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The immune response of naïve mice infected with the neuropathogenic schistosome *Trichobilharzia regenti*

Imunitní odpověď naivních myší infikovaných neuropatogenní schistosomou *Trichobilharzia regenti*

PH.D. THESIS / DIZERTAČNÍ PRÁCE

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ABSTRACT

Helminth neuroinfections represent a serious health issue, but the mechanisms of the host immune response often remain neglected despite the fact they might contribute to pathogenesis. This is partly due to the unavailability of clinical samples and the lack of suitable laboratory models. Herein, I focused on the characterization of several aspects of the immune response of mice infected with the neuropathogenic avian schistosome *Trichobilharzia regenti*.

After the percutaneous infection of mice (accidental hosts), most *T. regenti* schistosomula are entrapped and eliminated in the skin, but the parasite antigens initiating the protective immune reaction are not known. Our *in vitro* experiments revealed that *T. regenti* cathepsin B2, a cysteine peptidase used for the skin penetration, activates bone marrow-derived dendritic cells much stronger than the parasite homogenate, suggesting its role in initiating the mixed type1/2 host immune response. However, some schistosomula manage to escape from the skin and continue their migration to the spinal cord. Here they crawl preferentially within the white matter which we demonstrated by the robust 3D imaging techniques, ultramicroscopy and micro-CT. The invasion of the spinal cord is accompanied by striking hypertrophy of astrocytes and microglia. We showed that living schistosomula induce production of interleukin 6 in astrocyte cultures, but their homogenate or active isoforms of *T. regenti* cysteine peptidases trigger even stronger reaction, including the increased secretion of tumor necrosis factor α and nitric oxide by astrocytes and/or microglia. It seems that these glial cells actively participate in maintaining the neuroinflammation initiated by the infection. Finally, we examined the role of nitric oxide in the host immune response. Our data show that nitric oxide is produced early in the skin phase of the infection, but it does not directly kill the schistosomula. It rather continuously debilitates the parasite by disrupting its proteolytic machinery.

Taken together, the thesis markedly extends the knowledge of the host-parasite immune interactions between the neuropathogenic schistosomes and their accidental mammalian hosts. These novel data set a good starting point for further research on *T. regenti* neuropathogenicity and the impacts of helminth-caused neuroinflammation on the host. Such findings will be valuable not only in the field of parasitic neuroinfections but might also be appreciated in the research of (autoimmune) neurodegenerative diseases.

Key words: avian schistosomes, *Trichobilharzia regenti*, immune response, skin, spinal cord, astrocytes, microglia, nitric oxide, cysteine peptidases, 3D imaging.

ABSTRAKT

Helmintární neuroinfekce představují závažný zdravotní problém, ale mechanismy hostitelovy imunitní odpovědi často zůstávají opomíjeny, i když se mohou účastnit patogeneze. To je zčásti způsobeno nedostupností klinických vzorků, ale také nedostatkem vhodných laboratorních modelů. V této práci jsem se proto zaměřil na studium vybraných aspektů imunitní odpovědi myší nakažených neuropatogenní ptačí schistosomou *Trichobilharzia regenti*.

Většina schistosomul je u těchto náhodných hostitelů zastavena a eliminována časně po perkutánní infekci ihned v kůži. Není však jasné, jaké parazitární antigeny protektivní imunitní reakci spouštějí. Naše *in vitro* experimenty odhalily, že katepsin B2, cysteinová peptidáza používaná parazitem k penetraci kůže, aktivuje dendritické buňky odvozené z kostní dřeně mnohem více než kompletní parazitární homogenát. To naznačuje její úlohu ve spouštění počáteční imunitní odpovědi, která je polarizována směrem k typu 1/2. Některá schistosomula jsou však schopna kůži opustit a migrují dále do míchy. S využitím 3D zobrazovacích technik, ultramikroskopie a mikro-CT, jsme prokázali jejich preferenční lokalizaci v bílé hmotě. Přítomnost schistosomul v míše vyvolává výraznou hypertrofii astrocytů a mikroglií. Živá schistosomula u astrocytů indukují produkci interleukinu 6, jejich homogenát či aktivní isoformy cysteinových peptidáz však u astrocytů a/nebo mikroglií zvyšují i sekreci tumor nekrotizujícího faktoru alfa a oxidu dusnatého. To naznačuje aktivní zapojení těchto gliových buněk do udržování neurozánětu indukovaného infekcí. V poslední části práce jsme studovali vliv oxidu dusnatého na průběh infekce. Naše data ukazují, že k produkci oxidu dusnatého dochází v časné fázi infekce v kůži, avšak oxid dusnatý u parazita nevyvolává akutní cytotoxicitu. Spíše se zdá, že narušuje jeho proteolytický aparát, což vede k postupnému oslabování parazita.

Tato práce výrazně rozšiřuje znalosti o imunitních interakcích mezi neuropatogenní schistosomou a jejím náhodným savčím hostitelem. Nově získané poznatky představují dobrý výchozí bod pro navazující studium neuropatogenního působení *T. regenti* a vlivu neurozánětu na hostitele. Tato zjištění jsou cenným příspěvkem nejen pro studium parazitárních neuroinfekcí, ale mohou najít uplatnění i při výzkumu (autoimunitních) neurodegenerativních onemocnění.

Klíčová slova: ptačí schistosomy, *Trichobilharzia regenti*, imunitní odpověď, kůže, mícha, astrocyty, mikroglie, oxid dusnatý, cysteinové peptidázy, 3D zobrazování.

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ABBREVIATIONS

(r)TrCB1 (recombinant) *Trichobilharzia regenti* cathepsin B1

(r)TrCB2 (recombinant) *Trichobilharzia regenti* cathepsin B2

µCT micro-computed tomography

BBB blood-brain barrier

BMDC(s) bone marrow-derived dendritic cell(s)

CCL chemokine (C-C motif) ligand

CD cercarial dermatitis

CNS central nervous system

CSF cerebrospinal fluid

CXCL chemokine (C-X-C motif) ligand

DAMP(s) danger-associated molecular pattern(s)

DC(s) dendritic cell(s)

dpi day(s) *post* infection

ESP excretory/secretory products

GFAP glial fibrillary acidic protein

hpi hour(s) *post* infection

HSF soluble fraction of living schistosomula homogenate

IFN-γ interferon gamma

IL interleukin

iNOS inducible NO synthase

ISF interstitial fluid

LS living schistosomula

MHC major histocompatibility complex

MIP-1α macrophage inflammatory protein 1 alpha

MMPs matrix metalloproteases

NCC neurocysticercosis

NO nitric oxide

NT neurotoxocarosis

PAMP(s) pathogen-associated molecular pattern(s)

TGF-β transforming growth factor beta

TNF-α tumor necrosis factor alpha

1. INTRODUCTION

Parasitic helminths often invade the central nervous system (CNS) of mammals, including humans. Invasion of the CNS is either a natural part of the helminth somatic migration or it represents an unwanted, ectopic localization (Finsterer and Auer 2013). The clinical manifestation of helminth neuroinfections ranges from mostly asymptomatic to very severe which leads to sensory or cognitive deficits and seizures or epilepsy (Carpio *et al.* 2016, Vezzani *et al.* 2016, Garcia *et al.* 2019). Many factors, such as parasite burden, size, motility, or localization within the CNS, influence the course and outcome of the neuroinfection. Moreover, the host immune response affects helminth growth and survival but might also participate in pathogenesis (Adalid-Peralta *et al.* 2018). Thus, a better understanding of host-helminth immune interactions is essential to get a comprehensive insight into the biology of neuroinfections. It would allow us to develop better protective measures, treatment strategies, and diagnostic tools. Additionally, the lessons learned from the neuroinfections could be potentially utilized in the study of immunopathological processes associated with (autoimmune) neurodegenerative diseases (Fan *et al.* 2015).

In the theoretical part of the thesis, I briefly introduce the general concepts of the immune response in the CNS and address it specifically for selected helminth neuroinfections. Then I move to schistosomes (blood flukes) and summarize the current knowledge about *Trichobilharzia regenti*, the neuropathogenic schistosome used in the practical part of the thesis. Its inherent neurotropic behavior in birds and mammals (definitive and accidental hosts, respectively) makes *T. regenti* a natural model for exploration of the helminth-parasite interactions either in the CNS or in the periphery. Original results of the practical part are then presented within five articles published/accepted for publication in peer-reviewed journals.

1.1. The immune response in the CNS

The CNS has traditionally been regarded as an immune-privileged site due to its incapability to launch an immune reaction against alloantigens. They were believed to be virtually sequestered within the nervous tissue not being exposed to and recognized by the immune system. The unusual ability of implanted tumors or normal tissue grafts to survive in the brain parenchyma (Murphy and Sturm 1923, Medawar 1948, Widner and Brundin 1988) and ingenious barrier systems preventing unwanted leukocyte influx into the CNS (Reese and Karnovsky 1967, Brightman and Reese 1969, Saunders *et al.* 2014) have supported the view of the CNS immune privilege for decades. Furthermore, pathogen- or danger-associated molecular patterns (PAMPs or DAMPs, respectively) do not induce a strong innate immune response (Andersson *et al.* 1992, Schnell *et*

al. 1999, Locatelli *et al.* 2012) and bacterial or viral antigens injected into the parenchyma mostly remain unnoticed by the host adaptive immune response (Matyszak and Perry 1995, Stevenson *et al.* 1997, Matyszak and Perry 1998). Such immunologically quiescent conditions offer pathogens a suitable niche to establish latent infections (Forrester *et al.* 2018).

However, several contrasting observations, such as efficient detection of antigens implanted into the ventricular system (Murphy and Sturm 1923, Matyszak and Perry 1996, Stevenson *et al.* 1997), CNS antigen drainage into the deep cervical lymph nodes (Harling-Berg *et al.* 1989, Cserr *et al.* 1992) or the recent (re)discovery of meningeal lymphatic vessels (Louveau *et al.* 2015b), cast doubt on the paradigm of the immune privilege absoluteness. In this respect, the CNS should be considered immunologically "unique" (Louveau *et al.* 2015a) rather than "privileged" as the latter might be misunderstood or misinterpreted. Also, the term "brain-associated immune deviation" was introduced pointing to the inherent active maintenance of the suspended immune status in the CNS (Wenkel *et al.* 2000).

