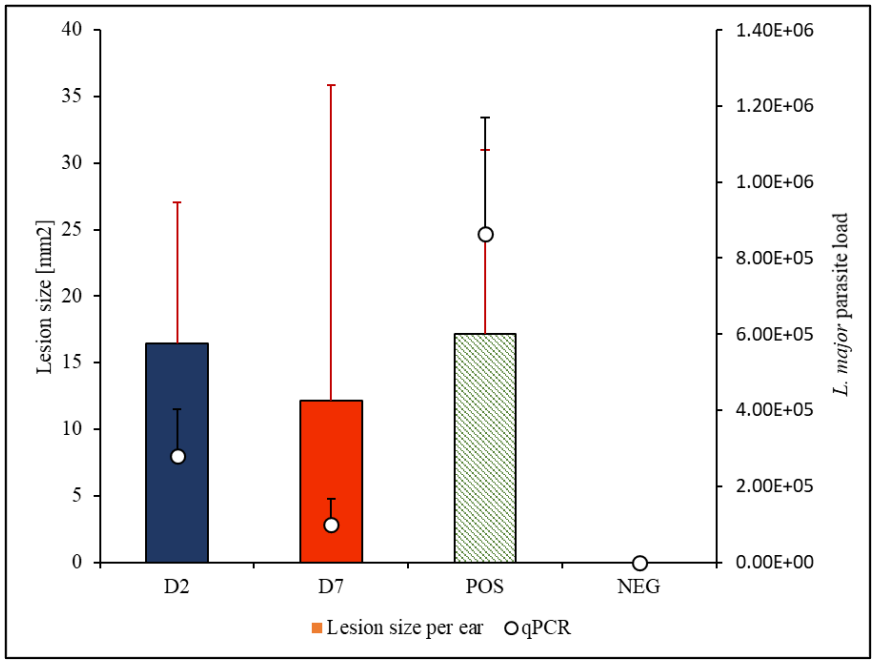


Fig. 13: The relationship between *L. major* load in the draining lymph node and lesion size, both parameters from the same ear. Bar charts represents the mean lesion size ± SD, dots represent mean *L. major* load in the draining lymph node ± SD.

4.1.3 Antibody response against salivary gland homogenate of *Phlebotomus duboscqi* and corpuscular antigen of *Leishmania major*

The antigen specific IgG antibody response was measured by ELISA with sera from mice obtained, after the immunization prior to infection (= preinfection) and after the infection (= postinfection). Tab.14 summarizes the mean number of blood fed *P. duboscqi* females per mouse for each immunization. Antigens used for the detection of specific antibodies were salivary gland homogenates of *P. duboscqi* and corpuscular antigen of *L. major*. Results are summarized in following graphs.

Tab. 14: The number of blood fed *P. duboscqi* sand flies per mice in each immunization. The data are expressed as mean ± SD. N.A. = not applicable

Mean number of blood fed sand flies – *P. duboscqi* per mice			
GROUP	**1. immunization**	**2. immunization**	**3. immunization**
D2	26 ± 3	15 ± 8	28 ± 7
D7	27 ± 2	28 ± 2	33 ± 2
POS	N.A.	N.A.	N.A.
NEG	N.A.	N.A.	N.A.

Fig. 14 shows the specific IgG antibody response against antigens from SGH of *P. duboscqi*. Preinfection levels at week 0 (W0) of anti-*P. duboscqi* antibodies of pre-exposed D2 and D7 group did not show significant difference from other nonimmunized POS and NEG groups ($p > 0.005$). At week 5 p.i. (W5), D2 and D7 group had significantly elevated levels ($p < 0.05$) of antibodies, in comparison to preinfection time point (W0) of the same group. Levels of anti – *P. duboscqi* antibodies of POS group which encounter SGH of *P. duboscqi* only once as a part of the infectious inoculum slightly increased from W0 to W5, however, this difference was not significant.

After 5 weeks of infection, pre-exposed D7 and D2 groups developed significantly higher antibody response to antigens from SGH of *P. duboscqi* compared to both nonimmunized and infected POS group and uninfected NEG control group ($p < 0.05$). The highest levels of anti – *P. duboscqi* antibodies were observed in D2 group (W5), where significant difference was measured also from group of mice infected one-week after the last immunization – D7 ($p = 0.008$).

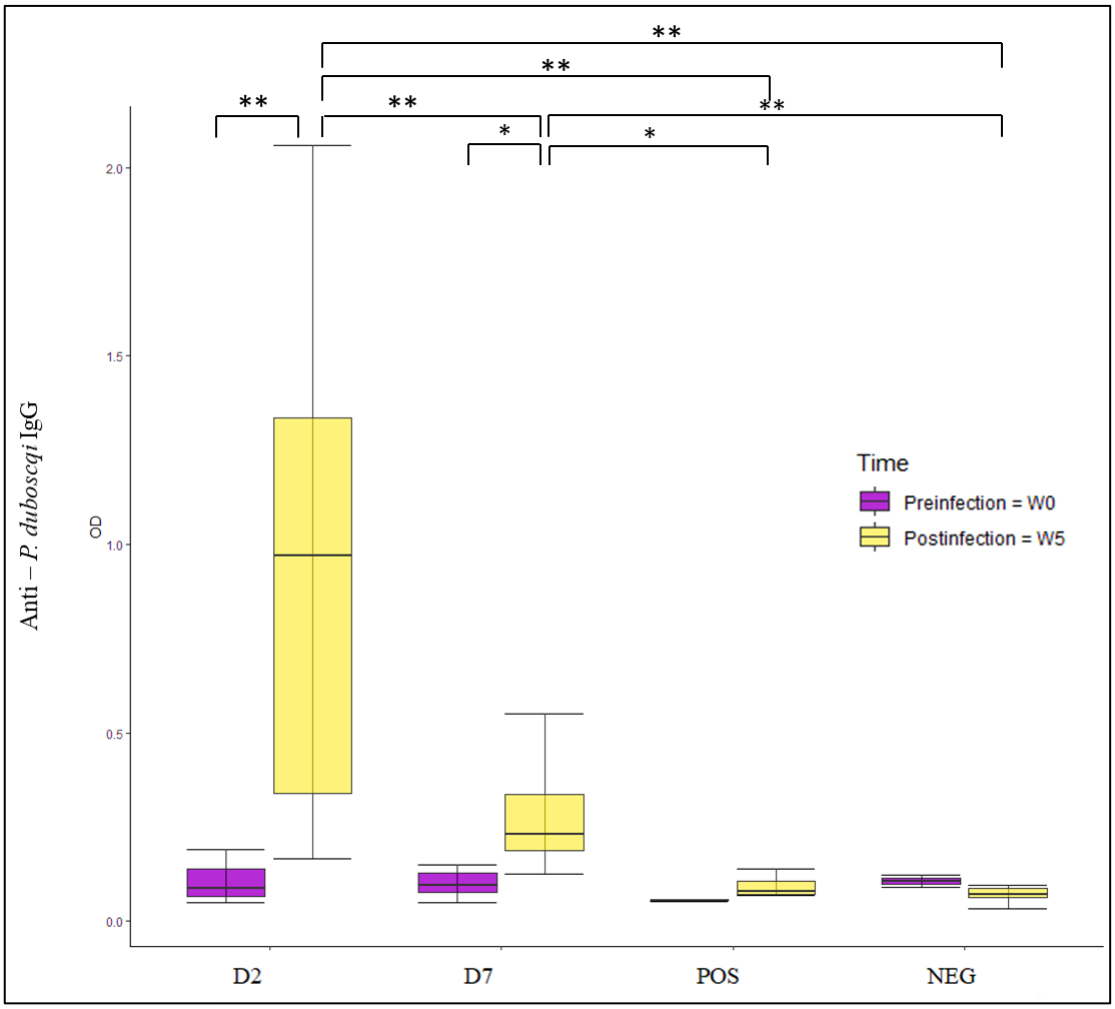


Fig. 14: Anti- *P. duboscqi* SGH IgG antibody levels after immunization and before infection = (W0) and post infection = (W5) according to the infectious group. * denotes significant difference $p < 0.05$, ** $p < 0.01$

Antibodies specific against corpuscular antigen of *L. major* also increased their levels during the 5 weeks of infection (**Fig. 15)**. There was no significant difference among groups at W0, which corresponds with the fact, that none of the groups was exposed to *L. major* antigens at that time point – at W0. The levels of anti – *L. major* IgG antibodies significantly elevated from W0 to W5 in pre-exposed D2 group ($p = 0.002$). Interestingly, no statistically significant increase in levels of specific antibodies was detected for control the POS group ($p > 0.05$).

Comparison of anti – *L. major* IgG antibodies at W5 among groups revealed significantly increased levels of antibodies for POS and D2 groups in contrast to noninfected control NEG group. D7 group did not significantly elevated levels of anti - *L. major* IgG during the 5 weeks of infection and showed significantly lower levels from D2 ($p = 0.037$) and POS ($p = 0.001$) group at W5. The overall highest levels of anti - *L. major* antibodies at W5 had the POS group, which significantly exceeded both D2 ($p = 0.008$) and D7 group ($p = 0.001$). Although there was only a smaller increase in anti-*L. major* IgG levels at W5 in contrast with control POS group, D2 group still showed significantly higher levels from the other pre-exposed group – D7 ($p = 0.037$).

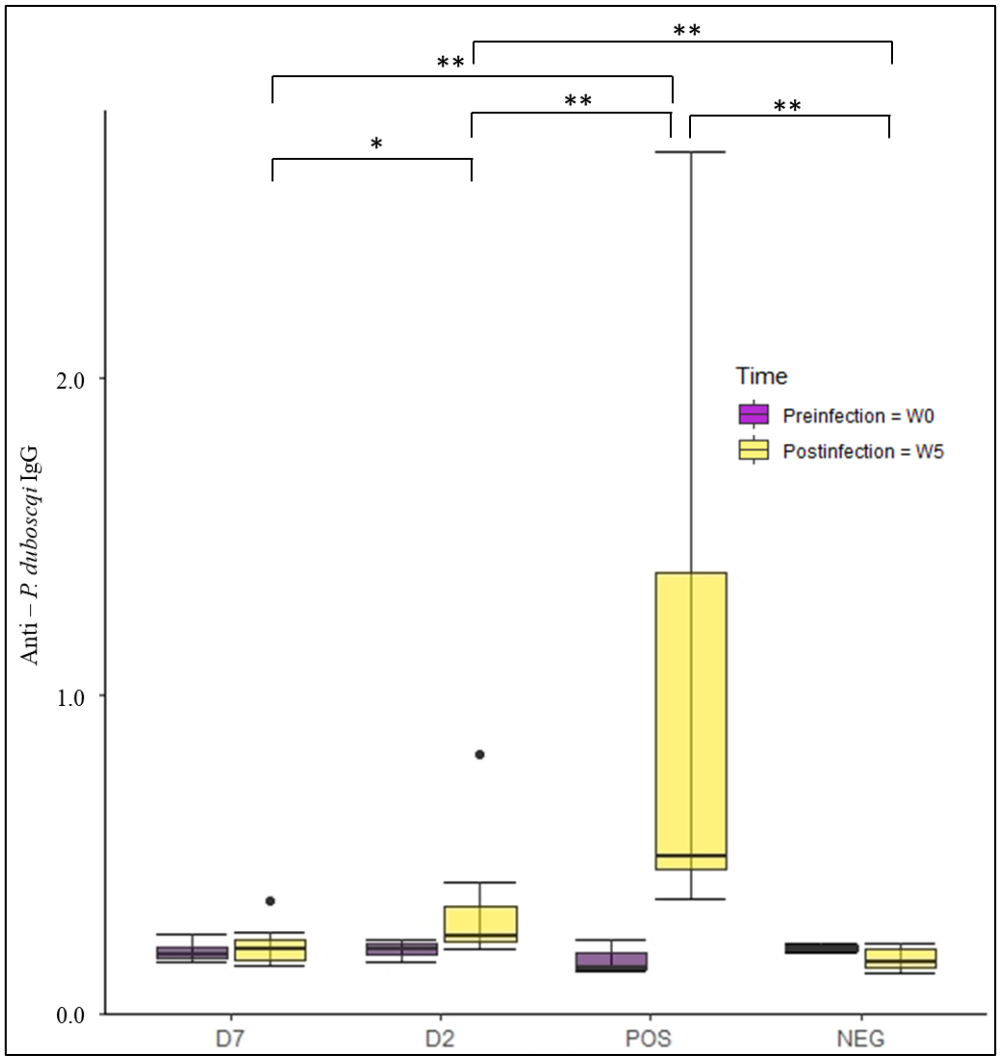


Fig. 15: Anti - *L. major* specific IgG antibody levels before infection after the immunization = W0 and at the end of the experiment = W5. * denotes significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

To investigate the relationships between levels of antigen-specific antibodies and other measured parameters, the correlation tests was performed. The strongest correlation was observed for anti-*L. major* with loads of *L. major* in the ear tissue ($r = 0.7$, $p < 0.0001$). Levels of anti-*L. major* IgG positively correlated also with parasite loads in the draining lymph nodes ($r = 0.42$, $p = 0.021$) and similar correlation was detected also with lesion size at week 5 p.i. ($r = 0.42$, $p = 0.016$). There was no significant correlation with levels of anti-*P. duboscqi* IgG ($p > 0.05$).

Anti- *P. duboscqi* IgG antibodies showed the strongest correlation, however still mild, with parasite loads in the draining lymph nodes ($r = 0.5$, $p = 0.007$). Low positive correlation was detected also in relation with *L. major* parasite loads in the ear tissues ($r = 0.36$, $p = 0.047$), similarly to lesion size at week 5 p.i. ($r = 0.38$, $p = 0.037$).

4.1.4 Cellular immune response analysed by flow cytometry

Adaptive immune response was analysed by flow cytometry based on selected T cell populations – $CD3^+$ T lymphocytes, $CD3^+CD4^+$ (T helper cells) and $CD3^+CD8^+$ (cytotoxic T cell) in the draining lymph nodes.

Results from the flow cytometry measurements are summarized in Fig. 16. Significantly higher ratio to all events had T cells population positive for $CD3^+$ of NEG group in comparison to pre-exposed infected D2 group ($p = 0.008$). Similarly, $CD3^+CD4^+$T cell population showed also significantly higher ratio to all events in NEG group from D2 group ($p = 0.013$). Other parameters did not differ significantly in between experimental groups ($p > 0.05$). All measured T cell populations followed similar trend in ratio to all cells per experimental group, regardless the lowest numbers detected for $CD3^+CD8^+$ T cells, exceeded approximately twice by $CD3^+CD4^+$ T cells numbers followed by overall highest rates for general population of $CD3^+$ T cells.

Correlation test revealed strong significant correlation between the ratio to all events of $CD3^+$ lymphocytes and parasite load of *L. major* in the lymph nodes ($r = 1$, $p < 0.0001$), followed by milder correlation with loads of *L. major* parasites in the ears ($r = 0.6$). In contrast with these results was correlation of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, where no significant correlation ($p > 0.05$) was detected for quantity of *L. major* parasites in both tissues.