Some of the specific features related to the CNS immunology are demonstrated below focusing on the recent advances in the field. Specifically, these topics are covered: the blood-brain barrier, the afferent and efferent communication of the CNS with the peripheral immunity and immunoregulatory mechanisms of the CNS parenchyma.

1.1.1. Specific features of the CNS immunity

The blood-brain barrier (BBB), separating the CNS parenchyma from the circulatory system, is the most striking anatomic feature related to the CNS immunity. In capillaries, the BBB is composed of (a) endothelial cells bound together by tight junctions, (b) pericytes incompletely covering the vascular endothelium, (c) the basement membrane coating the endothelium and pericytes, and (d) astrocyte foot projections forming *glia limitans* around the basement membrane and the pericytes (Sharif *et al.* 2018). Additionally, perivascular spaces inhabited by long-lived antigen presenting myeloid cells are found in the postcapillary venules (Owens *et al.* 2008, Faraco *et al.* 2017). Due to the hard-to-breach architecture, the BBB was regarded as the static physical barrier guarding the fragile CNS parenchyma against external invaders – either pathogens or immune cells. However, the BBB has essential physiological roles in healthy individuals as it regulates the transport into and out of the CNS to maintain parenchymal homeostasis. Hence, the term "neurovascular unit" has been established to highlight the dynamic and complex functions of this structure (Hawkins and Davis 2005, Villabona-Rueda *et al.* 2019).

Despite historical doubts, the CNS mutually communicates with the peripheral immune system. The afferent way (CNS → periphery), including the antigen drainage and presentation to naïve T cells, mostly relies on the soluble route. Specifically, the CNS antigens from the ventricular or subarachnoid spaces are drained by the cerebrospinal fluid (CSF) either (a) into the blood through arachnoid villi (especially in humans) (Upton and Weller 1985) or (b) into the deep cervical lymph nodes *via* the nasal and dural lymphatic vessels going through the cribriform plate of the ethmoid bone (especially in rodents) (Kida *et al.* 1993, Aspelund *et al.* 2015, Louveau *et al.* 2015b, Norwood *et al.* 2019). Interestingly, the CSF pathway does not take a significant amount of the parenchymal antigens contained in the interstitial fluid (ISF) which are rather drained through the perivascular spaces within vessel walls (Carare *et al.* 2008). The connection between the CSF and the ISF called "glymphatic system" has been proposed (Iliff *et al.* 2012) but its physiological and immunological importance is still a matter of a debate (Louveau *et al.* 2017, Abbott *et al.* 2018).

Considering the cellular afferent way, the healthy CNS was originally considered to lack classical dendritic cells (DCs) known from the peripheral tissues. It corroborated with the presumed immune privilege status (Hart and Fabre 1981). Later, the DCs capable of T cell priming were described both in the perivascular and parenchymal sites of the inflamed brain tissue (Fischer and Reichmann 2001). However, their origin and relationship to other populations in the CNS, such as microglia or macrophages, remained unclear or even controversial as well as their presence in non-inflamed tissue (Greter *et al.* 2005, Bulloch *et al.* 2008, Dando *et al.* 2016, Papadopoulos *et al.* 2020). The puzzle was recently solved thanks to the precise microdissection of the particular CNS compartments and application of the mass cytometry. The study revealed that conventional DCs are localized in the healthy leptomeninges and choroid plexus but are rarely found in the parenchyma (Mundt *et al.* 2019). However, myeloid populations associated with the healthy or inflamed CNS display enormous phenotype heterogeneity and functional diversity (Ajami *et al.* 2018, Mundt *et al.* 2019, Dando *et al.* 2019). This makes the traditional naming and characterization of the cell populations no less than challenging. A similar portion of uncertainty/controversy has accompanied the migration of the DCs from the CNS to the lymph nodes. Passage of the DCs from brains to the lymph nodes was suggested during neuroinflammation (van Zwam *et al.* 2009, Schiefenhövel *et al.* 2017) and they were also reported from the healthy meningeal lymphatic vessels (Louveau *et al.* 2015b). However, these observations need to be further clarified and validated.

The efferent way (periphery → CNS) includes the migration of peripheral immune cells into the CNS. Three major routes from blood to the CNS were identified: (1) across the choroid plexus to

the CSF, (2) through the meningeal vessels to the subarachnoid space filled with the CSF, and (3) *via* the perivascular spaces of the inflamed parenchymal postcapillary venules (Kerfoot and Kubes 2002, Ransohoff *et al.* 2003, Kivisäkk *et al.* 2003, Reboldi *et al.* 2009). While leukocytes (mainly CD4+ memory T cells) colonize the CSF under physiological conditions, the brain parenchyma is believed to lack leukocytes in young individuals without any kind of neuroinflammatory disease (Svenningsson *et al.* 1995, Kivisäkk *et al.* 2003, Engelhardt *et al.* 2017). T cells can be sometimes found within the perivascular spaces of healthy brains but are halted here if they do not recognize a cognate antigen displayed on the perivascular antigen presenting cells (Krakowski and Owens 2000, Greter *et al.* 2005, Herz *et al.* 2011). However, infiltration by leukocytes of the brain parenchyma was reported from aged healthy individuals. It presumably correlates with the aging-dependent breakdown of the BBB and can have pathological consequences (Gemechu and Bentivoglio 2012, Montagne *et al.* 2015, Ritzel *et al.* 2016, Erickson and Banks 2019).

Several immunoregulatory mechanisms are employed in the CNS parenchyma to prevent inflammation-related damage of the nervous tissue. These mechanisms target pro-inflammatory activities of immigrating peripheral leukocytes as well as resident parenchymal microglia. Neurons and astrocytes are responsible for most of the immunoregulatory mechanisms. For example, neurons themselves express relatively low amounts of major histocompatibility complex (MHC) class I molecules (Joly *et al.* 1991, Joly and Oldstone 1992). Consequently, fragments of intracellular proteins are less displayed which prevents cytotoxic T cell-mediated killing. On the other hand, it creates a suitable niche for intracellular pathogens, such as tick born encephalitis virus of *Toxoplasma gondii*, which preferably dwell in neurons (Bílý *et al.* 2015, Cabral *et al.* 2016). Additionally, neurons use several pathways to mitigate activation and pro-inflammatory secretion of microglia to reduce their neurotoxicity during neuroinflammation (Neumann *et al.* 1998, Mott *et al.* 2004, Cardona *et al.* 2006, Mizuno *et al.* 2011).

Anti-inflammatory properties can also be ascribed to astrocytes. For example, they attenuate the microglia-driven neuroinflammation and associated neuronal damage by secretion of regulatory cytokines (e.g., transforming growth factor beta, TGF-β) (Aloisi *et al.* 1997, Norden *et al.* 2014, Cekanaviciute *et al.* 2014). Furthermore, astrocytes might trigger apoptosis in infiltrating immune cells by expression of FasL (Bechmann *et al.* 1999, Kohji and Matsumoto 2000, Bechmann *et al.* 2002) or induce upregulation of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), the T cell "switch-off" receptor (Gimsa *et al.* 2004). Beyond the regulatory processes directly in the CNS parenchyma, a growing body of evidence suggests that gut microbiome significantly influences both astrocytes and microglia with potential consequences for the severity of the neuroinflammation (Erny *et al.* 2015, Rothhammer *et al.* 2016, Fung 2020).

1.1.2. The host-immune response against neurotropic helminths

Apart from the specific features of the CNS immunity, biology of the particular parasite species must be considered in studies of the host immune response to interpret the results in an appropriate context. For example, parasitic helminths are multicellular animals that can mechanically break the BBB architecture during the migration towards and within the CNS. This might facilitate uncontrolled infiltration of the CNS parenchyma by peripheral leukocytes and serum proteins (such as complement) which normally do not pass through the selectively semipermeable BBB (Abbott 2002). Of note, such a strategy is different from unicellular pathogens that usually cross the BBB more gently (Forrester *et al.* 2018).

Also, helminth migration within the host is not restricted exclusively to the nervous tissue even in the case of strictly neurotropic species. Indeed, they infect their vertebrate hosts percutaneously or perorally and proceed with a somatic migration including the CNS (Finsterer and Auer 2013). Consequently, the host immune system is already activated (after the initial peripheral encounter with the helminth) at the point when the CNS itself is invaded. The intensity and polarization/type of such peripheral reaction should be considered as it can significantly influence the CNS immune status and readiness (Hoogland *et al.* 2015, Hoogland *et al.* 2018, Huang *et al.* 2018, Tejera *et al.* 2019). Additionally, not all individuals usually make their way to the CNS. A part of them can be eliminated soon after the infection at the entry site (Kouřilová *et al.* 2004a) and some migrate through tissues other than the CNS, depending on the level of their neurotropism (Janecek *et al.* 2014). In both cases, the "out-of-CNS" individuals represent a plentiful source of antigens boosting the peripheral immunity which must be taken into account when interpreting data related to the CNS immune reaction.

The availability of suitable laboratory models largely dictates the orientation of the helminth neuroinfection research. Neurocysticercosis and neurotoxocarosis are hence in focus as their models have been successfully established and deeply explored (de Lange *et al.* 2019, Strube *et al.* 2020). On the contrary, this is not the case of many other human helminth neuroinfections (overviewed in Table 1, see page 6). For example, despite human schistosomosis is #1 helminth disease, a valid model representing its neurological form is lacking (Silva *et al.* 2002, Lambertucci *et al.* 2014, Tan *et al.* 2019). Thus, new *in vivo* model systems for studying helminth neuroinfections are needed to reveal the diversity of host-parasite immune interaction in order to develop better treatment and prevent immunopathological sequelae injuring the host. Knowledge gained for recently studied neuroinfections (see Sections 1.1.3–1.1.5) represents a good starting point in this field.