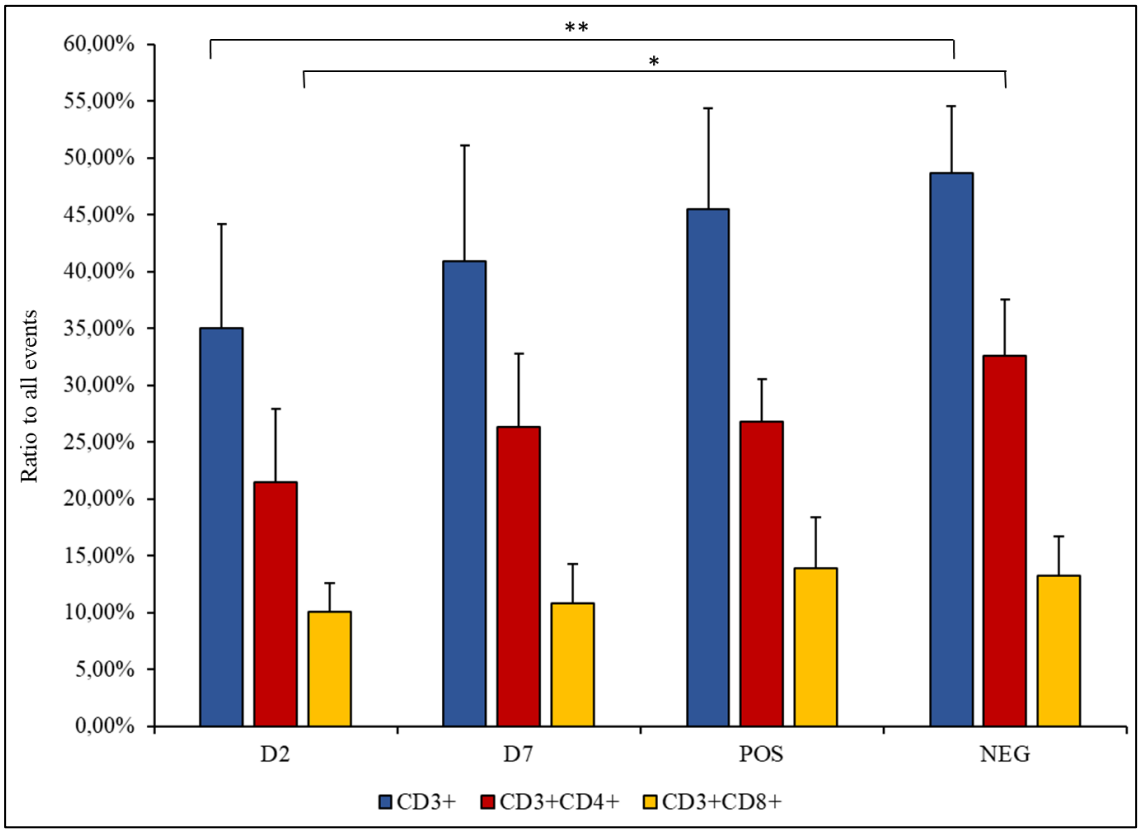


Fig. 16: Detected T cell subsets as mean of ratio to all events ± SD, including $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cell subsets * denotes significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.00$

4.2 EXPERIMENT no. 2: The effect of *Sergentomyia schwetzi* saliva on the course of the infection by *Leishmania major* inoculated with salivary gland homogenate of *Phlebotomus duboscqi*

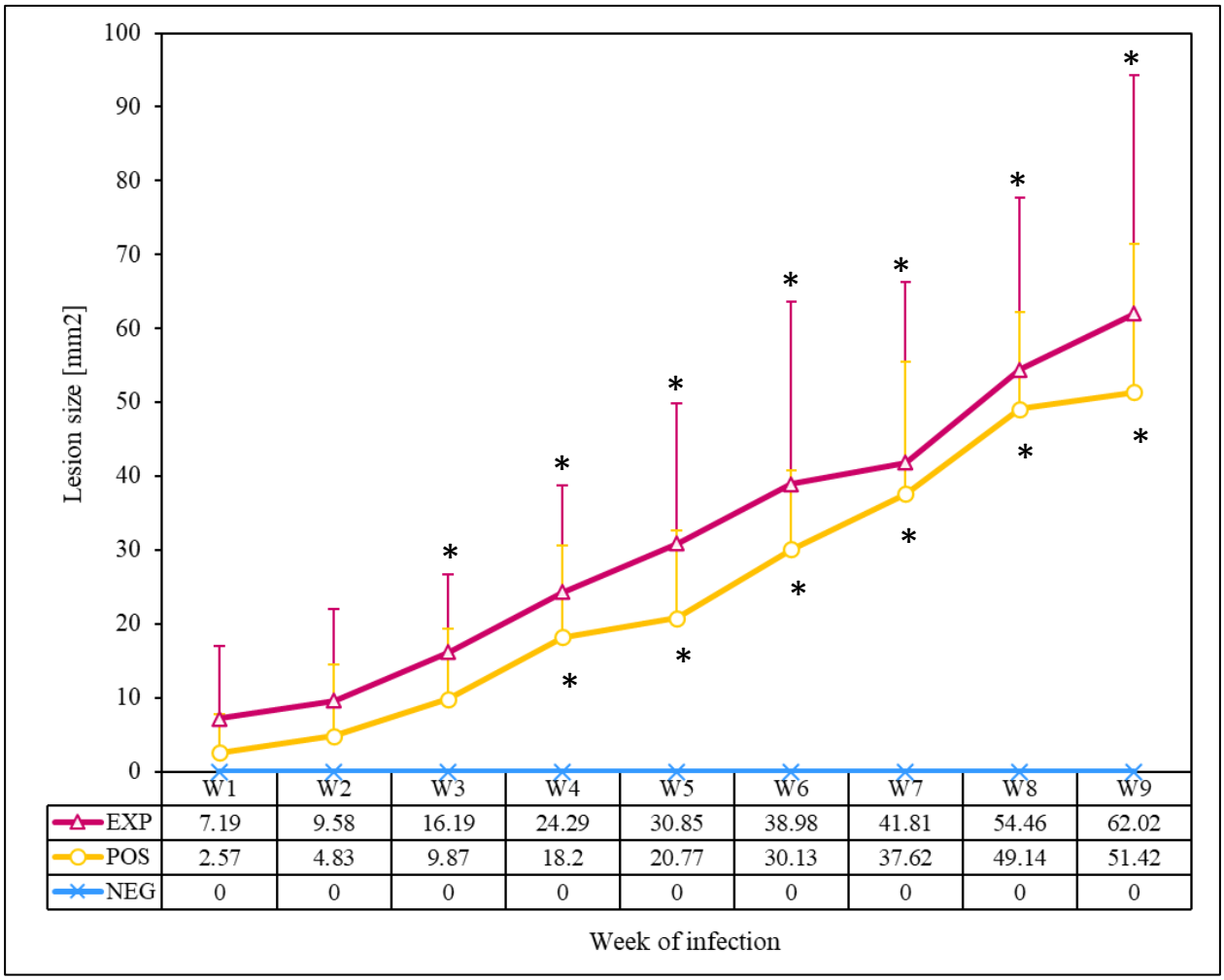
The aim of this part of the diploma project was to investigate the influence of pre-exposure to *S. schwetzi* saliva on the course of *L. major* infection inoculated along with saliva of its proven vector – *P. duboscqi*. The experiments were performed on BALB/c mouse strain according to the scheme described in detail in materials and methods (chapter 3.4.2). Results are presented by experimental group. Additionally, the independent experiments were compared as well, to reveal the effect of unintended different immunization scheme in replication 2A compared to replications 2B, 2C, as described in detail in materials and methods part (chapter 3.4.2).

Three different groups of BALB/c mice were used: EXP group consisted of mice pre-exposed to *S. schwetzi* bites followed by *L. major* + *P. duboscqi* SGH infection, POS mice represented non-exposed infected positive control, and in the NEG group, mice were non-exposed and uninfected. Effect of preexposure was evaluated by measurement of lesion area, *L. major* parasite load in two different tissues (ear pinna and its draining lymph node) by quantitative PCR, antigen-specific IgG antibodies (anti-*L. major*, anti-*P. duboscqi* and anti-*S. schwetzi*) by ELISA and the presence of selected immune cells populations in ear-draining lymph node by flow cytometry (markers for T cells, Th1/Th2, central memory T cells (TCM), and effector memory T cells (TEM).

4.2.1 *Leishmania* lesion size and its appearance

The ears of mice from all experimental groups – EXP, POS, and NEG were monitored weekly during the infection for the lesion's development, which was evaluated by lesion size and ulceration. Fig. 17 illustrates the development of the lesion over the period of 9 weeks.

Lesions in both infected groups (EXP, POS) started to occur from week one (W1) after the infection and grew gradually till the end of the experiment at week 9. The variability in lesion sizes for both infected groups were followed by changes in the phenotype of the lesions, manifested as red spots during the first two weeks and then changed from non-ulcerative to ulcerative, some of them showing a self-healing phenotype at the end of the infection (W9 – see fig. 18). The lesion size of EXP group was over all the largest from other two groups, with no significant difference compared to POS group ($p > 0.05$). POS group followed similar pattern in the lesion development as EXP group, being slightly bigger from fourth to ninth week p.i. The statistical analysis revealed significant difference between EXP and NEG groups from the third week onward ($p = 0.026$) and between POS and NEG groups from the fourth week onward ($p = 0.047$). There was no significant difference in lesion size between groups EXP and POS during the whole monitored period.



	W1	W2	W3	W4	W5	W6	W7	W8	W9
EXP	7.19	9.58	16.19	24.29	30.85	38.98	41.81	54.46	62.02
POS	2.57	4.83	9.87	18.2	20.77	30.13	37.62	49.14	51.42
NEG	0	0	0	0	0	0	0	0	0

Fig. 17: The development of the *L. major* lesions, counted as a sum of surface area of lesion from both ears. The graph shows mean ± SD * denotes significant difference at $p < 0.05$ of infected group compared to noninfected NEG control group.

Fig. 18 illustrates the lesion development in all groups at week 9 p.i. The figure shows the particular replication of the experiment – 2A and 2B, 2C. Ears from several mice from each group are presented, to demonstrate the variability even within the same replication.

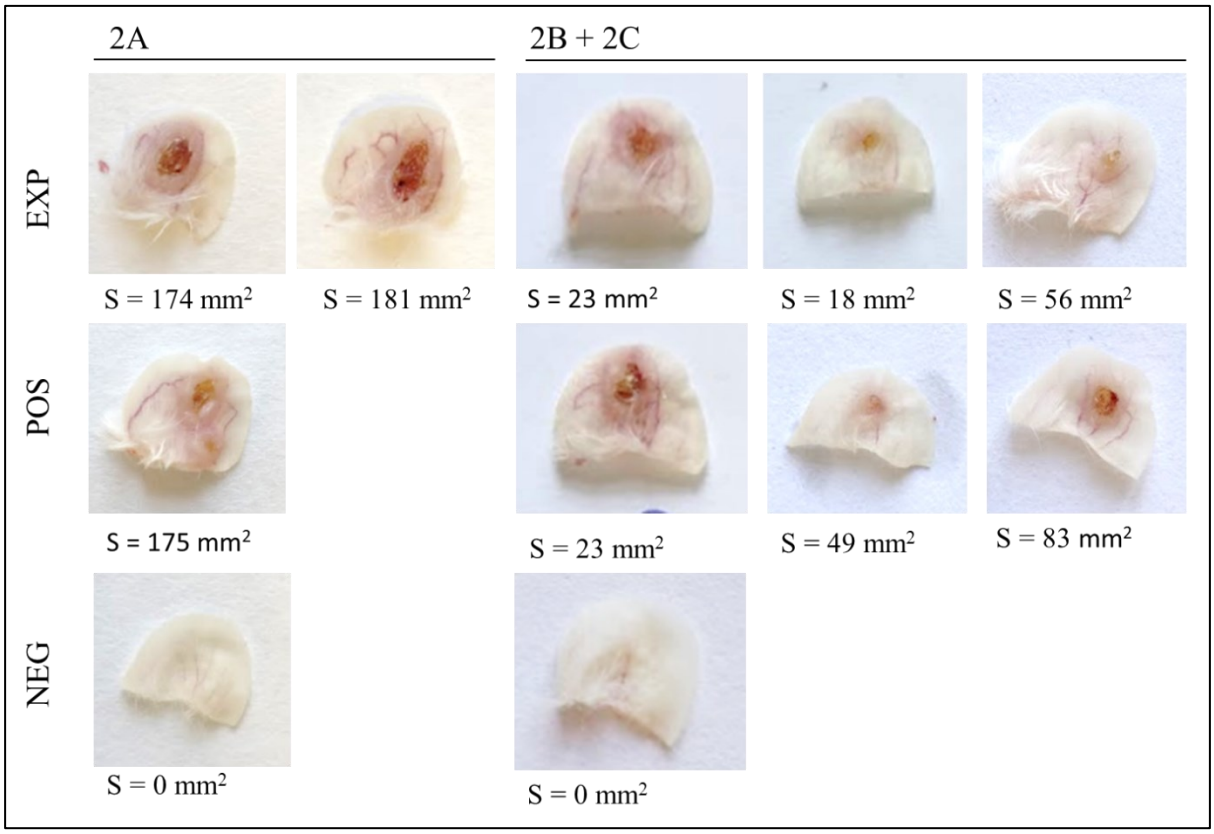


Fig. 18: Representative photos of the *Leishmania* lesions on the right ear pinnae at week 9 p.i. Photos 2A represents ears of mice from immunization scheme affected by covid restrictions, photos from 2B, 2C represent originally intended experimental design. Representative mouse ears from groups EXP, POS and NEG. S denotes for lesion size in mm^2.

The exposure of mice to sand fly saliva of *S. schwetzi* bites was different in EXP group between the three independent experiments 2A and 2B, 2C as mentioned in materials and methods. Taking this factor into account, we analysed the differences between groups separately in 2A and 2B, 2C. Data presented in the Fig.19 shows similar trends for all 3 groups (2A, 2B, 2C); the EXP and POS groups followed the same pattern in lesion size over the monitored period, with EXP group exceeding the lesion size of POS group in general. However, the lesion size of EXP group 2A was significantly larger than of EXP groups 2B, 2C from seventh week till the end of the experiment ($p < 0.05$). Thus, in further analysis we continue also with separated data of 2A vs. 2B, 2C.

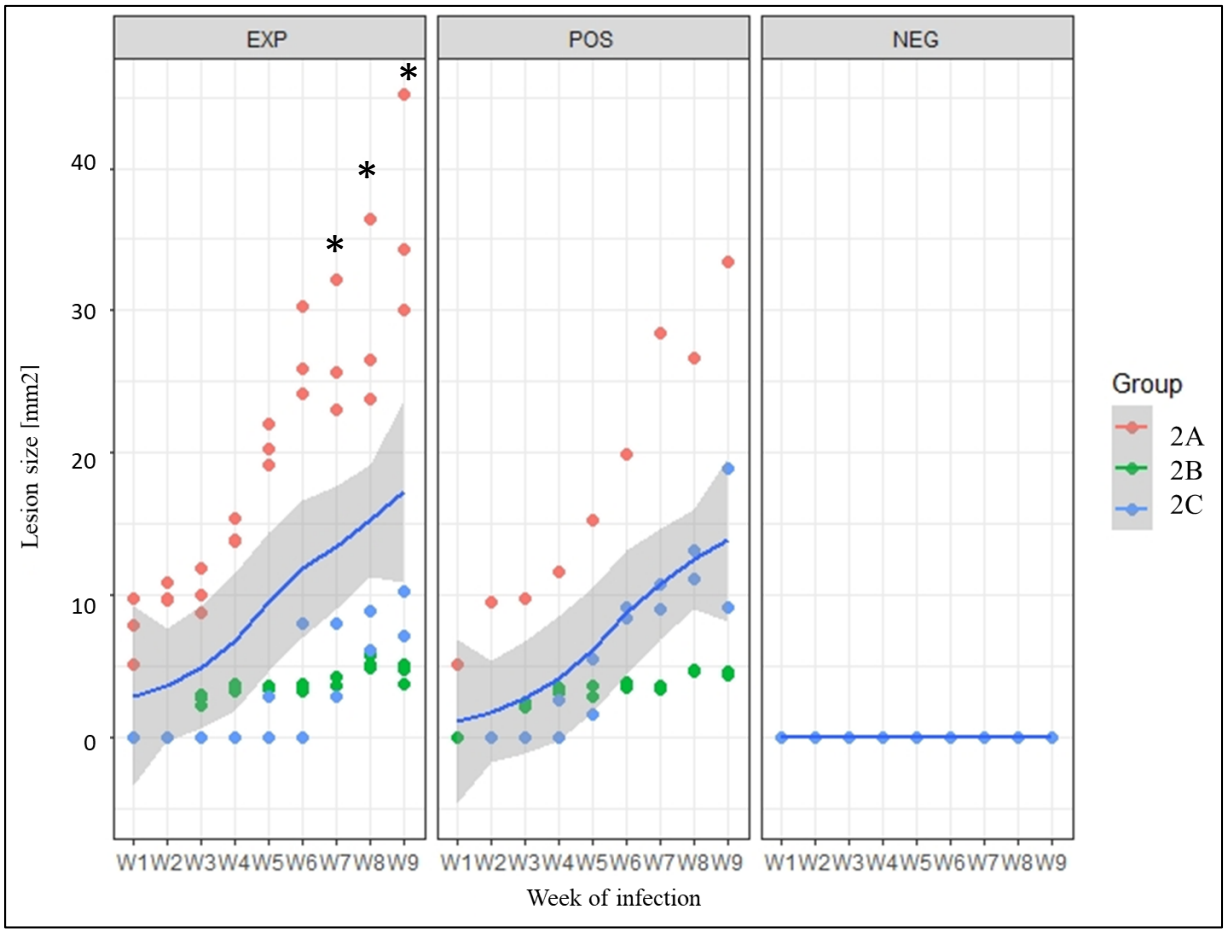


Fig. 19: Lesion size development during the 9-week period p.i. of infection. Blue line shows the means for all three independent replicates – 2A (full red circles), 2B (full green circles), 2C (full blue circles). * denotes significant difference between EXP vs. POS groups at $p < 0.05$.

There was a difference in numbers of mice having visible lesions during the first weeks p.i. across the group (Tab. 15). Lesion in mice from group 2A progressed the most rapidly, occurring already at week 1 (Fig. 19).

Tab. 15: Number of mice with visible lesions divided by total number of all experimental mice per group during the monitored period

	W1	W2	W3	W4	W5	W6	W7	W8	W9
EXP	3/8	3/8	6/8	6/8	7/8	7/8	8/8	8/8	8/8
POS	1/5	1/5	3/5	4/5	5/5	5/5	5/5	5/5	5/5
NEG	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

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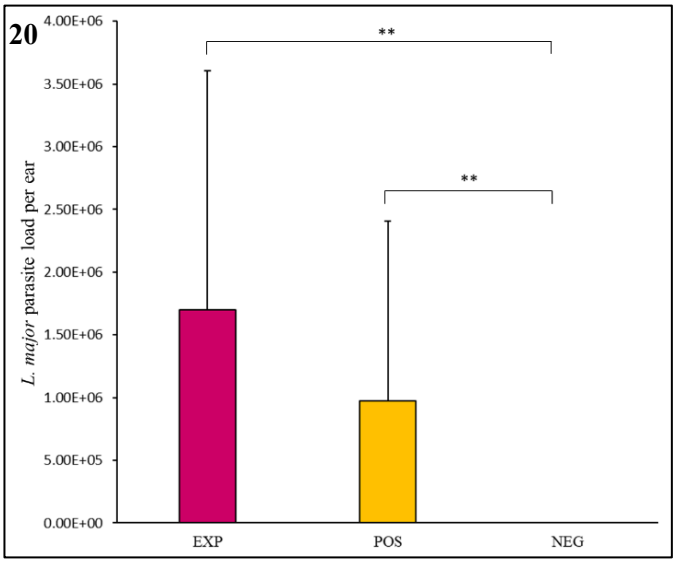
4.2.2 *Leishmania major* parasites quantification

In order to investigate the numbers of *L. major* in the ear and corresponding draining lymph node tissues, a quantitative analysis by qPCR was performed. *Leishmania* parasites were detected in ears and lymph nodes of mice from groups EXP and POS; no *Leishmania* was detected in the uninfected NEG group. The Fig. 20 and 21 show the parasite load in the right lymph node and right ear pinna, respectively, for all 3 independent replicates – 2A, 2B, 2C.