Table 1. An overview of helminths affecting the CNS of humans[1]. The species typically associated with the CNS pathology are marked by an asterisk (*) (Katchanov and Nawa 2010, Garcia *et al.* 2013, Finsterer and Auer 2013). A question mark (?) indicates expected, but not experimentally confirmed data.

Helminth	CNS invading stage	Mode of the CNS invasion
TREMATODES		
Paragonimus spp.	juveniles	migration within soft tissues (?)
Schistosoma spp.*	eggs	hematogenous dissemination
CESTODES		
Echinococcus spp.*	hexacanths	hematogenous dissemination
Spirometra spp.	plerocercoids	migration within soft tissues (?)
*Taenia solium**	hexacanths	hematogenous dissemination
NEMATODES		
Angiostrongylus spp.*	larvae (L3)	hematogenous dissemination
Baylisascaris spp. *	larvae (L3)	hematogenous dissemination
Gnathostoma spp.	larvae (L3)	migration within nerves or soft tissues (?)
Toxocara spp.*	larvae (L3)	hematogenous dissemination
Trichinella spp.	larvae (L1)	hematogenous dissemination

1.1.3. Neurocysticercosis

Neurocysticercosis (NCC) is perhaps the most common helminth infection of the human CNS. It is caused by hexacanths of the pork tapeworm *Taenia solium* which are disseminated into the CNS where they develop into cysticerci (cysts). Humans hence become the intermediate hosts. NCC affects 2.5–8.3 million people living mostly in rural areas of developing countries in America, Asia, and Africa. Of note, it is the most frequent preventable cause of epilepsy being responsible for 30% of epilepsy cases in those endemic areas (WHO 2019 [online]). The worldwide distribution and grave effects on human health make NCC #1 among helminth neuroinfections. Thus, enormous scientific efforts have been made to reveal the host immune response and its role in the NCC pathogenesis. The large availability of human clinical samples and the development of diverse animal models (Arora *et al.* 2017, de Lange *et al.* 2019) have significantly facilitated the research. As the topic is regularly and extensively reviewed (e.g., Garcia *et al.* 2014, Fleury *et al.* 2016, Gonzales *et al.* 2016, Prodjinotho *et al.* 2020, Garcia *et al.* 2020), the "big picture" of the host immune response will be introduced here to enable a comparison of NCC

[1] Admittedly, more species have been reported to infect the human CNS (e.g., *Fasciola hepatica*, *Strongyloides stercoralis*, filarial species etc.) (Garcia *et al.* 2013). However, such cases are scarce, and the species do not typically exhibit neurotropic behavior and neurological pathology.

with neurotoxocarosis and neuroschistosomosis presented later (see Sections 1.1.4 and 1.1.5, respectively).

The localization of the cysts within the CNS (parenchymal or extraparenchymal) and their viability (viable or degenerating/dying) define the host immune reaction and clinical outcome of the disease. The parenchymal cysts are usually small (<2 cm in humans), stationary and no significant inflammatory response develops around them as far as they are viable. It seems to be due to the active evasion/suppression of the host inflammatory reactions, possibly by induction of the tolerogenic dendritic cells or anti-inflammatory M2 macrophages/microglia (Rodríguez-Sosa *et al.* 2002, Terrazas *et al.* 2011, Sun *et al.* 2014, Chauhan *et al.* 2015, Quenum Zangbede *et al.* 2018). The parasite excretory/secretory products (ESP) are most likely responsible for these effects. A positive correlation between the number of the brain cysts (i.e., the amount of the released ESP) and the anti-inflammatory milieu supports this hypothesis (Tharmalingam *et al.* 2016). Astrogliosis, activation of microglia and a mixed Th1/Th2 response might occur early after the infection (Toenjes and Kuhn 2003, Mejia Maza *et al.* 2019) but it is soon shifted to Th2 which is associated with the asymptomatic course of the disease (Chavarría *et al.* 2003, Verma *et al.* 2011a).

As the cysts degenerate and die, either naturally or due to the anthelminthic treatment, they lose the ability to modulate the host immune response and their intrinsic antigens are also newly exposed to the immune system (Restrepo *et al.* 1998, Uddin *et al.* 2010, Amit *et al.* 2011). Consequently, strong neuroinflammation develops being accompanied by remarkable gliosis and infiltration of the peripheral leukocytes into the affected nervous tissue (Alvarez *et al.* 2002, Singh *et al.* 2015a, Mishra *et al.* 2016, Sampaio *et al.* 2020). The strong immune reaction is driven by pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-1β, and IL-6, but pro-inflammatory/regulatory IL-4, IL-10, and IL-13 can also be present to control the neuroinflammation (Restrepo *et al.* 2001, Sáenz *et al.* 2012). Antigens of scolexes and cyst membranes trigger the expression of a plethora of chemokines, such as chemokine (C-C motif) ligand (CCL) 2, chemokine (C-X-C motif) ligand (CXCL) 8 or CXCL10. Astrocytes and monocytes seem to be the major sources of these chemokines and those produced by recruited γδ T cells likely amplify the response. Indeed, depletion of γδ T cells significantly reduces the cellular infiltration into the CNS (Cardona *et al.* 2003, Uddin *et al.* 2005, Uddin *et al.* 2006, Uddin *et al.* 2010). The increased production of proinflammatory cytokines, chemokines and matrix metalloproteases (MMPs) results in the disruption of the BBB. It further facilitates the influx of the peripheral leukocytes and boosts the neuroinflammation (Alvarez and Teale 2007, Alvarez and Teale 2008, Prasad *et al.* 2009, Verma *et al.* 2011b, Marzal *et al.* 2014, Mahanty *et al.* 2015, Singh *et al.* 2015b). Calcified nodules continuously form around the

destroyed cysts and the adjacent nervous tissue exhibits noticeable astrogliosis indicating the tissue repair by a glial scar after the exacerbated neuroinflammation (Fleury *et al.* 2016).

Headache, dizziness, seizures, or even epilepsy are the main clinical manifestations of pathological processes in the CNS associated with the degenerating/dying cysts (Garcia *et al.* 1993, Ndimubanzi *et al.* 2010, Garcia *et al.* 2020). Substance P, a neuropeptide belonging to the tachykinin family, was identified as a mediator of the seizures making them potentially preventable or treatable by administration of the appropriate receptor antagonists (Robinson *et al.* 2012). It might be beneficial for the patients as corticosteroids or antiepileptics, currently administered along with the anthelminthic drugs to prevent seizures, could have unwanted side effects. This example clearly shows that the precise examination of neuroinfection-related immunopathology might bring important data for prevention or treatment.

NCC caused by the extraparenchymal cysts is much less explored as it is diagnosed less frequently despite it can have more severe clinical consequences (Chavarría *et al.* 2005). Indeed, the cysts localized in the CNS ventricles or subarachnoid spaces can grow and spread which increases morbidity and mortality of the extraparenchymal NCC (Baro *et al.* 2020, Prodjinotho *et al.* 2020). The clinical manifestation includes obstructive hydrocephalus, increased intracranial pressure, compression of the brainstem, and meningitis (Bazan *et al.* 2016). The patients suffering from the extraparenchymal NCC exhibit elevated parasite-specific IgG in the CSF, which has a pro-inflammatory cytokine profile accompanied by increased production of reactive oxygen species (Chavarría *et al.* 2005, Rodríguez *et al.* 2008). Contrary to the NCC associated with parenchymal cysts, the inflammation is triggered even if the cysts are viable, but the reason for this is not known. The possible explanation might be that the cysts are growing and produce more membrane antigens with pro-inflammatory effects that exceed the antigens with an immunoregulatory function (Garcia *et al.* 2020). Furthermore, the intimate contact of the cysts with the CSF and more intense draining of the parasite antigens towards the lymph nodes might fuel the host immunity. However, none of this has yet been proven.

1.1.4. Neurotoxocarosis

Neurotoxocarosis (NT; or cerebral toxocarosis) results from the invasion of the CNS by larvae of *Toxocara canis* or *T. cati*, ascarid intestinal roundworms frequently infecting dogs and cats, respectively. Humans and rodents represent paratenic hosts who become infected by ingestion of embryonated eggs. The parasites are not able to develop in the paratenic hosts and migrate throughout their body. It usually triggers a tissue inflammation in the affected organs, such as liver and lungs (*larva migrans visceralis*), eyes (*larva migrans ocularis*) or the CNS (NT). An

enigmatic “covert/common” toxocarosis, displaying nonspecific symptoms, has also been recognized in humans (Ma *et al.* 2018). Serological studies revealed a worldwide distribution of human toxocarosis, but the real number of people infected with *Toxocara* spp. is hard to reach as most of the cases are likely asymptomatic and remain unnoticed (Rostami *et al.* 2019, Nicoletti 2020). For example, only around 100 human cases of NT have been described in the past 70 years which is considered very low and pointing to underestimation and neglect of the disease (Nicoletti 2016, Deshayes *et al.* 2016, Nicoletti 2020). Indeed, the human samples are scarcely available for studying the CNS immune response, perhaps except for *post mortem* examinations. Hence, animal models of NT are the main source of information.

Mice are the most frequently used experimental hosts of *Toxocara* spp. which corroborates with their role of paratenic hosts even under the natural conditions (Antolová *et al.* 2013, Strube *et al.* 2013, Krücken *et al.* 2017). Three phases of murine toxocarosis can roughly be defined: (1) the acute phase (0–14 days *post* infection; dpi) associated with the initial somatic migration of the larvae mostly into the liver and lungs, (2) the subacute phase (14–28 dpi) characterized by the onset of the CNS neuroinflammation and the host behavioral changes, and (3) the chronic phase (after 28 dpi) when the behavioral changes and pathological processes progressively deteriorate (Janecek *et al.* 2014, Resende *et al.* 2015, Janecek *et al.* 2017, Ruiz-Manzano *et al.* 2019, Strube *et al.* 2020). The larvae invade the CNS as soon as 2–3 dpi (Janecek *et al.* 2014, Resende *et al.* 2015) and then continuously accumulate in the nervous tissue[2]. The species believed to prevail in human infections, *T. canis*, exhibits a stronger affinity towards the CNS in mice than *T. cati* and causes more severe pathology. Also, *T. canis* preferably migrates into the cerebrum while *T. cati* tends to gather in the cerebellum (Janecek *et al.* 2014).

Severe pathology accompanies the larval migration within the CNS. Specifically, hemorrhagic lesions, BBB impairment, parenchymal damage, neuronal death, and axonal injury accompanied by accumulation of β-amyloid precursor protein were recorded in mice. Demyelination and downregulation of myelin-associated genes also contribute to the CNS pathology (Liao *et al.* 2008a, Cardillo *et al.* 2009, Janecek *et al.* 2014, Heuer *et al.* 2015, Springer *et al.* 2019). The pathological effects are presumably associated with the mechanical tissue damage caused by the migrating larvae, but the impact of the host immune response cannot be excluded.

The larval migration activates astrocytes and microglia, the CNS resident immune cells. Astrocytes are hypertrophied as revealed by increased production of glial fibrillary acidic protein

[2] Nevertheless, other patterns of the CNS colonization, such as biphasic or peaking at the beginning of the chronic phase, were also suggested (reviewed by Strube *et al.* 2020).

(GFAP) and likely participate in the tissue and BBB repair. Microglia are often found as "gitter cells" phagocytosing myelin but their other than scavenger functions during the infection (e.g., anti-parasitic, immunoregulatory) have not been addressed yet (Othman *et al.* 2010, Janecek *et al.* 2014, Eid *et al.* 2015, Springer *et al.* 2019). Othman *et al*. (2010) suggested that nitric oxide (NO) production by "glial cells" might have detrimental effects on the nervous tissue. However, their hypothesis, based only on immunohistochemical staining of inducible NO synthase (iNOS), needs to be tested in appropriate experimental setup. Overall, further functional and phenotype analyses are needed to reveal the roles of astrocytes and microglia during NT.

Infiltration of peripheral leukocytes into the CNS parenchyma is rarely observed in mice (Liao *et al.* 2008a, Liao *et al.* 2008b, Othman *et al.* 2010, Eid *et al.* 2015) even though human NT often manifests as eosinophilic meningoencephalitis (Moreira-Silva *et al.* 2004, Fan *et al.* 2015). The specific reason for this is not known but is probably related to slightly different pathology and course of the infection in various mouse strains (Epe *et al.* 1994, Cox and Holland 2001). Indeed, Springer *et al*. (2019) recently observed perivascular cuffs containing eosinophilic granulocytes in C57BL/6 mice. Nevertheless, the larvae are not trapped by any of the immune cells and no granulomas are usually found in the mouse CNS (Liao *et al.* 2008a, Cardillo *et al.* 2009, Janecek *et al.* 2014, Springer *et al.* 2019). This could be explained by immunological non-reactivity of the larvae or better by immunomodulation/immune evasion of the host (Maizels 2013). Alternatively, the simple fact that the larvae are big (350–400 µm in length) and actively moving organisms should be considered as they might simply escape the host immune cells (Xinou *et al.* 2003). It agrees with scarce findings of formed, but "empty" granulomas in the CNS of NT human patients (Dent *et al.* 1956, Hill *et al.* 1985, Nelson *et al.* 1990). Collectively, these data indicate that cell-based immune response is not protective against NT either in mice or men.

Cytokines/chemokines direct the neuroinflammation during NT and so are co-responsible for the disease outcome. Corroborating with the generally "proinflammatory" picture of NT, upregulated expression of genes coding for IL-5, IL-6, TNF-α, IFN-γ, but also regulatory IL-10 were detected in the brains of infected mice at various timepoints (Hamilton *et al.* 2008, Othman *et al.* 2010, Eid *et al.* 2015). However, a comprehensive look at the cytokine/chemokine profile was missing until the microarray gene expression analysis of brains performed by Janecek *et al*. (2015) in the chronic phase. They revealed that IL-4, IL-5, IL-6, IL-13, and IL-19 were among the most differentially expressed cytokines being accompanied by an increased expression of many chemokines. These cytokine/chemokine expression data were recently challenged by a large-scale study measuring the real concentration of selected cytokines/chemokines in the affected CNS tissues (Waindok and Strube 2019). Interestingly, the levels of pro-inflammatory cytokines

(e.g., TNF-α or IFN-γ) never exceeded those measured in the healthy mice and continuously decreased during the infection. On the contrary, IL-4 and IL-5 rocketed in the acute and subacute phase while CCL11 (or eotaxin 1) and CCL3 (or macrophage inflammatory protein 1 alpha, MIP-1α) were elevated all the time which correlates with the previous expression data (Waindok and Strube 2019). Production of anti-inflammatory bioactive lipid mediators might help to control the inflammation (Waindok *et al.* 2019) which would be beneficial both for the parasite and the host.

The collapse of the CNS homeostasis, related to exacerbated neuroinflammation or disruption of neurotransmitters (Othman *et al.* 2010), is reflected in motoric dysfunction, reduced activity and anxiety or impairment of learning and memory in infected mice (Olson and Rose 1966, Hamilton *et al.* 2006, Janecek *et al.* 2017). Neurological malfunctions (e.g., extremity weakness, paresis, or cognitive disorders) appear also in NT human patients and their severity is dependent on the number and localization of the larvae (Finsterer and Auer 2007). NT was even speculated to be associated with the development of mental retardation or neurological disorders, such as Alzheimer's disease and epilepsy (Nicoletti *et al.* 2007, Nicoletti *et al.* 2008, Holland and Hamilton 2013, Fan *et al.* 2015, Gale *et al.* 2016, Chou *et al.* 2017). These hypotheses must urgently be tested in order to (re)evaluate the risk which human toxocarosis might pose to millions of people worldwide.

1.1.5. Neuroschistosomosis

Neuroschistosomosis (NS) arises from the lodging of *Schistosoma* spp. eggs within the CNS. The cerebral form is usually caused by *S. japonicum*, while *S. mansoni* and *S. haematobium* affect the spinal cord. The reason for this is the size and shape of the eggs – those of *S. japonicum* are smaller and have no protrusions which enable their dissemination *via* blood circulation as far as to the brain. On the contrary, bigger eggs of *S. mansoni* and *S. haematobium*, even with protrusions, usually get stuck lower in the spinal cord (Scrimgeour and Gajdusek 1985). However, the CNS involvement seems to be a bit unusual as it is reported from less than 5% of schistosomiasis patients (Watt *et al.* 1986, Ferrari and Moreira 2011, Ross *et al.* 2012).

The similar pattern is observed also in mice in which egg dissemination into the CNS is rare or even negligible event (Aloe *et al.* 1996, Silva *et al.* 2002, Fan and Kang 2003, Lambertucci *et al.* 2014, Alves Fidelis *et al.* 2018, Dang-Trinh *et al.* 2018, Carvalho *et al.* 2019). It makes the classically (i.e. percutaneously by cercariae) infected mice unsuitable models to study NS. Consequently, microinjection of the eggs directly into the CNS parenchyma or subarachnoid spaces has been applied in some pilot studies (Wang *et al.* 2011, de Carvalho *et al.* 2017, Tan *et al.*

2019). The microinjection-based approach does not recapitulate the natural course of the infection, but it was shown to work well for modeling intestinal and urogenital schistosomosis (Fu *et al.* 2012, Richardson *et al.* 2014, Mayer *et al.* 2017). Nevertheless, its application in the case of NS needs to be further validated since the protocols have not been standardized yet and do not provide sufficiently plausible and reproducible outcomes. The conclusions on the host immune response during NS are thus largely based on clinical and pathological findings from human patients. It is beneficial as it forestalls a bias possibly related to the use of animal models (Fallon 2000, Cheever *et al.* 2000). On the other hand, it rules out any kind of hypotheses verification by experimentation due to ethical reasons.

The pathogenesis of NS is far from being fully understood but the general features seem to be similar as in the peripheral organs, such as the liver. Specifically, deposition of the eggs in the CNS induces an inflammatory response leading to granuloma formation, which is orchestrated by CD4+ T cells (Ferrari *et al.* 2008). The granulomatous reaction developing around the eggs comprises three stages: (1) the necrotic-exudative stage – the granuloma is large and composed of eosinophils, lymphocytes, plasma cells, and microglia/macrophages, a zone of periovular necrosis is present, the adjacent nervous tissue is congested, edematous, and exhibits astrogliosis; (2) the productive stage – the granuloma is smaller and contains epithelioid and multinucleated giant cells, lymphocytes and plasma cells, no periovular necrosis is evident, egg shells are deformed; (3) the healing stage – the granuloma undergoes fibrotic changes and astrogliosis is present in the adjacent nervous tissue (Pittella 1997, Tan *et al.* 2019). The local inflammation and pressing the nervous tissue by periovular granuloma (the mass effect) are responsible for the pathology. Depending on the granuloma localization, the corresponding clinical form is (a) pseudotumoral encephalic or (b) spinal cord schistosomiasis presenting with (a) nystagmus, speech disturbances, motor weakness, and increased intracranial pressure or (b) lumbar and lower limb pain, muscle weakness and bladder dysfunction (Ferrari *et al.* 2008, Ferrari and Moreira 2011)[3]. Nonetheless, the NS might also remain asymptomatic if the granulomas are very few and sparsely distributed (Ferrari 2004, Ferrari *et al.* 2008).