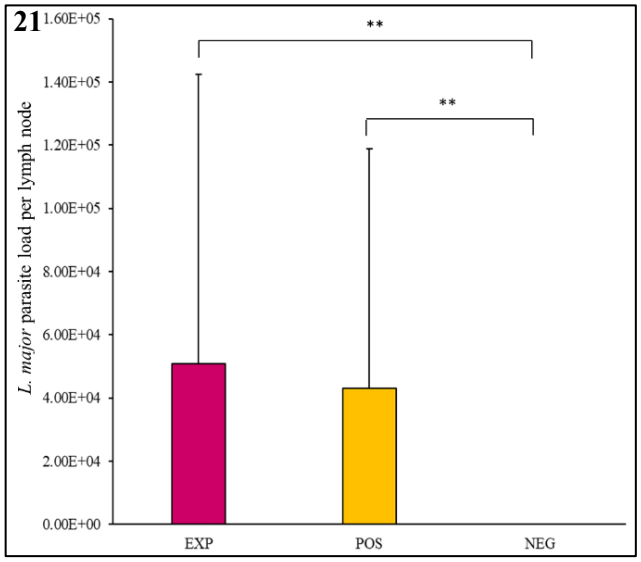


Fig. 20: Mean numbers of *L. major* parasites in right ear pinna. **Fig. 21:** Mean numbers of *L. major* parasites in right the lymph nodes ± SD. * denotes significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Although parasite load of EXP group exceeded POS group in both tissues - ears and lymph nodes, it was not significant ($p = 0.997$ and $p = 0.967$, respectively). Correlation analysis revealed strong relationship between the parasite load in ears with parasite load in the corresponding lymph node ($r = 0.808$, $p < 0.0001$).

Following Fig.22 and 23 highlight the variability between 2A and 2B, 2C groups, which was analysed due to the variable immunization scheme and significant difference in lesion size between these groups. Mice from group 2A had higher amounts of parasites located in ears for immunized group (Fig. 22) and in lymph nodes of positive control (Fig. 23), whereas most parasites in mice groups 2B, 2C were detected for POS group and EXP group in ears and lymph nodes respectively. Trends are opposite in group 2A in comparison to 2B, 2C for *L. major* parasite load in ears and lymph nodes, however these differences were not statistically significant.

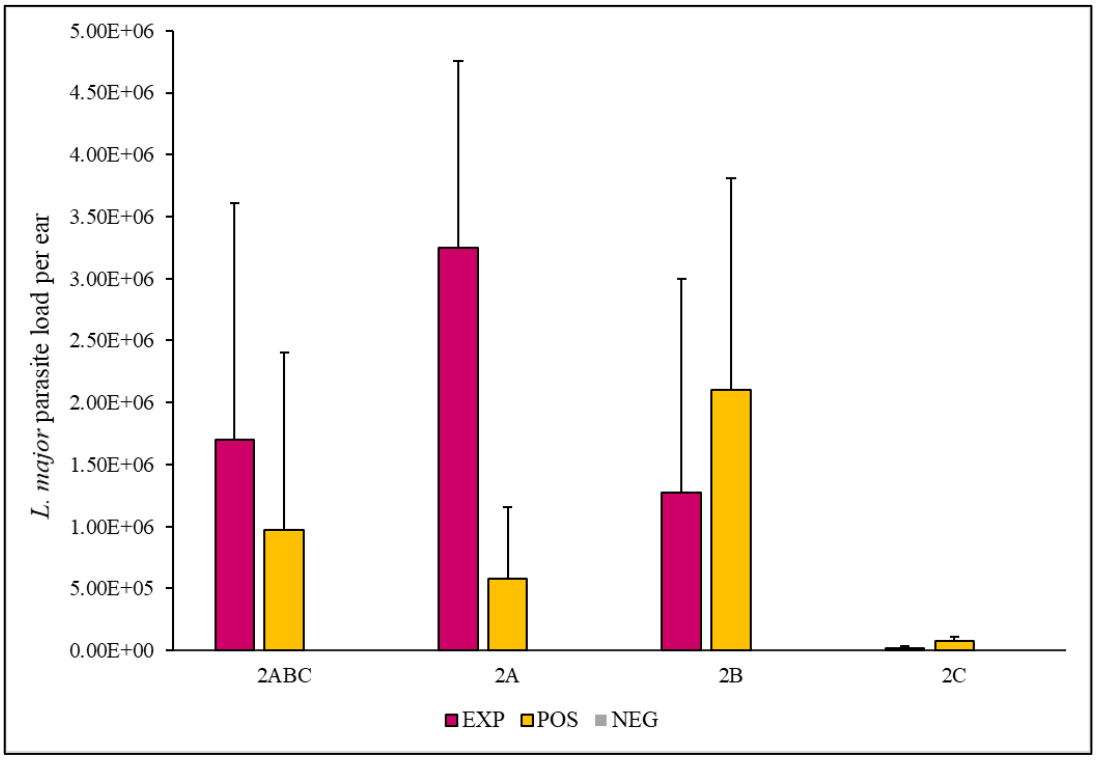


Fig. 22: Mean numbers of *L. major* parasites per ear ± SD according to the replication of experiment no.2. No significant differences were counted between replications.

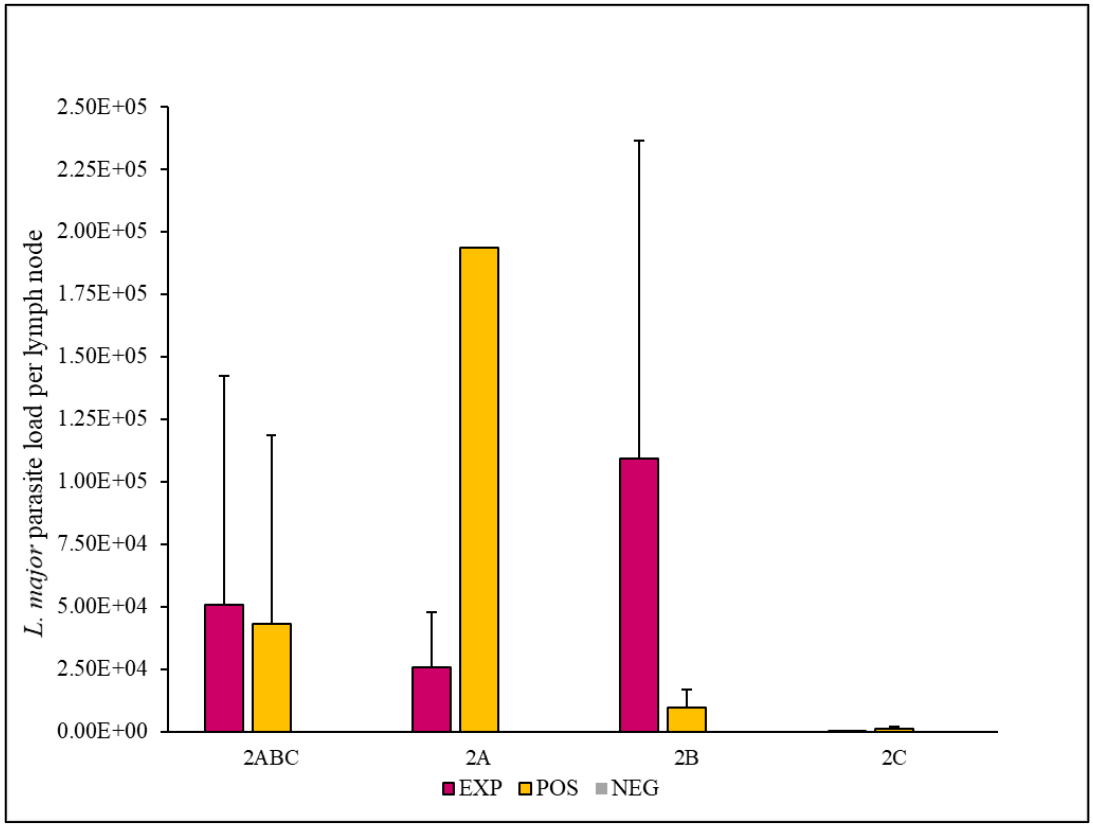


Fig. 23: Mean numbers of *L. major* parasites per lymph node ± SD according to the replication of experiment no.2. No significant differences were counted between replications

4.2.3 Comparison of parasite load with lesion size at 9 week post infection

The correlation test showed only mild significant correlation between the right ear lesion size and numbers of *L. major* in the right ear (r = 0.4, p = 0. 0011) as well as for numbers of *L. major* in right lymph nodes (r = 0. 28, p = 0.0041). Fig. 24 shows the lesion size together with corresponding numbers of *L. major* per ear and Fig. 25 shows the same data with numbers of *L. major* per the corresponding draining lymph node.

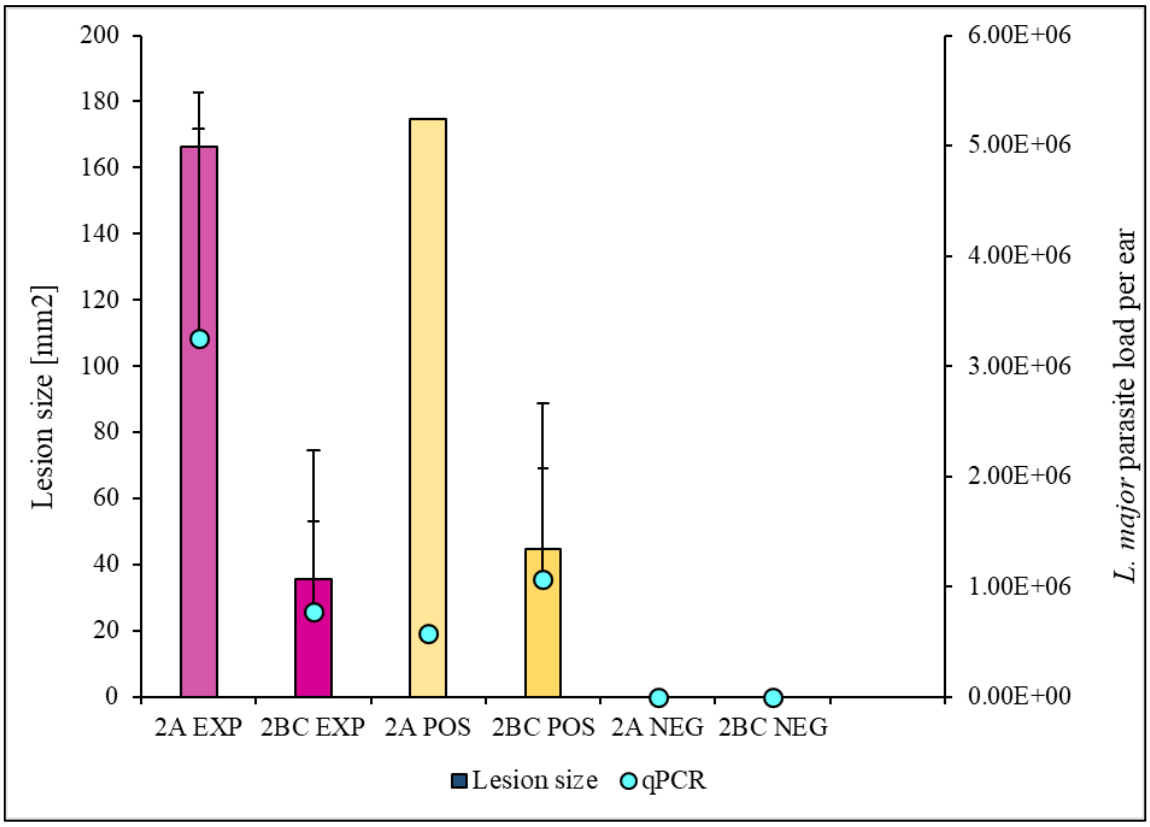


Fig. 24: Correlation of lesion size with numbers of *L. major* in corresponding ear (r = 0.703, p = 0. 0011). Bars represent mean lesion size ± SD, full circles represent mean parasite load ± SD in the indicated group.

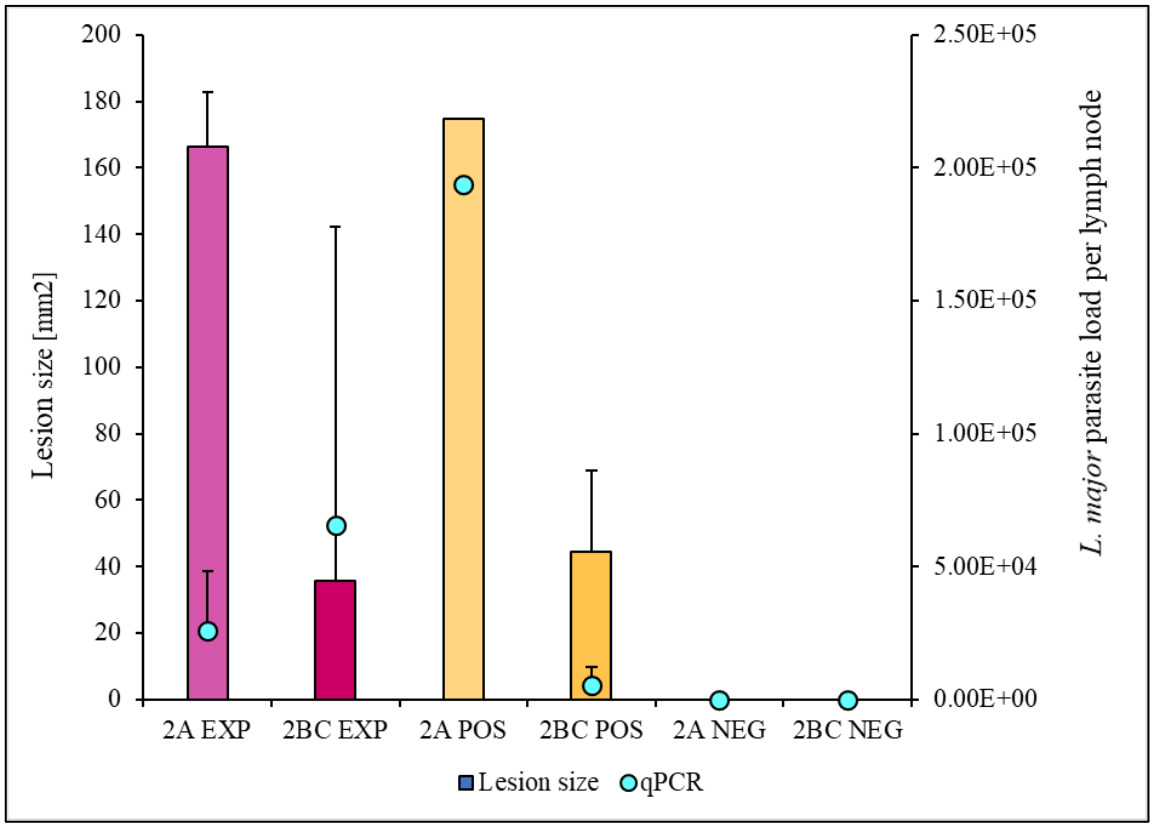


Fig. 25: Correlation of lesion size with numbers of *L. major* in draining lymph node (r = 0. 642, p = 0.0041). Bars represent mean lesion size ± SD, full circles represent mean parasite load ± SD in the indicated group.

4.2.4 Antigen specific antibody response against salivary gland homogenate of *Phlebotomus duboscqi* and *Sergentomyia schwetzi* and corpuscular antigen of *Leishmania major*

The detection of antibody response was performed by ELISA test with 3 different antigens – in particular SGH of *P. duboscqi* and *S. schwetzi* and corpuscular antigen of *L. major*. Antigen- specific IgG antibodies were measured at three different time points – before immunization in case of EXP group (preimmunization), after immunization and before infection with *L. major* (preinfection - W0) and at the end of the experiment – week 9 - post-infection (postinfection -W9). Mean numbers of blood fed *S. schwetzi* females per immunization are presented in Tab. 16.

Tab. 16: The number of blood fed *S. schwetzi* females during each immunization of mice from the EXP group. The data are expressed as mean ± SD. N.A. = not applicable

EXP GROUP	Mean number of blood fed - *S. schwetzi* females per mice				
	1. immunization	2. immunization	3. immunization	4. immunization	5. immunization
2A	26 ± 3	15 ± 8	8 ± 5	28 ± 7	17 ± 5
2B	27 ± 2	28 ± 2	33 ± 2	N.A.	N.A.
2C	28 ± 2	28 ± 2	28 ± 2	N.A.	N.A.

4.2.4.1 Antibody response against salivary gland homogenate of *Sergentomyia schwetzi*

Illustrated in the Fig. 26, anti-*S. schwetzi* IgG levels rose during the infection, the highest levels occurred in EXP group – 9 weeks post infection. EXP group developed significantly higher anti-*S. schwetzi* IgG from preinfection to postinfection time point, however, after 9 weeks of infection, anti-S. schwetzi IgG were not significantly elevated ($p > 0.05$) from POS and NEG group.

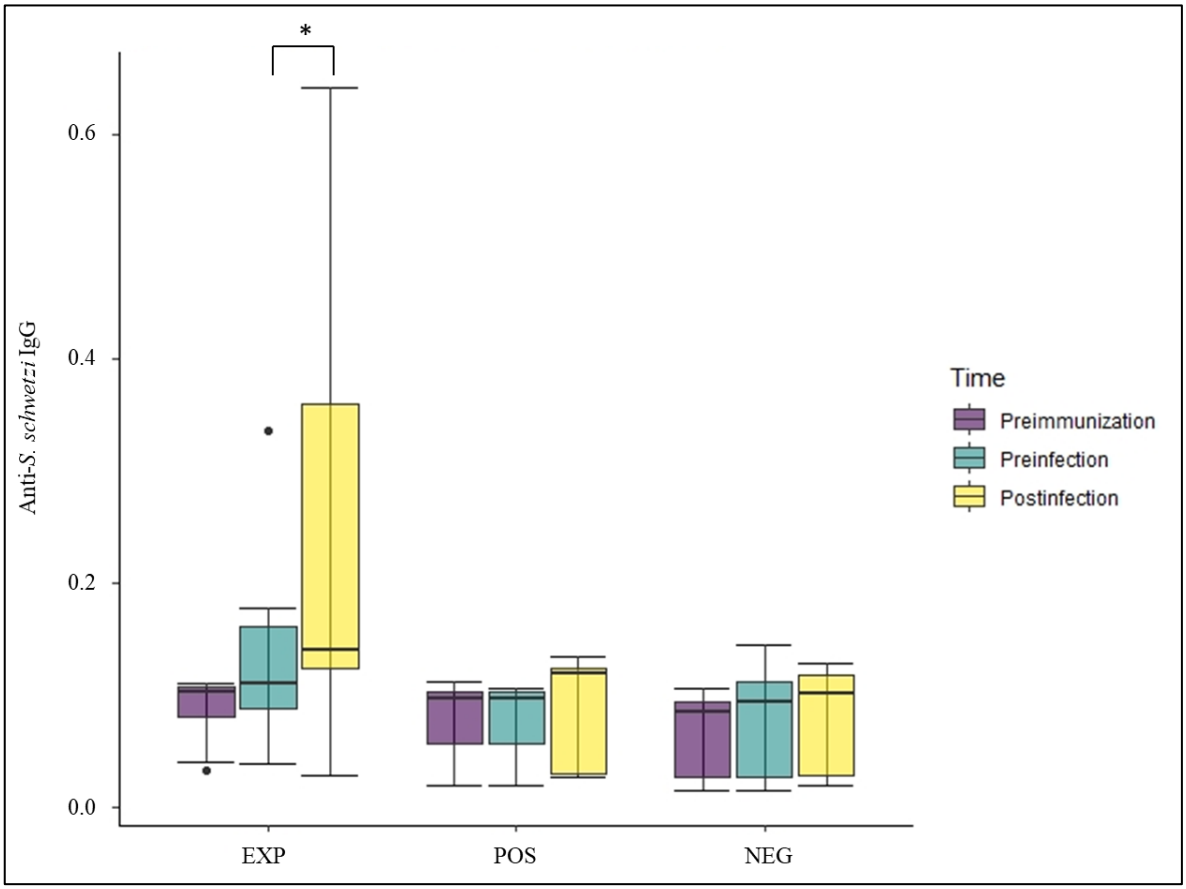


Fig. 26: IgG antibodies against SGH of *S. schwetzi* at indicated time points of the experiment. * denotes significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Variability in the immunization scheme of EXP group among 2A and 2B, 2C groups affected also the levels of IgG antibodies measured in preinfection and postinfection samples. Additional exposure of EXP mice to bites of *S. schwetzi* in group 2A resulted in significantly increased anti-*S. schwetzi* IgG level, in comparison to groups 2B, 2C at W9 (the postinfection time point) as is shown in Fig. 27.

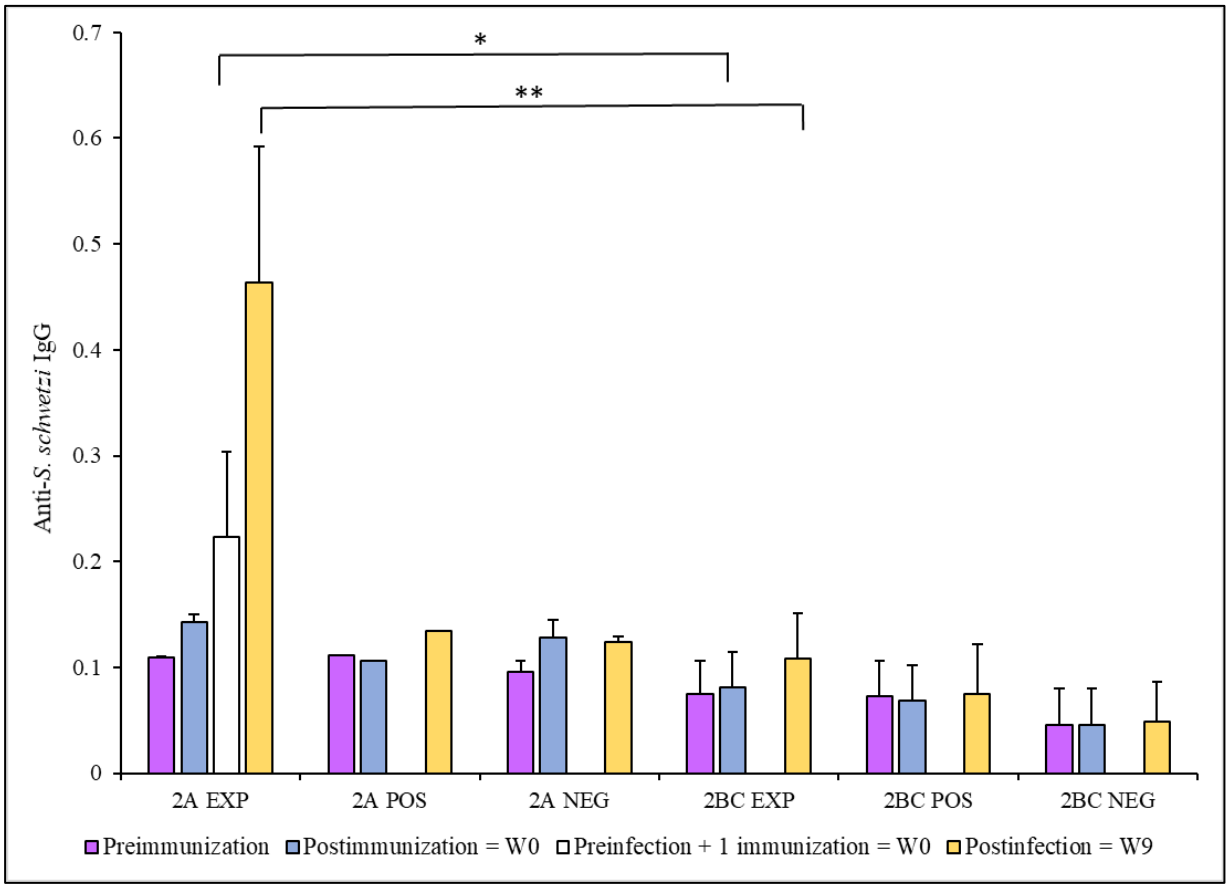


Fig. 27: Variability of IgG levels in additionally immunized 2A group in contrast with groups 2B, 2C ± SD, which were immunized in a different scheme. The statistical analysis was in this case not possible to perform for the POS group, due to small number of tested samples. * denotes for $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Anti – *S. schwetzi* IgG antibody levels significantly correlated with loads of *L. major* parasites in the ear ($r = 0.679$, $p = 0.0019$) and in the lymph nodes ($r = 0.572$, $p = 0.0132$) tissues. No significant correlation was detected in between anti–*S. schwetzi* IgG and lesion size ($p > 0.05$)

4.2.4.2 Antibody response against salivary gland homogenate of *Phlebotomus duboscqi*

The serum samples from mice 2ABC were also tested with antigens from SGH of *P. duboscqi* by ELISA test. Results are summarized in Fig. 28.

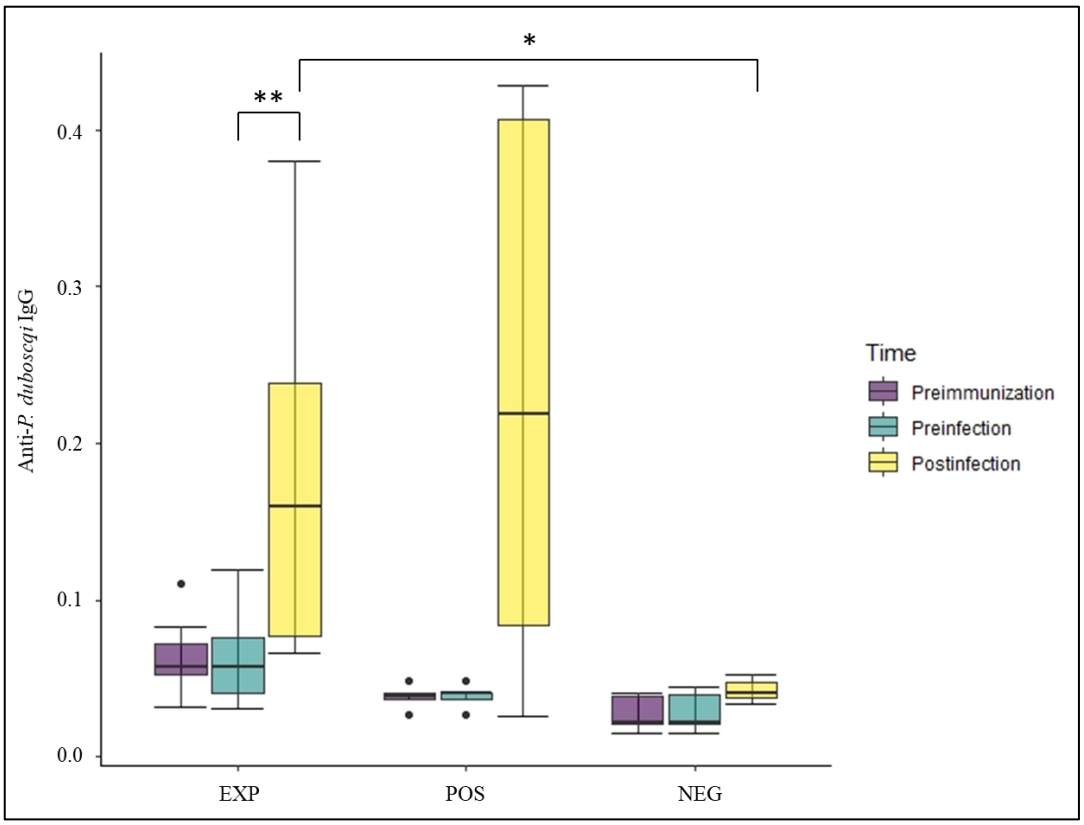


Fig. 28: IgG antibodies against SGH of *P. duboscqi* at indicated time points of the experiment, cross bars are indicating medians of shown data. * denotes for $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The infected groups (EXP and POS) were exposed to *P. duboscqi* SGH only once during the whole experiment, at the time of infection. Specific anti-*P. dubocqi* IgG levels were significantly elevated at W9 compared to W0 for the EXP group, but not in POS group, although it follows similar pattern as EXP group. EXP group had significantly higher levels of anti – *P. duboscqi* IgG compared to nonimmunized NEG group at W9. Other groups did not vary significantly from the NEG control group at any time point of the infection.

The analysis of difference between 2A and 2B, 2C groups did not revealed significant difference among IgG levels in EXP group Fig. 29.

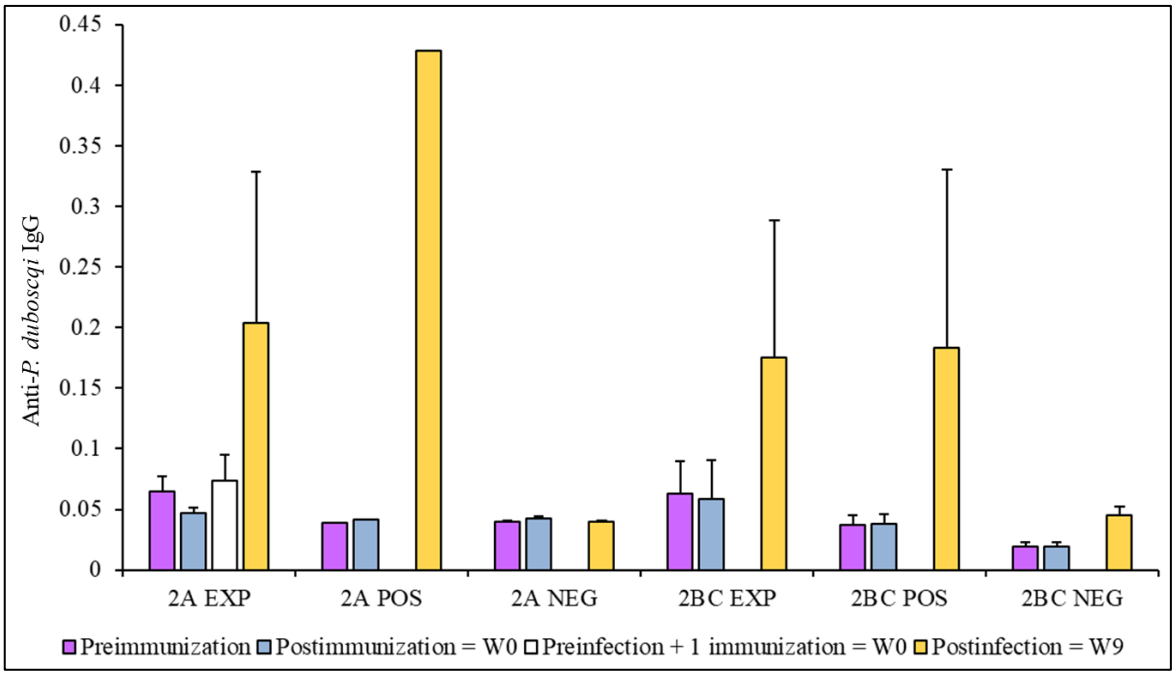


Fig. 29: The comparison of IgG levels to SGH of *P. duboscqi* between additionally immunized 2A group in contrast to groups 2B, 2C ± SD, which followed different immunization scheme. The statistical analysis was in this case not possible to perform for the POS group, due to small number of tested samples. * denotes for $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Correlation test with anti–*P. duboscqi* IgG levels revealed significant positive correlation with *L. major* parasite load in the ear ($r = 0.617$, $p = 0.0064$) and in the lymph nodes tissue ($r = 0.713$, $p = 0.0009$). Milder, although still significant, was also the correlation of anti – *P. duboscqi* IgG levels with lesion size ($r = 0.490$, $p = 0.0388$) at week 9 of the infection.

4.2.4.3 Antibody response against corpuscular antigen of *Leishmania major*

Levels of anti-*L. major* specific IgG significantly rose during the 9 weeks of infection only for EXP group ($p = 0.0078$). At week 9, both EXP and POS group showed significantly elevated levels of anti-*L. major* IgG in comparison to NEG group (Fig. 30). There was no significant difference between levels of anti-*L. major* IgG between EXP and POS group ($p > 0.05$). Analysis between groups 2A and 2B, 2C, did not show significant differences of anti-*Leishmania* IgG in additionally immunized group 2A in comparison to groups 2B, 2C (Fig. 31).