The cytokine milieu in the CSF of NT patients indicates the Th2 polarization. Specifically, IL-1β, IL-4, IL-6, IL-10, and IL-13 were elevated both in the CSF and serum of NT patients while TNF-α and IFN-γ were found to be significantly decreased (Ferrari *et al.* 2006, Sousa-Pereira *et al.* 2006,

[3] Additionally, acute schistosomal encephalopathy is recognized which is not necessarily associated with egg deposition within the CNS. It presents as vasculitis presumably mediated by eosinophils or immune complexes, but the specific pathogenesis remains unknown (Jauréguiberry *et al.* 2007, Ferrari and Moreira 2011).

Kruschewsky *et al.* 2016). The concentration of IL-4 and IL-6 was even higher in the CSF than serum which suggests intrathecal production of the cytokines and favoring type 2 immunity (Ferrari *et al.* 2006). Considering the cytokine milieu directly in the CNS parenchyma, no data are available for humans, but a recent study tried to address this issue in mice. However, their immunohistochemical images are unsatisfactory and together with inappropriate methodical controls do not allow one to make any conclusions (Carvalho *et al.* 2019).

1.2. Avian schistosomes, neglected relatives of human blood flukes

The family Schistosomatidae (Digenea) includes 14 genera of trematodes parasitizing in birds or mammals (Figure 1) (Horák *et al.* 2019). Human blood flukes (the genus *Schistosoma*) are the most prominent representatives of the family since they cause severe hepato-intestinal or urogenital disease to >140 million people living mostly in poor communities in (sub)tropical areas (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators 2018, McManus *et al.* 2018). However, avian schistosomes should also attract research and public attention due to their worldwide distribution (Lashaki *et al.* 2020) and pathogenicity in vertebrates, including humans.

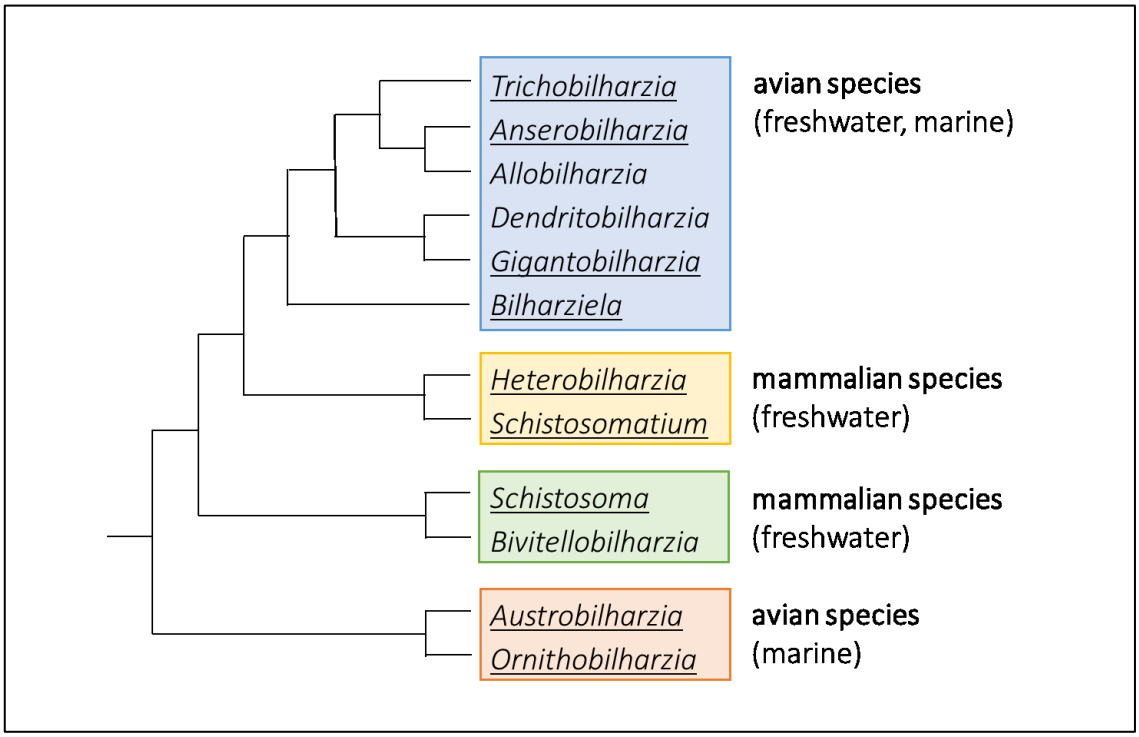

Figure 1. A cladogram of the Schistosomatidae family. Four clades are distinguished each using different definitive hosts (birds or mammals) and environments (marine or freshwater). Underlined genera are confirmed causative agents of human cercarial dermatitis. Adapted from (Horák *et al.* 2015).

Most importantly, avian schistosomes are causative agents of human cercarial dermatitis (CD) also known as swimmer's itch. It is an allergic disease developing after repeated exposure of skin to especially avian[4] schistosomes (Kolářová *et al.* 2013, Macháček *et al.* 2018). In the last decade, the number of reported CD outbreaks has markedly increased (Soldánová *et al.* 2013, Lawton *et al.* 2014, Horák *et al.* 2015, Gordy *et al.* 2018, De Liberato *et al.* 2019, Tracz *et al.* 2019, Gulyás *et al.* 2020). Hence, CD is regarded as a (re)emerging disease that has substantial economic consequences, especially if recreational areas are afflicted (Horák *et al.* 2015). Furthermore, it is recognized as an occupational hazard for people working in contaminated water (Harries 2004), such as rice farmers, hydrologists, environmental samplers, or lifeguards. To develop better protective measures and diagnostic or therapeutic tools, the intricate host-parasite interactions, specifically the host immune response, must be untangled.

Among avian schistosomes, the genus *Trichobilharzia* has become the most conspicuous. Accommodating >30 species, it is the largest genus of the Schistosomatidae family (Brant and Loker 2013) and it is reckoned as the primary etiological agent of CD (Kolářová 2007, Soldánová *et al.* 2013, Horák *et al.* 2015). Two *Trichobilharzia* species, differing in tissue tropism, are readily available for experimental work as their complete life cycle can routinely be maintained under the laboratory conditions: the viscerotropic *T. szidati* (Neuhaus 1952), and the neurotropic *T. regenti* (Horák *et al.* 1998a). Both species are valuable models for studying host-schistosome interactions and involvement of the latter into the "50 Helminth Genomes Project" (Coghlan *et al.* 2019) recently attested significance of the species and avian schistosomes in general.

According to the topic of the presented thesis, the biology of *T. regenti* will further be described with a special emphasis on the infection of and the development in accidental mammalian hosts.

1.2.1. Biology of *Trichobilharzia regenti*, the neurotropic avian schistosome

Trichobilharzia regenti, discovered in Southern Bohemia by Horák et *al*. (1998a), is the avian schistosome widely distributed across Europe (Picard and Jousson 2001, Rudolfová *et al.* 2007, Korsunenko *et al.* 2010, Jouet *et al.* 2010, Christiansen *et al.* 2016, Prüter *et al.* 2017, Marszewska *et al.* 2018). Recently, it was also introduced by migratory birds and game

[4] Admittedly, CD can also be triggered by human schistosomes, such as *S. mansoni*. However, the symptoms are usually not as severe as in the case of CD induced by avian schistosomes. Furthermore, they mostly develop only in naïve persons from non-endemic areas (Boros 1989, Langenberg *et al.* 2020). Outbreaks of CD among farmers caused by repeated exposure to mammalian schistosomes were reported from India and Nepal and were associated mainly with species parasitizing in domesticated animals (reviewed in Horák *et al.* 2015).

waterfowl to northern Iran (Ashrafi *et al.* 2018) and New Zealand (Brant and Davis 2011), respectively, which highlights its epidemiological significance.

The life cycle of *T. regenti* follows a general pattern typical for schistosomes, i.e., two hosts (invertebrate and vertebrate) are exploited and cercariae infect the definitive host by penetration of the skin (Figure 2). However, certain differences between the life cycle of *T. regenti* and other schistosomes, specifically the best-known human species, do exist. They are mentioned in the next paragraph and summarized in Table 2.

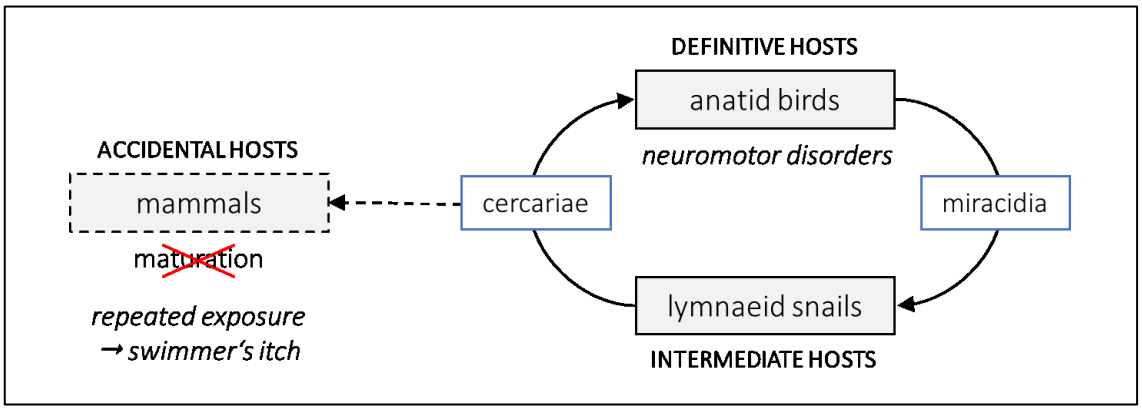

Figure 2. The life cycle of *Trichobilharzia regenti*. Different types of the hosts are depicted in grey boxes, the free-living infectious stages are outlined in blue. Major pathology caused to the vertebrate hosts is shown in italics.

Table 2: A comparison of life cycle features of *Trichobilharzia regenti* and *Schistosoma mansoni*. The first is an avian schistosome used in the thesis, the latter is the major human schistosome widely used as a model species.