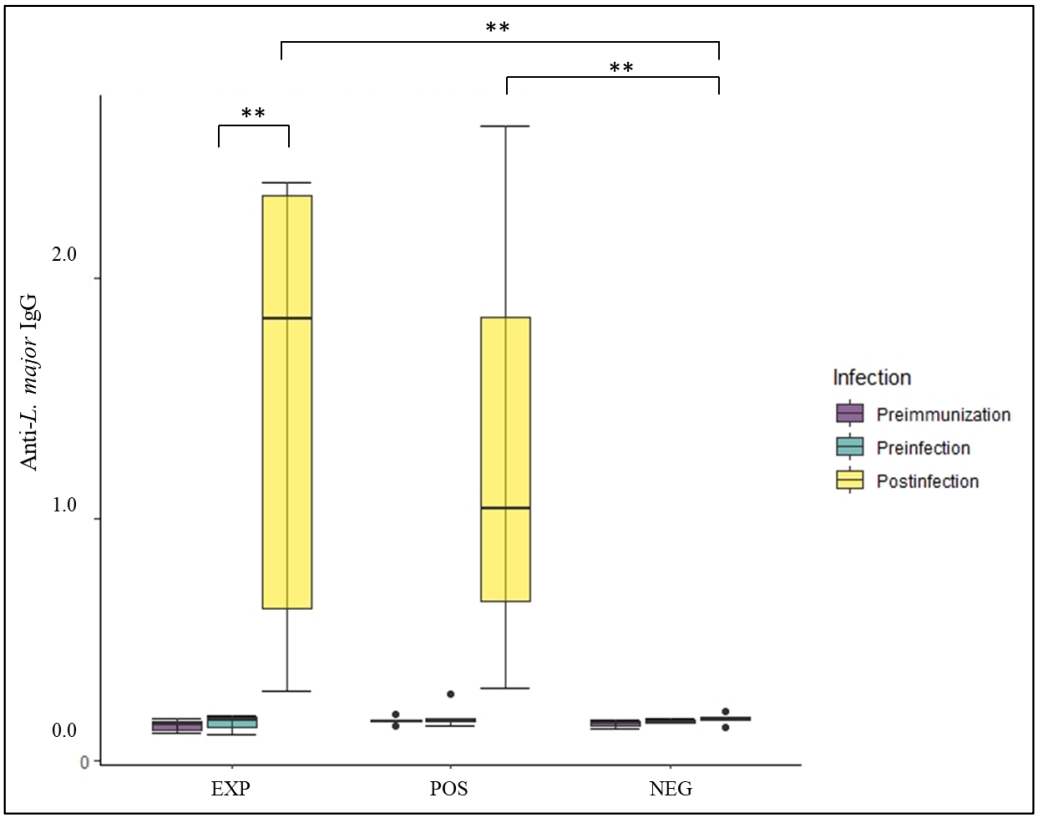


Fig. 30: IgG antibodies against corpuscular antigen of *L. major* at indicated time points of the experiment, cross bars are indicating medians of shown data. Significance is marked by * sign, where ns = non-significant and * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

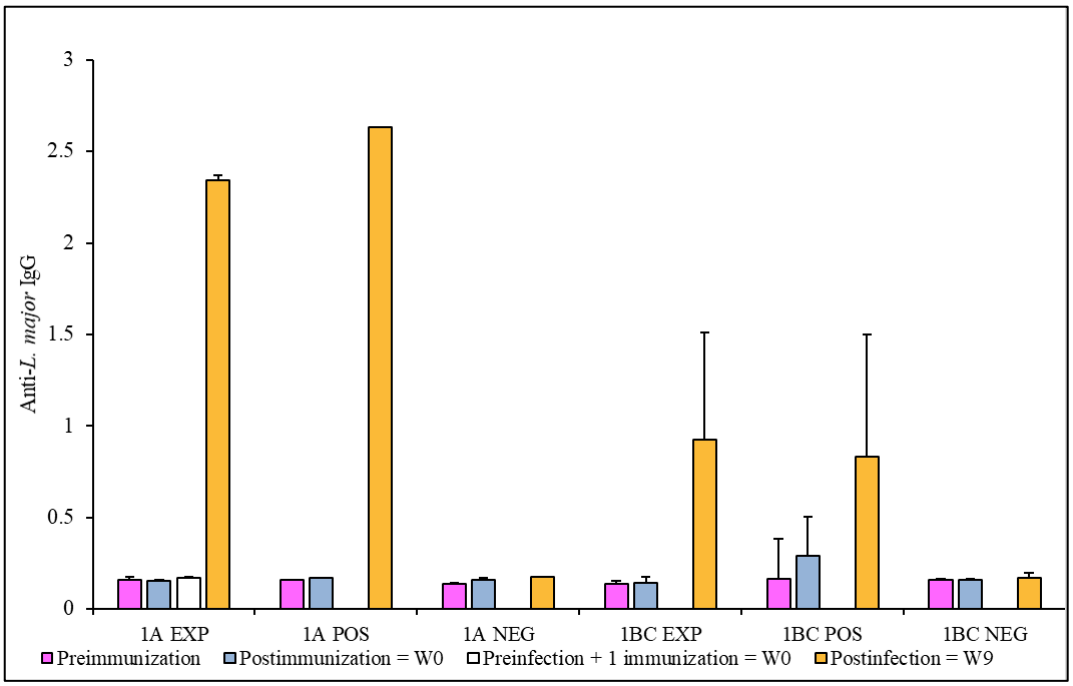


Fig. 31: The comparison of IgG levels to corpuscular antigen of *L. major* between additionally immunized 2A group in contrast to groups 2B + 2C, which followed different immunization scheme. The statistical analysis was in this case not possible to perform for the POS group, due to small number of tested samples. Significance is marked by * sign, where ns = non-significant and * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Anti–*L. major* specific IgG levels were tested also in terms of correlation with other measured parameters. Significant correlation was found within all other measure parameters, summarized in Tab. 17. The strongest correlation was found with *Leishmania* parasite load in the tissues and the least correlation was showed with anti-*P. duboscqi* IgG at week 9 p.i.

Tab. 17: Statistically significant correlation of anti-L. major antibodies with other parameters

	Ear lesion size area (W9)	*L. major* parasite load in the lymph nodes	*L. major* parasite load in the ear	Anti-*S.schwetzi* IgG antibodies (W9)	Anti-*P. duboscqi* IgG antibodies (W9)
Corelation coefficient (r)	0.76	0.87	0.88	0.75	0.65
P value	0.0002	< 0.0001	< 0.0001	0.0003	0.0035

Antigen specific IgG antibodies reached the highest levels at week 9 of infection, exceeding other measured time points – as preimmunization and preinfection, presented in the Fig. 32. Overall highest levels of anti-*L. major* antibodies IgG were observed for EXP and POS groups, however, they did not significantly differ between these two groups.

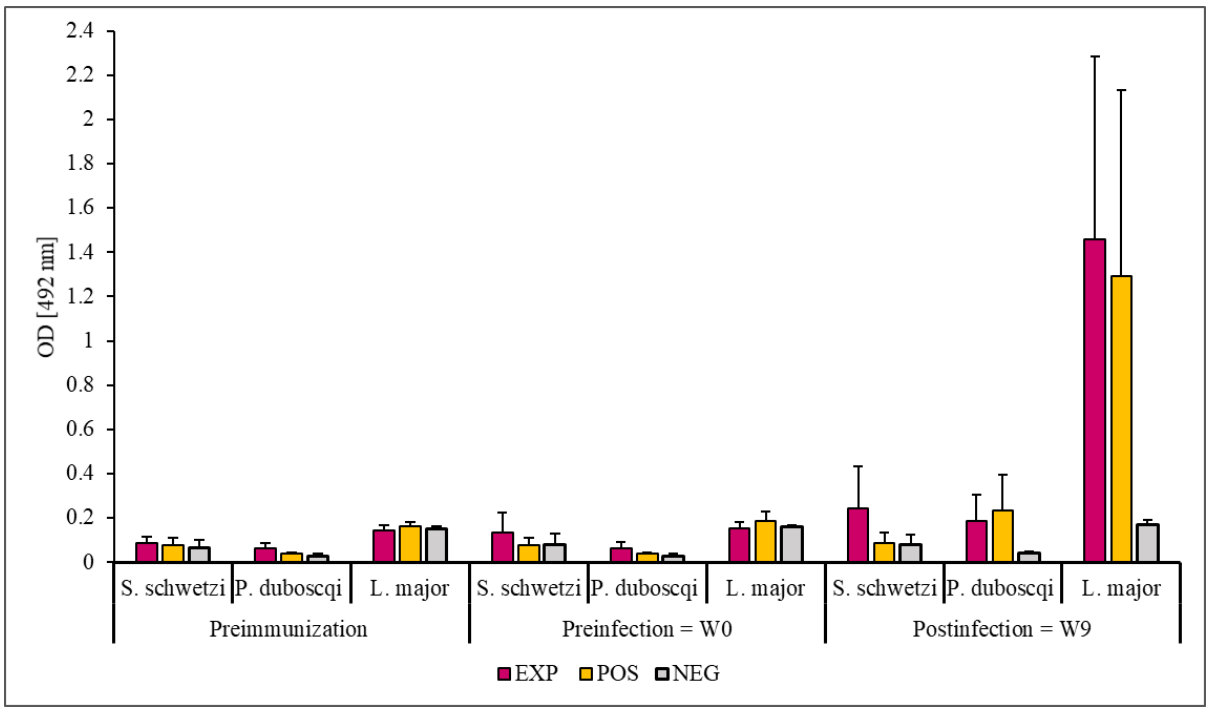


Fig. 32: Antigen-specific IgG response to *P. duboscqi, S. schwetzi, L. major* antigens during different time points of the experiment ± SD.

4.2.4.4 Specificity of antibody response against salivary gland homogenate

Serum from mice repeatedly exposed to only *S. schwetzi* bites were examined for cross-reaction with antigens from SGH of *P. duboscqi*. Similarly, serum from mice repeatedly exposed only to *P. duboscqi* bites were examined for its reactiveness with antigens from SGH of *S. schwetzi*. Results summarized in the Fig. 33 showed strong cross-reaction of anti-*P. duboscqi* IgG with the antigens from SGH of S. schwetzi, with protein of approximately 40 kDa. Mild cross-reaction of serum from *P. duboscqi* exposed mice was observed also with 30-40 kDa protein from SGH of *S. schwetzi*.

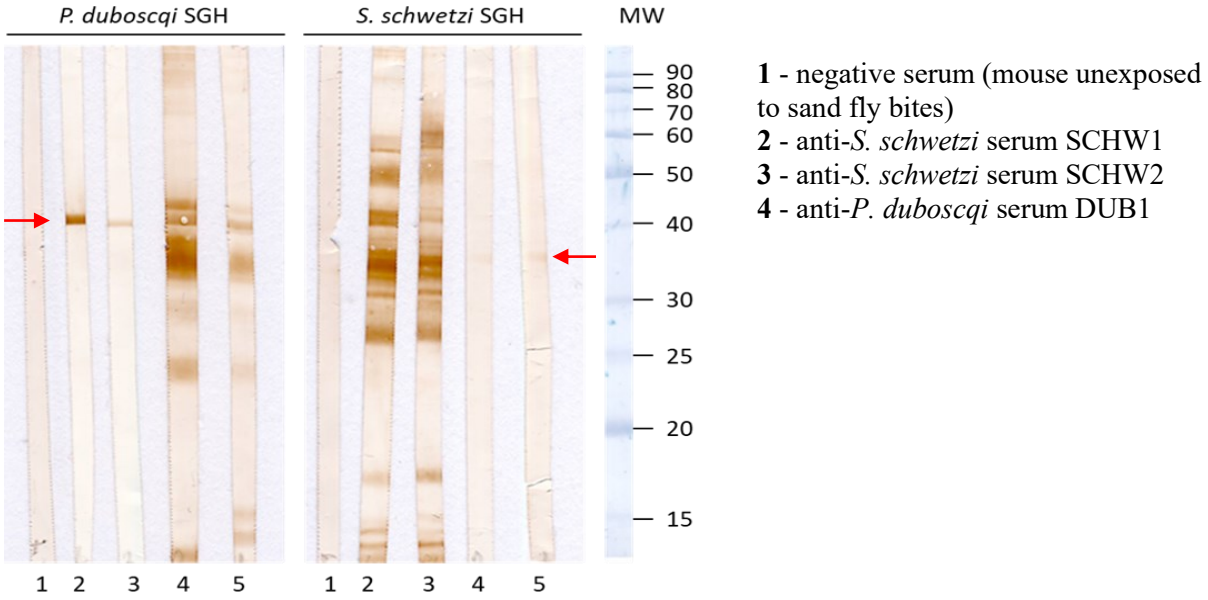


Fig. 33: Immunoblot of serum from *S. schwetzi* exposed and *P. duboscqi* exposed mice with SGH of S. *schwetzi* and with *P. duboscqi*. Red arrows highlight the cross-reactive bands of each serum. MW denotes for molecular weight.

4.2.5 Cellular adaptive immune response analysed by flow cytometry

4.2.5.1 Flow cytometry from draining lymph nodes

Lymph nodes draining the infected ears were subjected for the T cells populations analysis, similarly, to experiment no.1. Fig. 34 illustrates the mean percentage of lymphocytes $CD3^+$ T cells, helper T cells $CD3^+CD4^+$, cytotoxic T cells $CD3^+CD8^+$, memory T cells $CD3^+CD4^+CD62L^+$ and Th1 T cells $CD3^+CD4^+CD183^+$ subpopulations in the lymph node per experimental groups.

Nonimmunized and noninfected NEG control group did not significantly vary in ration to all events of selected T cell populations from both infected groups – EXP and POS, except lymphocyte loads, gated out by size and granularity. In that case, nonimmunized infected POS group had significantly higher ($p = 0.0079$) numbers of lymphocytes than NEG group, however, other measured parameters of NEG stayed below significant difference levels from other experimental groups ($p > 0.05$).

In POS group, significantly higher number of CD3+CD8+ cytotoxic T lymphocytes ($p = 0.0451$) was measured compared to the EXP group. None of these groups differ significantly from noninfected NEG controls.

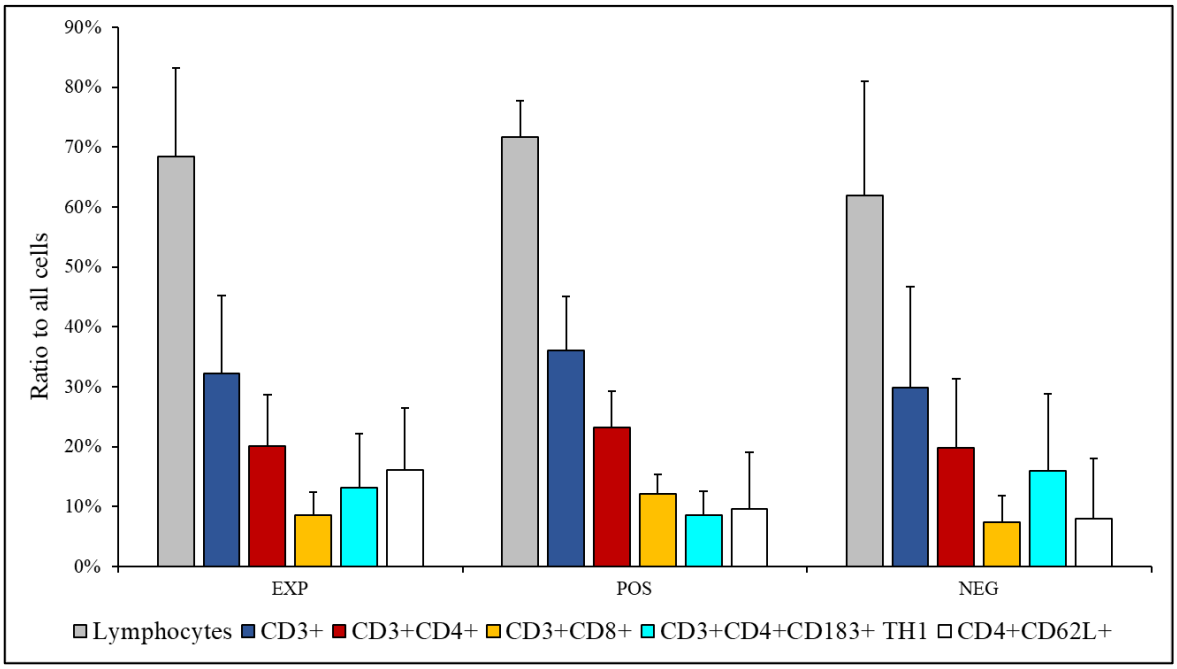


Fig. 34: Ratio to all cells of selected immune cell populations in draining lymph nodes divided according to the experimental groups. * denotes at $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

4.2.5.2 Flow cytometry from spleen

Similarly, to the draining lymph nodes, also spleen cells of mouse were measured for the selected immune cells populations. Comparison of selected immune cell populations in between experimental groups did not reveal any significant difference between ratio to all cells. Fig. 35 is summarizing these results.

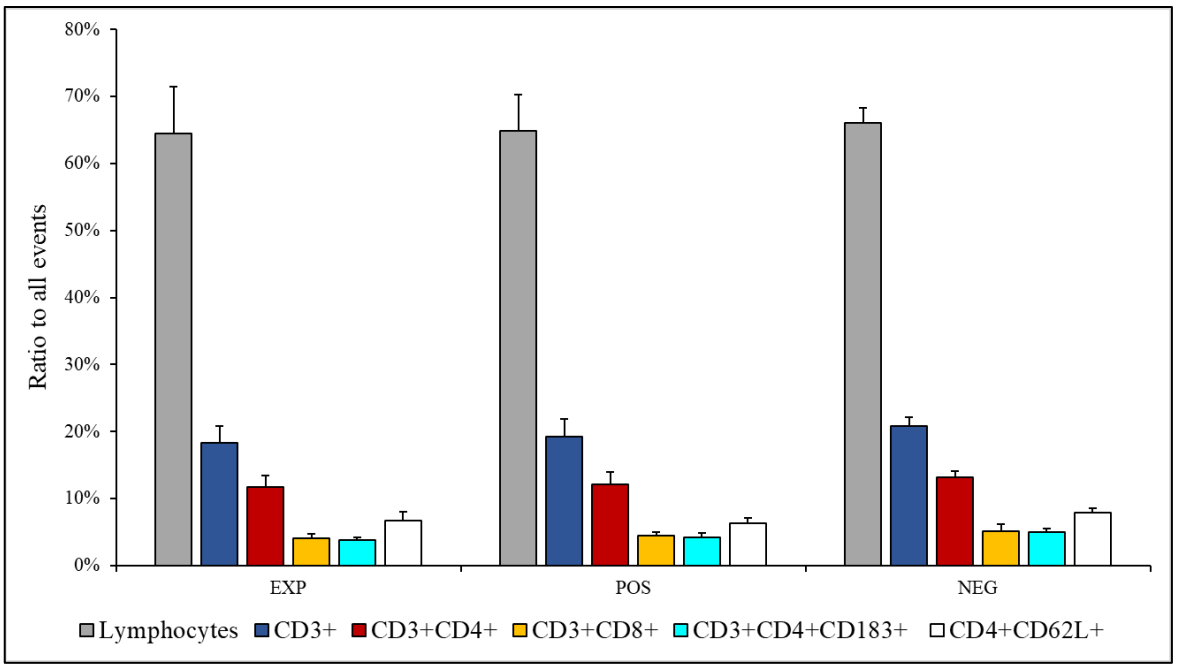


Fig. 35: Ratio to all cells of selected immune cell populations in draining lymph nodes divided according to the experimental groups.