	Trichobilharzia regenti	*Schistosoma mansoni*
Number of hosts	2	2
Intermediate hosts	*Radix* spp. (Lymnaeidae)	*Biomphalaria* spp. (Planorbidae)
Definitive hosts	anatid birds	primates, rodents
- mode of infection	percutaneous	percutaneous
- migration	nervous system, meninges	circulatory system
- nutrition	nervous tissue, blood	blood
- final site	nasal mucosa (extravascularly)	visceral veins (intravascularly)
- release	miracidia hatch from eggs directly in tissue	eggs excreted in feces, miracidia hatch in outer environment
Role of humans	accidental hosts	definitive hosts
Primary disease	cercarial dermatitis	hepato-intestinal schistosomiasis

First and foremost, *T. regenti* is the avian schistosome. Anatid birds, such as mallards, mute swans, greylag geese, or diving ducks, serve as definitive hosts (Jouet *et al.* 2010, Skírnisson *et al.* 2012). Contrary to human schistosomes, adults of *T. regenti* dwell in the nasal mucosa of infected birds. Here they copulate and lay eggs, being localized mostly outside of the blood vessels (Chanová and Horák 2007). Miracidia often hatch directly in the nasal tissue (Horák *et al.* 1998a) and are released into the water where they search for the intermediate hosts, lymnaeid snails of the genus *Radix* spp. (Horák *et al.* 1998a, Jouet *et al.* 2008, Huňová *et al.* 2012). After 5–6 weeks of intramolluscan development, ocellate furcocercariae leave the snails (Horák *et al.* 1998a), ready to seek and percutaneously infect the vertebrate host. No published data on host finding and recognition are available for *T. regenti* cercariae, but it is assumed that the stimuli are similar to those used by the closely related species *T. szidati*. It relies on physical (shadow, warmth) and chemical (skin cholesterol and ceramides) signals (Feiler and Haas 1988a, Feiler and Haas 1988b).

After attachment to the vertebrate host, cutaneous unsaturated fatty acids induce emptying of cercarial penetration glands and the released histolytic peptidases facilitate the creeping of the parasite through the skin (Haas and van de Roemer 1998, Mikeš *et al.* 2005). Lipid extracts of both duck and human skin trigger the penetration which suggests that there is no strong preference of cercariae towards the infection of birds (suitable definitive hosts) compared to the mammals (dead-end accidental hosts) (Haas and van de Roemer 1998). The inability to avoid such unfavorable penetration, possibly associated with a short cercarial lifespan, makes mammals "epidemiological sinks" of the parasite (Johnson *et al.* 2019).

After penetration of the skin, the newly transformed *T. regenti* schistosomula search for the peripheral nerves *via* which they migrate towards the spinal cord (Horák *et al.* 1999, Hrádková and Horák 2002). In the definitive hosts, schistosomula continue their migration through the spinal cord, the brain, and meninges to the nasal mucosa where they mature (Hrádková and Horák 2002, Chanová and Horák 2007); a day-by-day time course of the infection in ducks is shown in Table 3 (see page 17). The parasite migration via the CNS is frequently associated with leg paralysis and orientation/balance disorders of the infected birds (Horák *et al.* 1999). Not only is this neurotropic migration pattern completely different from that typical for human/visceral schistosomes[5] (Horák *et al.* 2002, Brant and Loker 2013, Nation *et al.* 2020) but it is also rarely seen among helminths in general (Kristensson *et al.* 2013).

[5] Prüter *et al*. (2017) recently found the visceral schistosome species *Bilharziella polonica* in the spinal cord and brain meninges of mallards from Germany. They suggested the neurotropic infection route analogous to *T. regenti*, but the parasites were not found in the nasal mucosa.

Table 3: Migration of *Trichobilharzia regenti* in experimentally infected domestic ducks. The blue cells indicate the distribution of living *T. regenti* life stages during the infection. Migration data were compiled from several studies (Horák et al. 1999, Kolářová et al. 2001, Hrádková and Horák 2002, Chanová and Horák 2007). Abbreviations: DPI, days *post* infection; PerNe, peripheral nerves; SynSC, synsacral spinal cord; ThoSC, thoracic spinal cord; CerSC, cervical spinal cord; MeOb, medulla oblongata; Cere, cerebellum; Hemi, cerebral hemispheres; Men, meninges; NasM, nasal mucosa.

DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Skin																									
PerNe																									
SynSC																									
ThoSC																									
CerSC																									
MeOb																									
Cere																									
Hemi																									
Men																									
NasM																									

The neurotropic behavior of *T. regenti* was demonstrated also in mice (Hrádková and Horák 2002). However, the course and fate of the infection are different than in ducks as mammals are unsuitable accidental hosts of *T. regenti*. The most prominent feature of such host-parasite incompatibility is effective halting and elimination of the vast majority (ca 90%) of the newly transformed schistosomula in the skin right after the penetration (Kouřilová *et al.* 2004a). The remaining schistosomula can escape to the CNS but most of them stay stuck in the thoracic and cervical spinal cord. Some schistosomula can reach the cerebellum or hemispheres, but the invasion of the brain is considered rather exceptional in immunocompetent mice (Horák *et al.* 1999, Hrádková and Horák 2002, Lichtenbergová *et al.* 2011, Chanová and Hrdý 2016); a day-by-day time course of the infection in mice is shown in Table 4 (see page 18). Furthermore, growth and development of schistosomula are suppressed in mice, possibly by the host immune response and/or the absence of some essential nutritional or stimulatory factors; the parasites never reach maturity (Blažová and Horák 2005).

Table 4: Migration of *Trichobilharzia regenti* in experimentally infected immunocompetent mice[6].
The blue cells indicate the distribution of living *T. regenti* life stages during the infection. Migration data were compiled from several studies (Kolářová *et al.* 2001, Hrádková and Horák 2002, Kouřilová *et al.* 2004a, Kouřilová *et al.* 2004b, Lichtenbergová *et al.* 2011, Chanová and Hrdý 2016, Bulantová *et al.* 2016). Abbreviations: DPI, days *post* infection; PerNe, peripheral nerves; LuSC, lumbar spinal cord; ThoSC, thoracic spinal cord; CerSC, cervical spinal cord; MeOb, medulla oblongata; Cere, cerebellum; Hemi, cerebral hemispheres; Men, meninges; NasM, nasal mucosa.

DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Skin																									
PerNe																									
LuSC																									
ThoSC																									
CerSC																									
MeOb																									
Cere																									
Hemi																									
Men																									
NasM																									

A significant role of the host immune response in the control of the infection in mice is supported by several observations. First, schistosomula migrate faster and more frequently to the CNS in immunocompromised SCID mice which consequently have a higher parasite burden in the CNS compared to immunocompetent strains (Hrádková and Horák 2002, Kouřilová *et al.* 2004b). Second, damaged and dead schistosomula are detected in the CNS of immunocompromised SCID mice at later timepoints (Lichtenbergová *et al.* 2011). Last, enhanced parasite trapping is observed in the skin of repeatedly infected immunocompetent mice which suggests the development of a protective immunity (Kouřilová *et al.* 2004a, Kouřilová *et al.* 2004b). Although several studies aimed to describe the immune response of mice infected with *T. regenti* (see Sections 1.2.2 and 1.2.3), the mechanisms responsible for parasite elimination either in the skin or the CNS remain unknown.

1.2.2. The skin phase of *T. regenti* infection in mammals

The skin is a primary physical barrier of the host which cercariae must breach. Histolytic enzymes are released from cercarial glands and facilitate penetration of the skin (Mikeš *et al.* 2005) (see

[6] Pooled data from infections of BALB/c and C57BL/6J mice are shown since the migration pattern seems to be similar in both strains. However, a thorough comparative study, not performed yet, should be done to verify these observations.

Section 1.2.4). Within the next 12–24 hours, cercariae transform into schistosomula. The process of transformation is accompanied by tail disposal, shedding of glycocalyx (Horák *et al.* 1998b, Řimnáčová *et al.* 2017), the formation of a double membrane covering the tegument (shown *in vitro*; Chanová *et al.* 2009) and switch from aerobic to microaerobic metabolism (Leontovyč *et al.* 2016). These morphological and biochemical changes support the parasite survival in the host. Even though the somatic migration was described for *T. regenti* (see above) as well as for other *Trichobilharzia* species (Olivier 1953, Haas and Pietsch 1991, Horák and Kolářová 2001, Chanová *et al.* 2007, Horák *et al.* 2008), most of the schistosomula are entrapped in the skin by leukocytic infiltrate. It demonstrates the importance of the initial skin immune response in the parasite control.

In naïve mice, penetration of the skin by *T. regenti* cercariae evokes tissue edema, vasodilatation, and the influx of inflammatory cells. Neutrophils are the first leukocytes infiltrating the site of infection as soon as 4–6 hours *post* infection (hpi) when schistosomula are localized mostly in the epidermis or at epidermal/dermal junction (Kouřilová *et al.* 2004b). Schistosomula then move deeper to the dermis and neutrophils and eosinophils accumulate around them 12–24 hpi. To a lesser extent, macrophages, degranulating mast cells, and CD4+ cells also appear in the inflammatory foci which usually dissipate by 4–8 dpi (Kouřilová *et al.* 2004a, Kouřilová *et al.* 2004b). The immune cell infiltration, not observed in immunocompromised SCID mice, is expected to stop and eliminate schistosomula (Kouřilová *et al.* 2004b). The skin inflammation is accompanied by local production of various cytokines, such as IL-1β, IL-6, IL-4, IL-10, IL12p40, or IFN-γ. These data from skin biopsies indicate a mixed Th1/Th2 response which was observed also in the skin draining lymph nodes (Kouřilová *et al.* 2004a).

The skin pathology and immune response markedly differ in mice repeatedly exposed to *T. regenti* cercariae. Pustules and abscesses appear in the epidermis and a mixture of leukocytes massively infiltrates the dermis (Kouřilová *et al.* 2004a). The inflammation, accompanied by perivasculitis, folliculitis, and parakeratosis, is much stronger, compared to naïve mice. It results in a more effective elimination of the schistosomula which are thus only rarely found in the CNS of repeatedly infected mice (Kouřilová *et al.* 2004a, Lichtenbergová *et al.* 2011). Histamine and Th2-associated cytokines, namely IL-4 and IL-10, are substantially produced in the skin early after the penetration. A shift towards Th2 response is later observed also in the skin draining lymph nodes (Kouřilová *et al.* 2004a). A systemic Th2 polarization in repeatedly infected mice is evident from elevated levels of parasite-specific IgG1 and IgE (Kouřilová *et al.* 2004a, Lichtenbergová *et al.* 2008b).

Repeated exposures of mice to cercariae of avian schistosomes, including *T. regenti*, are used as a model of CD. In humans, the disease is manifested by maculo-papulo-vesicular skin eruptions associated with edema and intensive itching. However, a generalized systemic reaction including fever, limb swelling, nausea, or diarrhea can also occur in some individuals (Kolářová *et al.* 2013). The course of CD and severity of the clinical signs and symptoms is related to the degree of host sensitization, i.e., they appear faster and are more pronounced in individuals with a history of CD (Gay *et al.* 1999, Macháček *et al.* 2018); this complies with the observations from mice (Kouřilová *et al.* 2004b). The histopathological image of CD in mice and humans also seems to be comparable, even following the similar timescale (Haemmerli 1953, Gay *et al.* 1999, Kouřilová *et al.* 2004b).

Taken together, the infection of mice/mammals with *T. regenti* induces immediate skin hypersensitivity followed by a late phase inflammatory response. The host immune response is responsible for arresting and elimination of the parasites in the skin, but the specific effector molecules/processes have not been identified yet. Also, the host immune response in the skin is not quite effective, especially if naïve mice are infected, and some schistosomula can continue their migration towards the CNS.

1.2.3. The CNS phase of *T. regenti* infection in mammals

Schistosomula that manage to leave the mouse skin migrate within the epineurium or inside the peripheral nerve fascicles towards the spinal cord which they enter *via* the spinal roots as soon as 2 dpi (Lichtenbergová *et al.* 2011). This early phase of migration through the nervous tissue is associated with no or only weak inflammation or focal edema (Kouřilová *et al.* 2004b). It is not clear whether this is due to immunomodulatory activities of the schistosomula or the generally delayed onset of the immune response in the CNS.

In the spinal cord, the inflammation develops by 6–7 dpi. Lymphocytes, plasma cells, and eosinophils infiltrate the perivascular areas, and inflammatory exudates sometimes appear also in the subarachnoid spaces (Kouřilová *et al.* 2004b). Neutrophils, activated microglia, and macrophages gather around the schistosomula or form a typical "rocket tail" infiltration behind them (Kouřilová *et al.* 2004b, Lichtenbergová *et al.* 2011). Schistosomula appear mostly in the gray and white matter, but they can be found also within the leptomeninges or in the central canal of the spinal cord (Kolářová *et al.* 2001, Kouřilová *et al.* 2004b, Lichtenbergová *et al.* 2011, Chanová and Hrdý 2016). They mechanically induce axonal injury and actively feed on myelin, but substantial demyelination is usually not observed (Lichtenbergová *et al.* 2011, Leontovyč *et al.* 2019). Activated astrocytes participate in tissue repair in the schistosomula migration tracks

(Faulkner *et al.* 2004, Sofroniew 2009, Lichtenbergová *et al.* 2011) which sometimes contain hemorrhages (Kouřilová *et al.* 2004b).

As the neuroinflammation escalates 14–21 dpi, the inflammatory lesions around already damaged schistosomula are found with an increasing frequency. Massive clusters of microglia, macrophages, eosinophils, neutrophils, and a few CD3+ lymphocytes enclose the parasites. Especially microglia and macrophages are believed to be responsible for their killing (Lichtenbergová *et al.* 2011), but it has not been proven yet. The intense neuroinflammation also seems to harm the adjacent tissue in which axonal damage or dystrophic/necrotic changes of neurons can be detected (Kolářová *et al.* 2001, Lichtenbergová *et al.* 2011). However, neuromotor disorders are usually not observed in immunocompetent mice. On the contrary, leg paralysis is often reported from immunocompromised mice, in which more frequent axonal injury, resulting from a higher schistosomula burden, is probably responsible for the pathology (Kouřilová *et al.* 2004b, Lichtenbergová *et al.* 2011).

Altogether, invasion of the murine CNS by *T. regenti* schistosomula triggers the neuroinflammation which effectively controls the infection. However, the immunostimulatory capacity of different parasite antigens is not known. Among others, a special attention should be paid to peptidases as they are released into the nervous tissue in large amount (see Section 1.2.4.). Both resident immune cells (microglia and astrocytes) and recruited leukocytes participate in the host immune response. However, their role in parasite clearance or regulation of the neuroinflammation has not been addressed. Additionally, the precise schistosomula tracking within the spinal cord matters should be performed as it might have an impact on the immune response and pathology. Finally, the effects of the neuroinflammation on dynamics of the peripheral immunity remain to be discovered.

1.2.4. Peptidases in the biology of *T. regenti*

Peptidases are multifunctional proteolytic enzymes essential to helminths, including *T. regenti*. They participate in penetration of the host, parasite somatic migration, ontogenetic development, protein digestion, and host-parasite immune interactions (McKerrow *et al.* 2006, Grote *et al.* 2018, Caffrey *et al.* 2018). Consequently, they are promising vaccine candidates and drug targets (Pearson *et al.* 2010, Ricciardi *et al.* 2016, Tallima *et al.* 2017, Stutzer *et al.* 2018, McKerrow 2018). Depending on the chemical nature of the groups responsible for catalysis, several peptidase catalytic types are recognized: aspartic, cysteine, glutamic, metallo, serine, and threonine (Barrett *et al.* 2013).

Cysteine peptidases are the most abundant type of proteolytic enzymes represented in the genome of *T. regenti* (Coghlan *et al.* 2019). Their activity can be detected in cercarial extracts (Mikeš *et al.* 2005, Kašný *et al.* 2007) and transcripts coding for cysteine peptidases are upregulated in schistosomula migrating through the spinal cord (Leontovyč *et al.* 2016, Leontovyč *et al.* 2019). Of all cysteine peptidases, cathepsins B seem to predominate being expressed mainly, but not exclusively, in schistosomula and adults. It suggests their important role during invasion of and survival in the vertebrate hosts (Mikeš *et al.* 2005, Dolečková *et al.* 2010, Leontovyč *et al.* 2019). Two representatives, *T. regenti* cathepsins B1 and B2 (TrCB1 and TrCB2, respectively), have been identified and functionally characterized so far (for a summary see Table 5).

Table 5: A comparison of cathepsin B1 and B2 (TrCB1 and TrCB2, respectively), cysteine peptidases of *Trichobilharzia regenti*. A question mark (?) indicates the expected localization/function.

	TrCB1	TrCB2
Localization	gastrodermis (schistosomula, adults?)	penetration glands (cercariae, schistosomula?)
Substrates	myelin basic protein, albumin, IgG, fibrinogen, collagen, myosin	keratin, collagen, elastin, myelin basic protein, fibrinogen
Biological function	digestion, immune evasion?	skin penetration, tissue migration
Isoforms	active: TrCB1.1–TrCB1.4 inactive: TrCB1.5, TrCB1.6	no isoforms found

TrCB1 is a digestive peptidase localized in the gastrodermis of *T. regenti* schistosomula (Dvořák *et al.* 2005). In this manner, it is similar to the blood-processing cathepsins B1 of adult *S. mansoni* and *S. japonicum* (Caffrey and Ruppel 1997, Sajid *et al.* 2003). However, the substrate preference of TrCB1 is different: it efficiently degrades myelin basic protein while hemoglobin is a poor substrate. Perhaps, it is an adaptation of *T. regenti* to the specific migration route and nutrition mode (nervous tissue) which are different from blood-feeding human schistosomes (Dvořák *et al.* 2005). Recently, other substrates, such as albumin, IgG, fibrinogen, collagen, or myosin, were identified (Dvořáková *et al.* 2020) but the biological relevance of their hydrolysis remains to be evaluated. Interestingly, TrCB1 has six isoforms but two of them (TrCB1.5 and TrCB1.6) are inactive due to the substitution of the catalytic cysteine by glycine (Dvořák *et al.* 2005). Anyway, these inactive isoforms are highly expressed in migrating schistosomula (Leontovyč *et al.* 2016). Their function is not fully understood but the inactive peptidases are generally believed to (a) regulate the activity of active isoforms by competing for substrates or inhibitors (Merckelbach *et al.* 1994, Dvořák *et al.* 2005) or (b) alter the host immunity (Bergström *et al.* 2009, Reynolds *et al.* 2014).

TrCB2 is a peptidase localized in the cercarial post-acetabular penetration glands and presumably participates in the penetration of the vertebrate skin. It is supported by effective degradation of skin proteins (e.g., keratin, collagen, or elastin) by TrCB2 (Dolečková *et al.* 2009). By these features, it is similar to cathepsin B-like peptidases of *S. japonicum* (Dvořák *et al.* 2008, Ingram *et al.* 2011). However, it differs from cathepsin B2 of *S. mansoni* which is found in the tegument of adults and has not yet recognized function (Caffrey *et al.* 2002). By acting in the skin penetration process, TrCB2 rather resembles *S. mansoni* cercarial elastase which is, however, a serine peptidase (Salter *et al.* 2000). Apart from cercariae, TrCB2 is highly expressed also in schistosomula. They use it as a histolytic enzyme enabling migration in the nervous tissue since TrCB2 can degrade myelin basic protein as well (Dolečková *et al.* 2009, Dolečková *et al.* 2010, Leontovyč *et al.* 2016).