5 DISCUSSION

Repeated exposure of humans and animals to bites of uninfected sand flies has been shown to elicit the development of delayed type hypersensitivity, described in several studies also as harara syndrome in humans (Theodor 1935, Belkaid *et al.* 1998, Belkaid *et al.* 2000, Vinhas *et al.* 2007, de Moura et al. 2007, Oliviera *et al.* 2013). The characterization of DTH response in response to sand fly bites is not consistent through the studies. General characteristics describes DTH as nodular reaction at the bite site, increased blood flow and recruitment of immune cells, mainly mononuclear phagocytic cells and T cells (Belkaid *et al.* 1998, Belkaid *et al.* 2000, Kamhawi *et al*. 2000).

The aim of the first part of presented project was to investigate the effect of expected ongoing DTH reaction on *L. major* infection. As is the name delayed type hypersensitivity referring to, the major visible and detectable reaction and cellular influx are occurring from 24-72 hours post repeated sand fly saliva sensitization, usually peaking at 48 hours (Belkaid et al. 1998, Oliveira *et al.* 2006, Oliveira *et al.* 2013). This reaction is considered to be the major mechanism behind the protective effect of sand fly saliva, when DTH reaction targeted towards antigens from sand fly saliva is skewing the skin microenvironment in the direction of anti-*Leishmania* effector mechanism (Belkaid *et al.* 1998*,* Kamhawi *et al.* 2000, Rohousova *et al.* 2011). Belkaid *et al.* (2000) proposed in their study that the aforementioned changes in the host tissues after repeated exposition to bites of *P. papatasi* were significant up to 5 days post sensitization with the saliva when measured on human volunteers (Belkaid *et al.* 2000). To our knowledge, so far published studies focused on the relationship of sand fly saliva – *Leishmania* – host immune response, were based on the same experimental schedule, where the infection was performed not earlier than one-week after the last immunization, meaning the time, when DTH reaction elicited by previous immunization might vanished (Belkaid *et al.* 2000, Kamhawi *et al.* 2000, de Moura *et al.* 2007, Rohousova *et al.* 2011). Therefore, in the first part of this diploma project, we performed the *Leishmania* infection at the first 48 hours after the last immunization, at the time of expected peak of DTH reaction from the last immunization.

This part included 3 groups of mice – D2 group (infection was performed 48 hours post last sand fly saliva immunization), D7 (infection one-week post last immunization), and POS group (infection with SGH without immunization. In agreement with previous research studies focused on the effect of sand fly saliva on *Leishmania* infection, lesion size and the severity were proved to be lowered in pre-exposed mice in comparison with nonimmunized control group (Belkaid *et al.* 1998, Rohousova *et al.* 2011). This feature of sand fly saliva exposure was considered as one of the main factors leading to the protective effect (Valenzuela *et al.* 2001). The inoculation of *L. major* used in our experiments included also SHG of sand fly used for preexposure - *P. duboscqi*, which was expected to serve as additional eliciting booster of ongoing DTH response, thus eliciting stronger reaction.

The analysis of the lesion size at week 5 – at the end of the experiment – showed overall smaller lesions in mice pre-exposed to sand fly bites (D2 and D7) in comparison to unexposed mice infected with *L. major* inoculum containing also SGH of *P. duboscqi* (POS). Additionally, mice from D7 group that were infected in the usual timepoint, one-week post immunization, had the smallest lesions from all groups similar to other studies focused on the protective effect (Belkaid *et al.* 2000, Rohousova *et al.* 2011, Kamhawi *et al.* 2000, Lestinova *et al.* 2015). This is in accordance with expected development of protective effect, which has been for this combination of vector and parasite *P. duboscqi – L. major* already described

by Rohousova *et al.* 2011. Another factor supporting the establishment of the protective effect in D7 group is the significant difference in lesion size from POS group at week 5 of the infection. Interestingly, lesions of mice from D2 group did not significantly differ from the POS group, nor from D7 group and the lesion size was even slightly larger from the other pre-exposed D7 group. These results are in contrast with our primary hypothesis, that the ongoing DTH response at the bite site might even enhance the protective effect against the infection and therefore support the host-protective immune response. On contrary, it seems that the ongoing anti-saliva immune response counteract the protective effect provided by immunization in D7 group. Our experiments did not include the immunological evaluation of establishment of DTH response at 2 days post immunization, which might contribute to the result analysis of our data and in is planned in our future experiments. Our preliminary experiments on BALB/c mice repeatedly bitten by *P. papatasi* showed that the bite site at day 2 p.l.i. is highly populated by immune cells with activated neutrophils and almost no classically activated macrophages, while at day 7 p.l.i. the bite site showed only mild influx of immune cells, no activated neutrophils but significant amount of classically activated macrophages (Polakova 2022 - Appendix no. 2). One ear from infected mice was also examinate for the presence of *L. major* parasites, while the other was fixated for immunohistochemical staining, with focus on neutrofiles and M1 macrophages. These samples were processed in collaboration with doc. Bardůnek Valigurová and Mgr. Poláková from Masaryk University in Brno. Their results are presented in Appendix no. 1.

One of the main parameters evaluating the protective effect is also the measurement of parasite load in corresponding tissue. The relationship of lesion size and number of parasites in the tissues in cutaneous *Leishmania*sis has not yet been conclusively confirmed (Saldanha *et al.* 2017), however, pre-exposed mice tend to have lower load of parasite (Belkaid *et al.* 1998, Kamhawi *et al.* 2000, Rohousova *et al.* 2011). We observed similar trend within our results from ear tissues, where nonimmunized infected POS group had 8-fold higher parasite loads in the ears than the pre-exposed D7 group. Result of D2 group showed again nonsignificant difference from the POS group ($p > 0.005$). These findings lead us to conclude that the protective effect has not been fully developed among mice from D2 group, moreover, infection performed during the first 48 hours post last immunization could diminish the effect of pre-exposure.

Although causative agents of cutaneous leishmaniasis - *L. major* - is known to disseminate in susceptible BALB/c mice (Laskay *et al.* 1995), preliminary experiments of our project did not show the presence of parasites in the spleen or bone marrow of exposed and infected mice (data not shown) and therefore we focused our analysis on the ear and the draining lymph nodes tissue.

The overall lower loads of *Leishmania* parasites in the lymph node tissues in comparison to data from ears corresponds with the non-visceralizing general feature of cutaneous *Leishmania*sis. Surprisingly, the highest loads of *L. major* in the draining lymph nodes were detected for D2 group, showing even up to 27-fold higher loads than D7 and 30-fold higher loads than control POS group. Although this is in contrast with previous studies, in which a trend in decrease of *L. major* parasite loads in the draining lymph nodes of pre-exposed mice is described (Rohousova *et al.* 2011, Lestinova *et al.* 2015), here we also hypothesise, that this differences in the distribution of parasites might be connected to the DTH effect of sand fly saliva. DTH was expected to be present at the bite site during the infection at 48 hours p.l.i., and it is characterized by significant immune cellular influx (Belkaid *et al*. 1998, Kamhawi *et al.* 2000, Vinhas *et al.* 2007, Polakova, 2022). *Leishmania* are intracellular parasites using mainly immune cells as their host cells (Burza *et al.* 2018). Therefore, higher loads of parasites inside lymph nodes detected for the pre-exposed D2 group,

might be a consequence of increased cell flow during the DTH response and therefore also higher distribution in the host. Additionally, there has been described an evidence of a syndrome based on DTH reactions to repeated exposure to sand fly bites in humans named harara. People facing this syndrome had randomly appearing papules not only on the side of the bites, but also on the other parts of the body. This is supporting the hypothesis, that increased flow of cells during the ongoing DTH response creates rather systematic response (Theodor *et al.* 1935).

The pre-exposure to sand fly saliva was performed in a way to evoke natural conditions, when sand flies were let to feed on the experimental mice freely. Although this might more reflect situations from endemic areas of sand flies and *Leishmania*, the total number of blood fed sand fly females is not under control. Therefore, the result analysis also includes the correlation test of sum of fed sand fly females per mice from all immunizations per mouse with the other measured parameters. Positive mild correlation was found between the number of fed sand flies and parasite loads in the draining lymph nodes. This supports the abovementioned hypothesis, that the pre-exposure to sand fly saliva and, subsequently, the host response towards it affects the distribution of parasite in the tissues.

Additionally, our results showed mild correlation ($r = 0.605$, $p = 0.0002$) between the lesion size and numbers of *L. major* in the corresponding ears within all groups, in which quantity of *Leishmania* in the ears also positively correlated ($r = 0.595$, $p = 0.0003$) with parasite numbers detected from lymph nodes. These results support previous findings that preexposure to sand fly saliva leads to better control of the infection, since the pre-exposed mice had overall lower parasite load in the tissue. However, timing of the infection seems to determine the final disease outcome, especially in the mean of presence of parasites in the tissues (Kamhawi *et al.* 2000, Lestinova *et al.* 2015).

Exposure to the antigens from sand fly saliva and from *L. major* parasites induces the humoral response in the host (Rohousova *et al.* 2005, De Moura *et al.* 2007). IgG specific to *Leishmania* parasites are not protective but rather may cause an exacerbation of the disease. Anti-*Leishmania* IgG have been shown to be able to create immune complexes, which induce IL-10 production at the expense of IL-12 production by activated macrophages, thus supporting parasite proliferation (Miles *et al*. 2005). Lesion size has been also shown to correlate with the levels of anti-*L. major* IgG antibodies, when the pre-exposed mice, having the smaller lesion size, showed also the lowest levels of anti- *L. major* antibodies (Rohousova *et al.* 2011, Lestinova *et al.* 2017).

The effect of anti-saliva specific antibodies on the outcome of *Leishmania* infection has not yet been fully understood. Rohousova *et al*. (2011) in their study hypothesise, that antibody response to sand fly saliva might correlate with the skin damage, as their result showed higher anti-*P. duboscqi* antibodies in mice with more severe lesions. Different study including human participants showed, that patients with more severe *Leishmania* lesions had increased levels of anti-*P. papatasi* salivary antigens antibodies, although the authors hypothesise that this could be connected also to the different genetical background, cumulative exposure to sand fly bites, or different parasite strains from that area (Mondragon-Shem *et al.* 2015). Higher levels of sand fly saliva specific antibodies have been reported to be connected with the higher probability of *Leishmania* infection (Rohousova *et al.* 2005, Kammoun-Rebai *et al.* 2017). However, this has been mostly connected to the higher exposure to sand fly bites and therefore also increased chance of *Leishmania* infection (Rohousova *et al.* 2005, de Moura *et al.* 2007).

Anti-*L. major* specific antibodies were significantly elevated only for infected groups – D2, POS at week 5 p.i., with the highest levels measured for the POS group. Moreover, these levels positively correlated with lesion size and loads of parasites in the right ears and in the draining lymph nodes. Similar results were showed in a study by Lestinova *et al.* (2015), where 7 weeks post infection the lowest levels of anti-*Leishmania* specific IgG were found in pre-exposed group, positively correlating with lesion size and parasite quantity. Supported by other studies *(*Miles *et al.* 2005*,* Rohousova *et al.* 2011*),* our results indicate that *Leishmania* specific IgG antibodies can be implicated in severity of the disease, contributing to the parasite survival. However, to distinguish whether the increased levels of anti-*L. major* IgG are connected only to the higher loads of parasites in the tissues and do or do not contribute to the final disease outcome should be performed.

Both D2 and D7 groups were pre-exposed to the sand fly bites of *P. duboscqi* females in a similar schedule prior to the infection. Levels of anti-*P. duboscqi* antibodies were measured at 2 different time points: (1) after the immunization – prior to the infection and (2) at the week 5 of the infection, to analyse their relationship with *Leishmania* infection. Preinfection levels did not significantly differ between experimental groups including naïve NEG control group, although there was a trend of increased levels in pre-exposed groups. This complies with the fact, that anti-sand fly saliva antibodies usually reach detectable levels from about fourth week of immunization. Significant increase in anti – *P. duboscqi* antibodies was measured for both pre-exposed groups D2 and D7 and week 5 of the infection, where overall highest levels was measured in group infected 48 hours after the last immunization - D2 group, exceeding to almost three times D7 group antibody levels.

Lesion size and loads of parasites in the ear tissue of D2 group did not significantly differ from non-immunized infected POS group, where no protective effect was elicited. Elevated levels of anti-*P. duboscqi* specific antibodies were found in both pre-exposed groups, which were exposed to the *P. duboscqi* saliva during the immunization, and through the infectious inoculum, where SGH of *P. duboscqi* was presented. Levels of anti-*P. duboscqi* specific antibodies have been previously found to correlate with the levels of anti-*Leishmania* antibodies (Rohousova *et al.* 2011). Analysis of the antibody response against *P. duboscqi* in our experimental groups, did not reveal such a correlation. Surprisingly, the highest levels of anti-*P. duboscqi* IgG were found in D2 group, significantly different from the D7 group ($p = 0.008$). Levels of anti-*P. duboscqi* antibodies were shown to correlate also with the number of blood fed females per mouse during the immunization. This does not reflect the outcome of the infection, but rather the immune response to exposure to sand fly saliva itself, as it was shown in previous studies (Vlkova *et al.* 2012, Martin-Martin *et al.* 2015, Marzouki *et al.* 2015, Sima *et al.* 2016).

Furthermore, to get general overview about the ongoing immune events in the experimental groups, the adaptive immune response in terms of selected T cell subsets response was examined. Analysed populations were lymphocytes, $CD3^+$ T cells, helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells in the draining lymph nodes. In case of this experiment, we were not able to examine the spleen, as originally planned. Both $CD4^+$ and $CD8^+$ T cell subsets have been shown to play an important role during the infection (Belkaid *et al.* 2001, Tomiotto-Pellisster *et al.* 2018). Briefly, Th1 subset of $CD4^+$ T cells plays crucial role in fighting the *Leishmania* infection, by production of IFNγ and thus promoting the proinflammatory response especially in macrophages, resulting in their leishmanicidal activities. $CD4^+$ T cells populations are presented from the first weeks of infections, mainly represented by effector T cells during the initial phase

and later on accompanied by $CD4^+$ memory T cells (Scott and Novais, 2016). Additionally, anti-sand fly saliva DTH response is based on $CD4^+$ T cells as described in the introductory part (Belkaid *et al.* 2000). The role of the $CD8^+$ T cells in *Leishmania* infection is rather secondary, supporting the $CD4^+$ T cells in Th1 establishment, especially during the low dose infection. However, their role in high dose infection is not the key one (Uzonna *et al.* 2004, Belkaid *et al.* 2002, Okwor *et al.* 2014). Results from our T cells populations measurements revealed significant difference only for $CD3^+$ and $CD3^+CD4^+$ cell populations between D2 and NEG group, D2 group having 0.7-fold lower rate of $CD3^+$ and 0.65-fold lower rate of $CD3^+CD4^+$ cells than NEG group. These finding are in contrast with other studies showing the employment and increase of $CD4^+$ population in acute and chronic infections (Uzonna *et al.* 2004, Filipe-Santos *et al.* 2009, Peters *et al.* 2014). It is important to mention that the analysis have been performed in the draining lymph node tissue and not in the tissue primarily affected by sand fly feeding and *Leishmania* infection – the ear pinnae. Ear pinnae were preferentially used for *Leishmania* quantification and for immunohistochemistry that was focused on the detection of Leishmania host cells – neutrophils and macrophages (Polakova 2022 – Appendix no. 1). In the follow-up studies, we plan to analyse the leishmania lesion site also by flow cytometry, focusing on both myeloid cells and lymphocytes.

Study by Okwor *et al.* 2014 also pointed out the effect of low vs. high dose of infection affecting the final $CD4^+$ T cell role during the infections. High doses of parasites (2x10^6 *L. major* per infectious inoculum) were connected to stronger $CD4^+$ T cell proliferation in the draining lymph nodes 14 weeks p.i. in comparison to low dose infection (1x10^3 *L. major* per infectious dose) (Okwor *et al.* 2014). Mice in experiment no. 1 were infected with 10^4 *L. major* per infectious dose, corresponding more with the low dose infection.

There is no evidence that sand fly saliva or *Leishmania* parasites itself would be able to abrogate the already elicited DTH reaction and protective effect (Belkaid *et al.* 1998, Belkaid *et al.* 2000). Therefore, to conclude the first part of presented project, timing of the infection post last immunization affects the disease outcome and immune response against *Leishmania* infection. Infection at 48 hours p.l.i., during the peak of expected DTH reaction, does not boost the protective effect elicited by repeated preexposure to bites of uninfected sand flies. Moreover, protective effect seems to be diminished by that timing of infection, which might be due to the increased flow of immune cells, enabling the spread of parasites in the tissues, together with the absence of the effector M1 macrophages. Additional experiments are needed to describe this effect in more details, e.g. deeper analysis of anti-saliva immune response at 48 hours post last bite or infection site analysis during the first hours and days of infection.

The second part of the diploma project was aimed to reveal the effect of preexposure to bites of *S. schwetzi* on the outcome of the *L. major* infection injected along with *P. duboscqi* SGH. These two phlebotomine species are phylogenetically distant, however, they are both man-biting and sympatrically distributed in Sub-Saharan Africa (Anjilli *et al.* 2011, Pigott *et al.* 2014) thus humans from this area may experience bites from both phlebotomine species. Accordingly, the aim of this experiment was to simulate naturally occurring conditions and its potential influence on *Leishmania* infection.

The cross-protective effect of sand fly saliva on the outcome of *Leishmania* infection has been proved so far for only few closely related species combinations (Thiakaki *et al.* 2005, Tavares *et al.* 2011, Lestinova *et al.* 2015). Such experiments have proved induction of the protective effect by *P. papatasi* bites against

infection along with *P. duboscqi* saliva (Lestinova *et al.* 2015) and by *Lu. longipalpis* SGH against *L. intermedia*-mediated infection (Tavares *et al.* 2011), although the effect was milder than with the usage of same-species saliva for pre-exposition and infection.

Experiment no.2 was performed with 3 independent replicates (2A, 2B, 2C) and 3 experimental groups were compared – EXP (pre-exposed to *S. schwetzi* bites and infected with *L. major* inoculum containing SGH of *P. duboscqi*), control POS group (infected with *L. major* and SGH of *P. duboscqi*, without previous exposition) and NEG naïve group (nonimmunized, noninfected). The infection was performed 48 hours p.l.i., similarly to D2 group in experiment no. 1. However, in the case of experiment no. 2, the experimental design was planned before the results analysis from experiment no. 1, thus originally aiming to achieve protective DTH effect of *S. schwetzi* saliva on the immune system at the time of infection. Therefore, we were not aware of the actual exacerbative effect as a result of infection performed within the first 48 hours p.l.i. in comparison to infection one-week p.l.i. as observed in experiment no. 1.

The analysis of the lesions size during the 9 weeks infection period, did not reveal any significant differences between the independent replicates. Overall trend was towards larger lesion size in EXP group, showing a kind of exacerbating effect on *Leishmania* infection. Additionally, lesion development in EXP group started to occur sooner than in POS group, already since the first week post infection. These results are similar to those observed by Thiakaki *et al.* (2005), where mice preimmunized with saliva of *P. papatasi* or *P. sergenti*, meaning species phylogenetically distant from saliva used in the infectious inoculum (*Lu. Longipalpis*) developed lesion as large in size area as control non-preimmunized group, although there was no difference at the timing of lesion occurrence.

As already mentioned before, three independent experiments were performed to obtain enough data for statistical analysis. Unfortunately, 2A replicate was affected by covid restrictions, changing the originally aimed time schedule of immunization from three pre-exposures to *S. schwetzi* bites in one-week intervals followed by infection 48 hours p.l.i, into three pre-exposures - 17 weeks break - booster immunization and infection 48 hours later p.l.i. Taking these differences in the immunization scheme into consideration, data from 2A replicate were analysed as separate experiment, compared with results from 2B+2C as true replicates. Interestingly, EXP group from the 2A replicate showed significantly larger lesions area from week 7 till the end of the experiment at week 9 p.i., than EXP group from 2B+2C replicates. Statistical analysis of inter-replicates variability for the EXP group and the POS group was not possible to perform due to the low numbers of mice in each replicate (Tab. 3).

Parasite burden in tissues of mice pre-exposed to heterologous sand fly saliva did not show lower numbers in comparison with control unexposed group (Thiakaki *et al.* 2005). Comparing loads of *Leishmania* parasites in the ears and lymph nodes tissues of EXP vs. POS group within data from all three replicates supported results from Thiakaki *et al.* (2005), also showing that no significant differences were found between pre-exposed (EXP) vs. nonexposed (POS) groups. On the other hand, strong correlation was found between the numbers of parasites in the ears and lymph nodes, similar to the results from experiment no. 1. The comparison of 2A with 2B+2C groups did not reveal any significant differences among the parasite load in the tissue of those replicates, although there was an interesting trend of the opposite *L. major* distribution between ears and lymph nodes among mice groups and replications (See at Fig. 22 and 23). Mice from EXP group of the 2A replicate had lower parasite burdens compared to the POS group in the draining lymph node, which contrasted with parasite loads of EXP and POS groups from 2B+2C

replicates. Additionally, 2A replicate showed higher numbers of *L. major* parasites for EXP group in comparison to POS group in ear tissues, again in contrast with data from 2B+2C replicates, POS group having the higher parasite loads compared to the EXP group.

Number of blood fed sand flies cannot be controlled during the natural way of immunization by sand fly bites. Based on the results from experiment no.1, where positive correlation was found between the parasite loads in the lymph nodes and the number of blood fed sand flies per mice, similar analysis was performed also for experiment no. 2. Firstly, repetitions 2A vs. 2B+2C differ in the number of immunizations, thus the sum of number of blood fed females per mice between 2A and 2B+2C groups was compared. Although the mice from 2A group experienced extra immunizations with *S. schwetzi*, the total sum of fed females per mouse was similar between mice from 2A and mice from 2B+2C group. Therefore, we hypothesise, that different loads and distribution of parasites between 2A and 2B+2C replicates is not connected to the total number of blood fed sand flies, however, the number of mice per 2A repetition is not sufficient for a better statistical analysis. Correlation test revealed again mild correlation between the *L. major* parasite load per lymph node and ear tissue and the sum of blood fed females per mouse.

Leishmania parasite burden in the tissues, has been shown to correlate with the lesion size (Rohousova *et al.* 2011, Lestinova *et al.* 2015). Our results are alike these previous findings, where positive correlation was found between the lesion size and *L. major* parasite load in lymph nodes as well as ears, and parasite numbers also correlated between analysed tissues. Additionally, data obtained from quantification of *L. major* parasites in the tissues support the conclusion, that pre-exposure to *S. schwetzi* sand flies did not elicit protective effect to *Leishmania* infection supplemented with SGH of *P. duboscqi*.

Levels of sand fly specific saliva antibodies are highly dependent on the frequency of exposure and timing of the measurement since the last exposition (Clements *et al.* 2010, Vlkova *et al*. 2012, Lestinova *et al.* 2017). To illustrate this, anti-*P.papatasi* sand fly saliva IgG were reported to encounter a steady increase in mice after repeated exposure for at least 24 weeks p.l.i., till the end of the experiment (Vlkova *et al.* 2012). The same pattern was observed for anti-*P. perniciosus* saliva IgG antibodies in mice, which levels rose during the period of sand fly saliva immunization and then reached stable levels persistent for at least 24 weeks after the last exposure (Martín-Martín *et al*. 2015). On the other side, levels of anti-*P. argentipes* specific IgG antibodies decreased rapidly within the first 30 days without exposure to sand fly bites in patients with visceral Leishmaniasis in endemic areas (Clements *et al.* 2010). To our knowledge, there is no research focused of the kinetics of antibody response to *S. schwetzi*.

Due to the different immunization scheme of EXP group in replicate 2A, the influence of additional saliva antigen exposure on the levels of anti-saliva specific IgG connected with a different timing and following infection could be observed. EXP group from additionally immunized 2A replicate had significantly higher ($p = 0.0005$) levels of anti-*S. schwetzi* specific antibodies at week 9 p.i. in comparison to 2B+2C groups.

From results of experiment no. 2., we can conclude that anti-*S. schwetzi* IgG antibodies are also facing steady growth during the repeated exposure of mice to their bites. Increased levels were already detectable one-week after the last exposure, however significant difference from pre-exposed levels was not observed until week 9 p.i. in EXP group. This agrees with the immunization scheme, where the only group exposed to the *S. schwetzi* saliva was EXP group. Presented study was however not focused on the

kinetics of specific IgG antibodies against *S. schwetzi* saliva, which means that significant levels could be reached earlier than 9th week post infection and further research for such an analysis should be performed.

Significant correlation with levels of *S. schwetzi* anti-saliva IgG was found for parasite burden in ears and lymph nodes, together with levels of anti-*L. major* antibodies. This is in accordance with results from experiment no. 1 and study by Rohousova *et al.* (2011), where different timing of infection after pre-exposure affected the load distribution of parasites in the tissues. Since the levels of anti-saliva specific antibodies are dependent on the frequency and timing after the last exposition as mentioned before, here we could hypothesise that the pre-exposure to sand fly saliva even to phylogenetically distant species elicited immune response of the host, however these changes do not necessarily result in the protective or enhancing effect.

Levels of anti-*P. duboscqi* IgG antibodies also significantly rose during the 9 weeks of infection, reaching up to almost same levels as anti-*S. schwetzi* IgG. The highest levels were observed for the POS group, although there was no significant difference to EXP group. Similar results were observed also by Lestinova *et al.* (2015), where group pre-exposed to *P. papatasi* bites and infected with SGH of *P. duboscqi* had overall higher levels of anti-*P. duboscqi* antibodies than the control unexposed group. Possible explanation for that observation might be, that B cells producing antibodies were already primed and exhausted by preexposure to *S. schwetzi* salivary antigens in the EXP group, which had the overall highest levels of anti-*S. schwetzi* antibodies at week 9 p.i.

Different immunization scheme of EXP mice in 2A experiment did not affect the levels of anti-*P. duboscqi* specific IgG, which corresponds with the fact, that *P. duboscqi* saliva was used only as a part of infectious inoculum. The positive correlation of levels of anti-*P. duboscqi* at week 9 was found with levels of anti-*S. schwetzi* IgG and anti-*L. major* IgG. These data suggest that anti-saliva immunity against unrelated species can affect the production of antibodies against *Leishmania* parasites, in a so far unknown mechanism.

Part of the experiment no. 2 was also focused on the analysis of adaptive cellular immune response performed by flow cytometry. Initial aim was to detect different memory T cells (central memory T cells) and helper T cell subpopulations (Th1, Th2, and Th17) in the experimental groups, however, due to the consistent problems with marker for CD197 and CD196 during the analysis, the results presented in this project include only populations identified with reliable markers.

Memory $CD4^+$ T cells are playing key role during the long-term infection and confer the protection against reinfection. Several memory T cells populations are connected to *Leishmania* infection, including skin resident memory T cells and effector and central memory T cells, appearing already during the initial phase of the infection (Zaph *et al.* 2004, Colpitts and Scot, 2010, Glennie *et al*. 2012). Skin memory T cells possess the ability to confer protection even without the presence of parasites, however, insufficiently without the stimuli from the central memory T cells (Colpitts and Scot, 2010). Central memory T cells are mainly found within the lymph nodes, characterized by expression of $CD4^+CD62L^{HIGH}IL\text{-}7R^{HIGH}$ (Woodland and Kohlmeier, 2009, Colpitts and Scot, 2010). We were able to detect $CD3+CD4^+CD62L^+$ population within our samples in both lymph nodes and spleen tissue, however, no statistical difference was observed among groups. For the future experiments, identification of SGH specific memory T cells, would help to uncover long-term immune response in pre-exposed host. Similarly, the Th1 cell subset analysis, characterized by $CD3^+CD4^+CD183^+$ markers, did not significantly differ among experimental

groups. We plan to repeat these analyses after solving the methodological problems such as finding the reliable dilution of CD197 and CD196 markers. In the follow-up experiments, we also plan to analyse the ear pinnae tissue for the immune cells presented at the site of leishmania lesion, to reveal the effector mechanisms behind.

To conclude the second part of the project – experiment no. 2 – pre-exposure of mice to the saliva of *S. schwetzi* did not confer protection against *L. major* infection inoculated with SGH of phylogenetically distant phlebotomine species *P. duboscqi*. This outcome could be connected to variability in composition of sand fly saliva of genus *Phlebotomus* and genus *Sergentomyia*, as described by Polanska, (2020). Previous study by Spitzova *et al*. (2020) showed, that same classes of molecules presented in the genus *Phlebotomus*, do not possess the same features as molecules from genus *Sergentomyia*. Results from our experiments might reflect this variability among species, on the other side also showing, that exposure to bites of *S. schwetzi* did not significantly overexacerbate the *L. major* infection. Additionally, partial cross-reactivity was observed between anti-*S. schwetzi* antibodies and *P. duboscqi* SGH, that may further contribute to the higher levels of detected anti-*P. duboscqi* IgG in mice preexposed to *S. schwetzi* compared to unexposed POS group.

Timing and frequency of immunization by *S. schwetzi* highly effected the intensity of immune response, however these changes were not efficient enough to provoke different outcome of the infection compared to unexposed group. This experiment has been performed on a limited number of mice and more data are needed to obtain better-supported results.

6 CONCLUSIONS

The presented project includes two connected experiments, investigating the effect of preexposure to sand fly saliva on the subsequent *Leishmania* infection.

Aim of the experiment no. 1 was to determine the relationship between the timing of last exposure to sand fly saliva and *Leishmania* infection. According to our results, infection performed at 48 hours post last exposure to *P. duboscqi* bites (D2 group), did not confer protective effect on *Leishmania* infection, as observed in the other experimental group infected one-week post last exposure (D7 group). This effect was evaluated mainly by comparison of lesion size and parasite load in the ear and lymph node tissues, showing no significant differences between D2 group and control nonimmunized infected POS group in both parameters. The immunology parameters such as levels of anti-*P. duboscqi* and anti-*L. major* specific antibodies were also altered by the infection timing, D2 group showing overall higher levels of antigen specific antibodies compared to D7 group. Further analysis of the adaptive immune T cell response is needed to reveal the immune mechanism behind this interesting phenomenon. In summary, the results of experiment no. 1 suggest that the disease outcome is influenced by the ongoing anti-saliva immune reaction at the site of *Leishmania* transmission. The timespan between the last exposure to vector saliva of the sensitized host and the *Leishmania* transmission might be an important, yet neglected, factor affecting the establishment leishmaniasis outcome by changing the skin immune environment.

The second part of this project was focused on the specifity of saliva-mediated protective effect between phylogenetically distant sand fly species, one species used for the immunization and different one for the infection. Here we conclude that pre-exposure to *S. schwetzi* bites did not elicit protective immune response, when the SGH of *P. duboscqi* was used along with *L. major* in the infective inoculum. Pre-exposed mice (EXP) did not significantly differ from the control nonimmunized POS group in lesion size area, nor parasite load in the ear and lymph node tissues. Accordingly, levels of anti-sand fly saliva and anti-*L. major* antibodies were not significantly different between those infected groups. Flow cytometry analysis of T cells populations among experimental groups did not show any significant differences among groups, however, additional experiments are planned.

Results of this project indicate for the first-time a detrimental role of timing of the *Leishmania* infection during the early time points after the last immunization by sand fly bites and prove no effect of pre-exposure to *S. schwetzi* on the course of *L. major* infection performed with SGH of *P. duboscqi*. Both situations can occur in Leishmaniasis endemic areas, and the understanding of such relationships might affect the control programs, including vaccine development.

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8 APPENDIX

V rámci **Experimentu č. 1** byla část vzorků zpracována ve spolupráci s kolegy z Masarykovy university v Brně. Cílem této přílohy je doplnit výsledky uvedené v této diplomové práci o další údaje a ukázat tak tento experiment v celé jeho šíři.

Design experimentu je detailně popsán v materiálu a metodice této diplomové práce. Myším byly odebrány vzorky tkání 5. týden po infekci. Jeden ušní boltec z každé myši byl převeden do zinkové fixáže (BD Pharmingen, kat. č. 552658) a odeslán ke zpracování do laboratoře doc. Bardůnek Valigurové (Masarykova universita, Brno). Vzorky zde byly odvodněny, zality do parafínu, nakrájeny a obarveny hematoxylinem-eosinem (obrázek č. 1) či inkubovány s protilátkami proti neutrofilům a klasicky aktivovaným M1 makrofágům (obrázek č.2), které byly vizualizovány fluorescenčně značenou protilátkou pomocí mikroskopu Olympus IX81 vybaveného konfokální jednotkou FluoView 500. Detailní popis zpracování vzorků pro mikroskopickou analýzu tkáně ušního boltce je uveden v diplomové práci Karolíny Polákové (Poláková 2022), ze které jsou převzaty i následující obrázky (včetně číslování obrázků).

Na obrázcích jsou řezy ušním boltcem myší v různém zvětšení. D7 je skupina myší infikovaná Leishmania major v odstupu 7 dnů od poslední imunizace sáním Phlebotomus duboscqi (prezentované snímky jsou z myši č. 12), myši ve skupině D2 byli infikované L. major v odstupu 2 dnů od poslední imunizace (na snímcích jsou vzorky z myši č. 1), jako pozitivní kontrola (POS) sloužily myši infikované bez předchozí imunizace (myš č. 7) a jako negativní kontrola (NEG) sloužily myši neimunizované a neinfikované (myš č. 14). V tabulce č. 1 je pak uvedena plocha leishmaniové léze v 5. týdnu po nákaze u mikroskopicky analyzovaného ušního boltce (levé ucho) a plocha leishmaniové léze spolu s náloží parazitů v kontralaterálním ušním boltci té samé myši (pravé ucho).

U myši ze skupiny D7 jsme detekovali zvýšené množství klasicky aktivovaných makrofágů a téměř žádné aktivované neutrofily (obrázek č. 2). U myší ze skupin POS a D2 bylo zastoupení těchto buněčných populací opačné, bylo pozorováno vyšší zastoupení neutrofilů na úkor klasicky aktivovaných makrofágů.

V rámci spolupráce s kolegy z Masarykovy University v Brně jsme provedli také předběžnou mikroskopickou analýzu tkáně ušního boltce u opakovaně pobodaných myší v časech, které odpovídají době infekce, tedy 48 hodin po posledním sání a týden po posledním sání.

Pro tento pokus byly myši opakovaně vystaveny sání flebotomem druhu *Phlebotomus papatasi* (jedná se o druh blízce příbuzný druhu *P. duboscqi* a potvrzeného přenašeče *L. major*), a to jednou za 7-14 dní v rámci péče o tuto kolonii flebotomů na kat. parazitologie PřF UK. Sání probíhalo dlouhodobě zhruba 1 rok. Myši byly uvedeny do celkové anestezie a po celou dobu bylo dbáno na komfort zvířat (dostatečná teplota v místnosti, vlhčení očí). Uspané myši byly umístěny do klece se samicemi flebotomů a ponechány 60 minut ve tmě v pokojové teplotě. Po každém sání zvířata opět nabyla vědomí a byla vrácena do označené chovné nádoby. Na konci pokusu byly myši uvedeny do hluboké celkové anestezie a usmrceny v intervalu 48 hodin (skupina D2, n = 2) nebo 1 týden (skupina D7, n = 2) po posledním sání. Odebrané ušní boltce byly převedeny do zinkové fixáže (BD Pharmingen, kat. č. 552658) a odeslány ke zpracování do laboratoře doc. Bardůnek Valigurové (Masarykova universita, Brno). Vzorky zde byly odvodněny, zality do parafínu, nakrájeny a obarveny giemsou, hematoxylinem-eosinem, inkubovány s protilátkami proti neutrofilům a klasicky aktivovaným M1 makrofágům, které byly vizualizovány fluorescenčně značenou protilátkou pomocí mikroskopu Olympus IX81 vybaveného konfokální jednotkou FluoView 500 (obrázek č.3). Detailní popis zpracování vzorků pro mikroskopickou analýzu tkáně ušního boltce je uveden v diplomové práci Karolíny Polákové (Poláková 2022), ze které jsou převzaty i následující obrázky (včetně číslování obrázků).

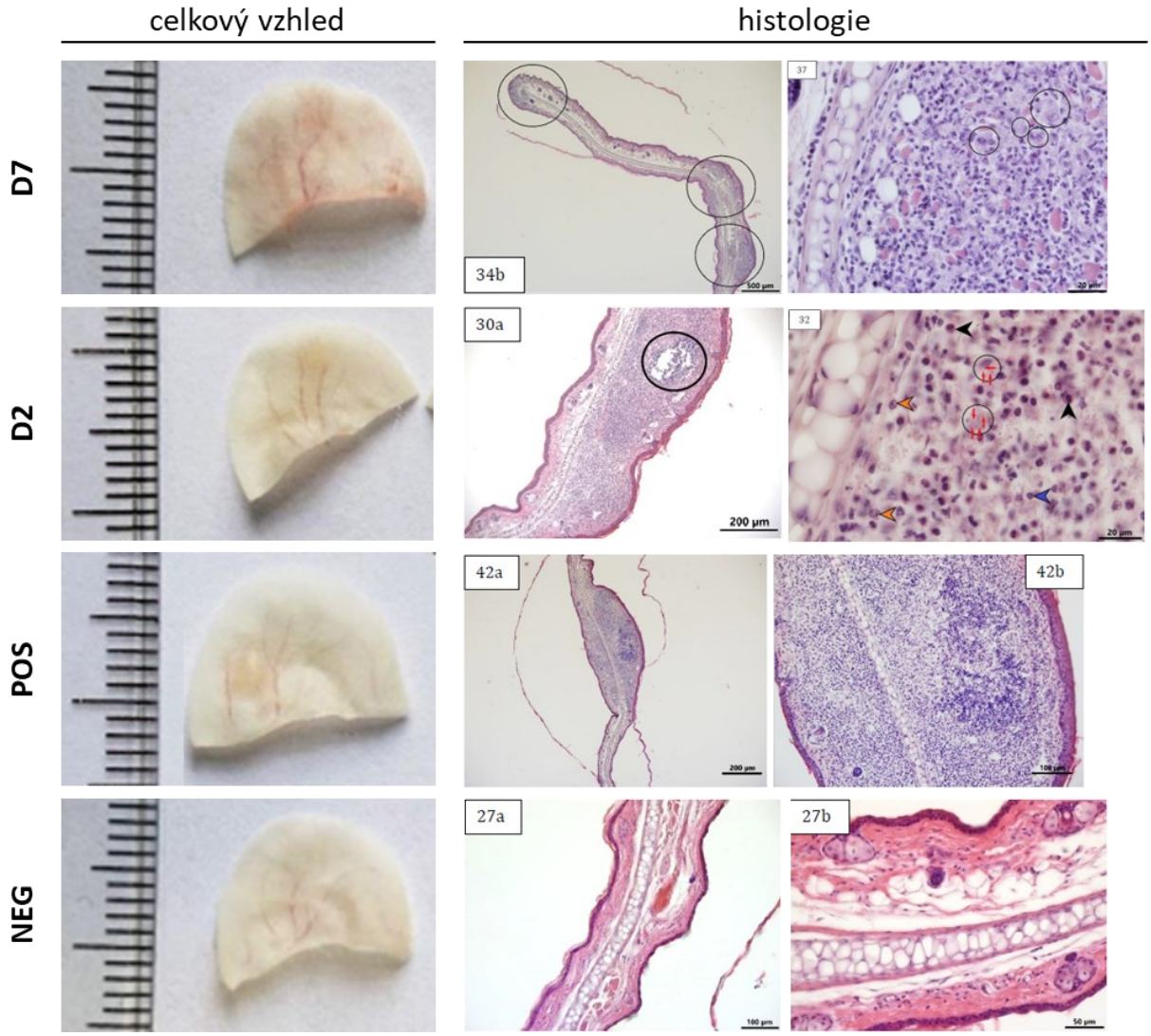
U myší ze skupiny D2 jsme pozorovali zvýšené množství imunitních buněk v tkáni, větší množství aktivovaných neutrofilů a téměř žádné M1 makrofágy. U myší odebraných ve větším časovém odstupu od posledního sání (skupina D7) bylo v tkáni přítomno menší množství imunitních buněk, téměř žádné aktivované neutrofily, ale zato jsme pozorovali vyšší zastoupení M1 makrofágů.

POUŽITÁ LITERATURA

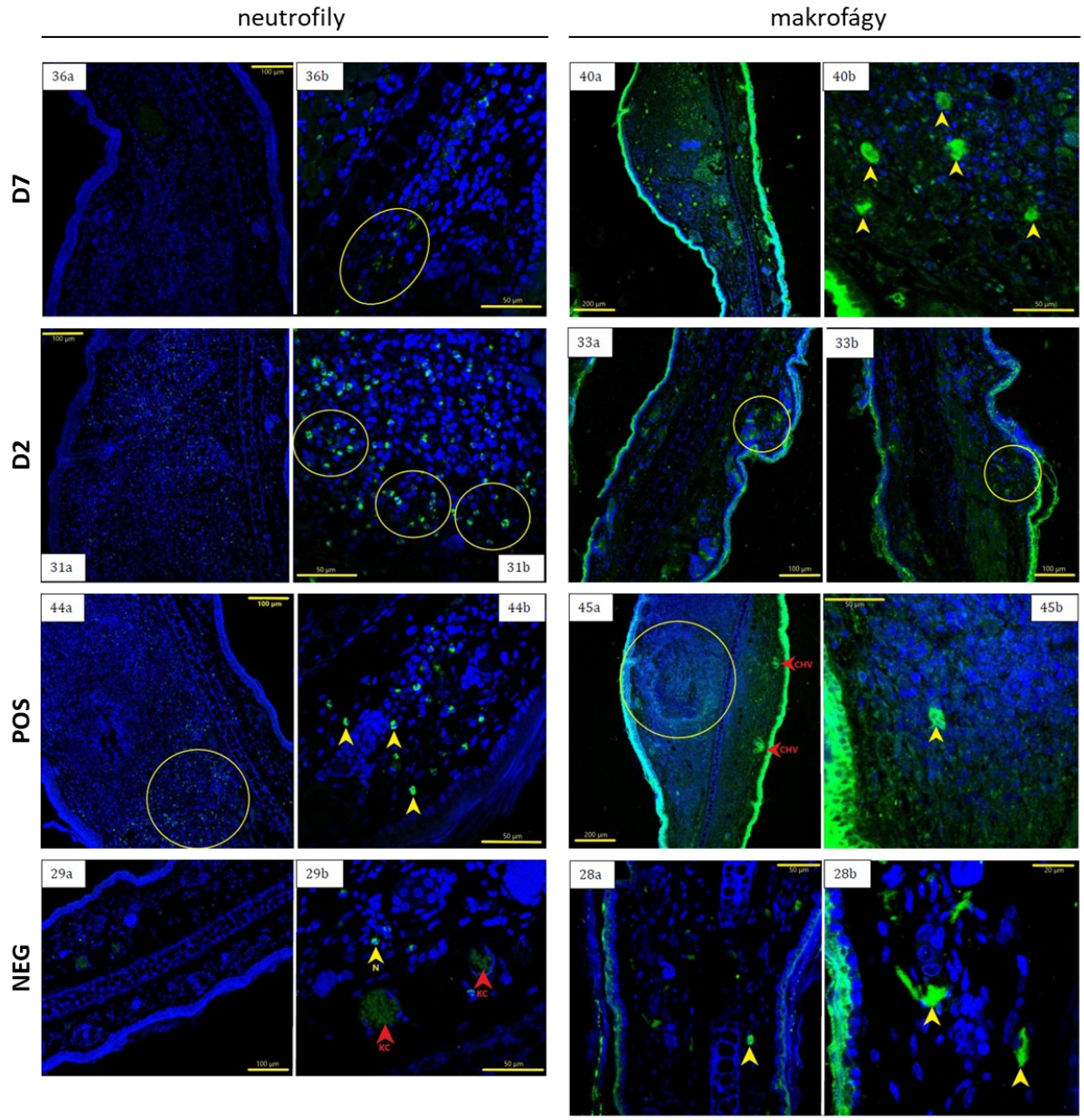
Bc. Karolína Poláková (2022): Vliv časné imunitní odpovědi u myší opakovaně pobodaných flebotomy na průběh kožní leishmaniózy. Diplomová práce. Ústav botaniky a zoologie, Přírodovědecká fakulta, Masarykova univerzita. 96 stran. Vedoucí práce: doc. RNDr. Andrea Bardůnek Valigurová, Ph.D.

Tabulka č. 1: Parametry leishmaniové infekce měřené v 5. týdnu infekce, v době odběru vzorů tkání. Upraveno dle Poláková 2022.

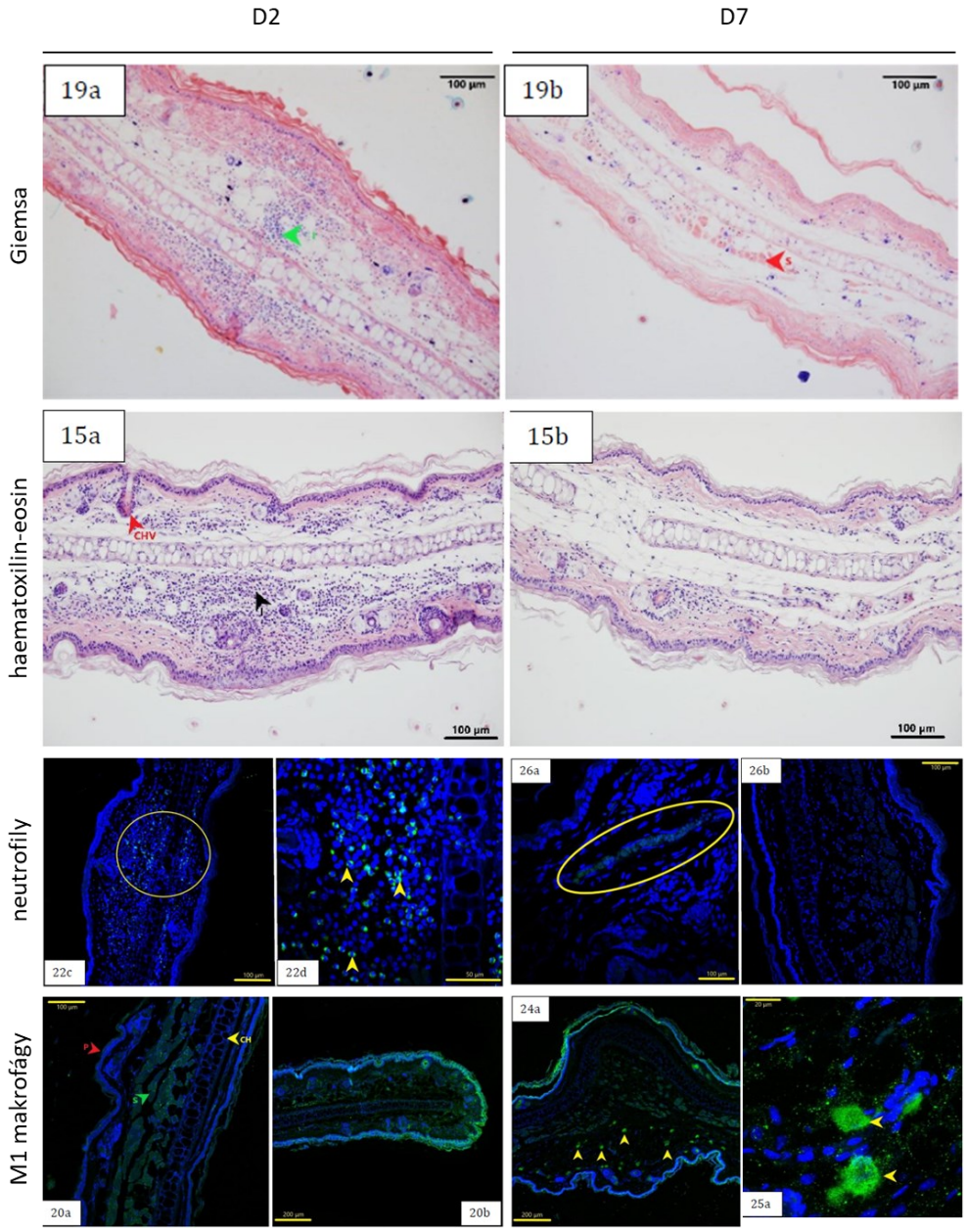
skupina		**D7**	**D2**	**POS**	**NEG**
č. vzorku		12	1	7	14
levé ucho	**plocha léze (mm2)**	113,04	10,17	25,28	0,00
pravé ucho	**plocha léze (mm2)**	78,50	12,01	16,49	0,00
	množství leishmanií (qPCR)	591	456	74 400	0



Obrázek č. 1: Celkový vzhled ušního boltce. Histologické preparáty barvené hematoxylinem-eosinem. 37 a 32 - makrofágy (některé jsou zakroužkované) s amastigoty leishmanií (červené šipky), 30a – nekrotizovaný buněčný materiál v centru léze (zakroužkováno). Upraveno dle Poláková 2022.



Obrázek č. 2: Imunohistochemické značení neutrofilů a klasicky aktivovaných makrofágů. Neutrofily byly značeny primární polyklonální protilátkou proti myeloperoxidáze (Invitrogen, kat. č. PA5-16672) a klasicky aktivované makrofágy primární polyklonální protilátkou proti inducibilní NO syntáze (Invitrogen, kat. č. PA3-030A). Sekundární protilátka byla v obou případech konjugovaná s FITC (Sigma-Aldrich, kat. č. F 0382, zelená). Buněčná jádra byla podbarvena Hoechst 33342 (modrá). **Neutrofily** (myeloperoxidáza): 36b, 31b a 44a – shluky aktivovaných neutrofilů (některé jsou zakroužkované), 44b - žluté šipky označují aktivované neutrofily, 29b - detail oblasti s aktivovanými neutrofily (jeden z nich je označen žlutou šipkou), červené šipky označují cévy s erytrocyty (autofluorescence). **Klasicky aktivované makrofágy** (iNOS): 40b, 45b, 28a a 28b - žluté šipky označují klasicky aktivované makrofágy, 33a a 33b - oblasti s menší lézí vykazující místy slabší fluorescenční signál (příklad zakroužkován), 45a - celkový pohled na lézi s nekrotickou tkání (zakroužkováno), červenými šipkami jsou označeny chlupové váčky (CHV). Upraveno dle Poláková 2022.



Obrázek č. 3: Mikroskopická analýza ušního boltce opakovaně pobodaných myší odebraného 2. a 7. den po posledním sání *P. papatasi.* Histologické preparáty byly barvené giemsou (19a, 19b) a hematoxylinem-eosinem (15a a 15b). Zelená šipka (19a) – shluky zánětlivého infiltrátu prostupujícího chrupavkou, S – svaly, CHV – chlupový váček, černá šipka (15a) - shluky zánětlivého infiltrátu. U imunohistochemických preparátů byly neutrofily značeny primární polyklonální protilátkou proti myeloperoxidáze (Invitrogen, kat. č. PA5-16672) a M1 makrofágy primární polyklonální protilátkou proti inducibilní NO syntáze (Invitrogen, kat. č. PA3-030A). Sekundární protilátka byla v obou případech konjugovaná s FITC (Sigma-Aldrich, kat. č. F 0382, zelená). Buněčná jádra byla podbarvena Hoechst 33342 (modrá). **Neutrofily** (myeloperoxidáza): 22c - shluk aktivovaných neutrofilů v domnělém místě vpichu flebotoma (zakroužkovaná oblast), 22d - detail infiltrátu s aktivovanými neutrofily (některé jsou označené šipkami) v místě sátí flebotoma, 26a (fixace ve 4% PFA) - značená oblast představuje krevní cévu. **M1 makrofágy** (iNOS): 20a (fixace ve 4% PFA) - S = svalová vlákna, CH = elastická chrupavka, P = pokožka, 24a - buněčný infiltrát s aktivovanými makrofágy (některé jsou označené žlutými šipkami), 25a - detail aktivovaných makrofágů (označené žlutými šipkami). Upraveno dle Poláková 2022.