Cathepsins B also play an important role in the host-parasite immune interactions. In the case of human schistosomes, they are naturally immunogenic and possibly applicable in immunodiagnostics (Ruppel *et al.* 1987, Ruppel *et al.* 1990, Li *et al.* 1996). Nevertheless, this is probably not the case of *T. regenti* cathepsins B which do not seem to elicit a specific antibody response in ducks, mice, or humans (Lichtenbergová *et al.* 2008a, Turjanicová *et al.* 2015). It was speculated that a 34-kDa antigen recognized by sera from mice repeatedly exposed to *T. regenti* and by those from patients with CD could be TrCB1 (Lichtenbergová *et al.* 2008b). However, the antigen was later identified as a glycolytic enzyme glyceraldehyde-3-phosphate isomerase (Kašný *et al.* 2009). Regarding effects on the cellular immunity, *T. regenti* peptidases released during the skin penetration were proposed to be the major allergens triggering the immediate inflammatory response (Kouřilová *et al.* 2004a). Of note, allergenic/Th2-inducing capacity was shown for cysteine peptidases of other parasites, including schistosomes (Furmonaviciene *et al.* 2000, Pulendran *et al.* 2010). Furthermore, *S. mansoni* cathepsin B1 was recently demonstrated to induce all Th1, Th2, and Th17 responses which suggests that these secreted enzymes might have a broad immunogenic capacity (Soloviova *et al.* 2019). However, the immunostimulatory properties of TrCB1 and TrCB2 and their impact on T cell polarization remain unknown.

Altogether, *T. regenti* is the avian schistosome that deserves research attention as it can be broadly used to study host-parasite interactions:

1) It is a suitable comparative model for human schistosomes. They have a lot in common but still differ significantly in certain features which could tell us a lot about the diversity of life strategies within the Schistosomatidae family.

2) It is an established model used to trigger CD under laboratory conditions. Due to the increasing number of human CD cases, there is an urgent need to develop better diagnostic and therapeutic tools for which it will be indispensable.
3) It is a promising model for studying multicellular neuropathogens. Exploration of factors driving its neurotropism and neuropathogenicity might be useful not only in the field of neuroinfections, but also in neurodegenerative diseases.

2. AIMS OF THE THESIS

Despite >20 years of research on *T. regenti* in mammals, essential knowledge gaps (highlighted in the foregoing literature review) remain in our understanding of the host immune response. In the thesis, we focused on the characterization of several aspects of the host immune response of naïve mice infected with the neuropathogenic schistosome *T. regenti*. The specific aims were:

- Characterize the parasite-specific antibody response within four weeks after the infection and the effect of *T. regenti* antigens on bone marrow-derived dendritic cells.
- Examine the response of murine astrocytes and microglia exposed to *T. regenti* antigens with a special emphasis on the production of cytokines and NO.
- Elucidate the role of NO in the host immune response against *T. regenti*.
- Assess the suitability of fluorescence tracers and 3D-imaging techniques for studying *T. regenti* migration and the host immune response in the CNS of mice.

3. LIST OF PUBLICATIONS

Materials & methods used in the thesis as well as the thesis results are included in five publications listed below in chronological order. My contribution to each publication is clearly stated in *italics*.

PUBLICATION #1

Bulantová J, Macháček T, Panská L, Krejčí F, Karch J, Jährling N, Saghafi S, Dodt H-U and Horák P (2016) ***Trichobilharzia regenti* (Schistosomatidae): 3D imaging techniques in characterization of larval migration through the CNS of vertebrates.** *Micron* **83**: 62–71.

My contribution: Performing experiments (intravital fluorescent staining of cercariae), data analysis and interpretation, writing the associated parts of the manuscript.

PUBLICATION #2

Macháček T, Panská L, Dvořáková H and Horák P (2016) **Nitric oxide and cytokine production by glial cells exposed *in vitro* to neuropathogenic schistosome *Trichobilharzia regenti*.** *Parasit Vectors* **9**: 579.

My contribution: Performing all experiments (except for preparation of recombinant proteins), data analysis and interpretation, writing the manuscript, being the corresponding author.

PUBLICATION #3

Dvořáková H, Leontovyč R, Macháček T, O'Donoghue AJ, Šedo O, Zdráhal Z, Craik CS, Caffrey CR, Horák P and Mikeš L (2020) **Isoforms of cathepsin B1 in neurotropic schistosomula of *Trichobilharzia regenti* differ in substrate preferences and a highly expressed catalytically inactive paralog binds cystatin.** *Front Cell Infect Microbiol* **10**: 66.

My contribution: Performing experiments (effects of TrCB1.6 on astrocytes, microglia, and RAW 264.7 macrophages), data analysis and interpretation, writing the associated parts of the manuscript.

PUBLICATION #4

Majer M, Macháček T, Súkeníková L, Hrdý J and Horák P (2020) **The peripheral immune response of mice infected with a neuropathogenic schistosome.** *Parasite Immunol* **42**: e12710.

My contribution: Performing experiments (sample collection, splenocyte stimulation, cytokine, and antibody ELISA), data analysis and interpretation, writing the associated parts of the manuscript.

PUBLICATION #5

Macháček T, Šmídová B, Pankrác J, Majer M, Bulantová J and Horák P (2020) **Nitric oxide debilitates the neuropathogenic schistosome in mice, partly by inhibiting its vital peptidases.** *Parasit Vectors* accepted.

My contribution: Designing the study, performing experiments (aminoguanidine treatment, peptidase activity, in vitro *preparation and treatment of schistosomula, assessment of schistosomula viability), data analysis and interpretation, writing the manuscript, being the corresponding author.*

In the printed version of the thesis, the publications are placed right after this page. In the electronic version of the thesis, they are uploaded as a separate appendix with restricted access not to infringe on the publishers' rights.

PUBLICATION #1

Bulantová J, Macháček T, Panská L, Krejčí F, Karch J, Jährling N, Saghafi S, Dodt H-U and Horák P (2016)

Trichobilharzia regenti (Schistosomatidae): 3D imaging techniques in characterization of larval migration through the CNS of vertebrates.

Micron 83: 62–71

DOI: 10.1016/j.micron.2016.01.009

PUBLICATION #2

Macháček T, Panská L, Dvořáková H and Horák P (2016)

Nitric oxide and cytokine production by glial cells exposed *in vitro* to neuropathogenic schistosome *Trichobilharzia regenti*.

Parasit Vectors **9**: 579

DOI: 10.1186/s13071-016-1869-7

(open access available)

PUBLICATION #3

Dvořáková H, Leontovyč R, Macháček T, O'Donoghue AJ, Šedo O, Zdráhal Z, Craik CS, Caffrey CR, Horák P and Mikeš L (2020)

Isoforms of cathepsin B1 in neurotropic schistosomula of *Trichobilharzia regenti* differ in substrate preferences and a highly expressed catalytically inactive paralog binds cystatin.

Front Cell Infect Microbiol 10: 66

DOI: 10.3389/fcimb.2020.00066

(open access available)

PUBLICATION #4

Majer M, Macháček T, Súkeníková L, Hrdý J and Horák P (2020)

The peripheral immune response of mice infected with a neuropathogenic schistosome.

Parasite Immunol 42: e12710

DOI: 10.1111/pim.12710

PUBLICATION #5

Macháček T, Šmídová B, Pankrác J, Majer M, Bulantová J and Horák P (2020)

Nitric oxide debilitates the neuropathogenic schistosome in mice, partly by inhibiting its vital peptidases.

Parasit Vectors accepted

4. SUMMARY & CONCLUSIONS

The presented thesis aimed at the characterization of several aspects of the immune response of mice infected with the neuropathogenic schistosome *Trichobilharzia regenti*. The parasite serves as a suitable comparative model for human schistosomes and was established as a trigger of CD. It is also a promising tool for studying multicellular neuropathogens due to its strong inherent affinity towards the nervous tissue. However, the host-parasite immune interactions have insufficiently been explored, especially in naïve, previously uninfected mammals.

The penetration of the mouse skin by *T. regenti* cercariae triggers an influx of inflammatory cells that accumulate around the newly transformed schistosomula. This initial phase of the infection is well characterized histopathologically, especially in repeatedly infected mice suffering from CD, and resembles the reaction mounted against human schistosomes (Incani and McLaren 1984, Kouřilová *et al.* 2004a, Kouřilová *et al.* 2004b). Antigens present in parasite glycocalyx, ESP, and perhaps extracellular vesicles are expected to initiate the host immune response in the case of human schistosomes (Perona-Wright *et al.* 2006, Paveley *et al.* 2009, Kuipers *et al.* 2020), but no data have been available for bird species. Thus, we tested the effect of *T. regenti* antigens on murine bone marrow-derived dendritic cells (BMDCs) (Majer *et al.* 2020 = PUBLICATION #4).

The cercarial homogenate, containing a mixture of surface and somatic antigens, only marginally affected maturation status and inflammation-promoting phenotype of BMDCs. The reason might be that dendritic cells do not normally encounter damaged schistosomula during the initial phase of the infection. They should rather recognize ESP released into the host tissues during the skin penetration as suggested but not tested by Kouřilová et al. (2004a). Accordingly, we demonstrated that the recombinant cysteine peptidase rTrCB2 activated mouse BMDCs and induced expression of *Ccl5*, *Cxcl10*, *Il12*, *Il33*, and *Il10* (Majer *et al.* 2020 = PUBLICATION #4). CCL5 could promote the production of IL-6 which was present in the skin early after *T. regenti* infection (Fischer *et al.* 2001, Kouřilová *et al.* 2004a) and CXCL-10 might boost Th1 response (Bonecchi *et al.* 1998, Khan *et al.* 2000, Vasquez *et al.* 2008). The cytokine profile displayed by rTrCB2-stimulated BMDCs, including IL-10, IL-12, and IL-33, suggests the mixed type 1/2 response, which agrees with the already published data (Kouřilová *et al.* 2004a). The mixed response is also supported by the equal levels of parasite-specific IgG1 and IgG2 associated with Th2 and Th1, respectively (Finkelman *et al.* 1988, Majer *et al.* 2020 = PUBLICATION #4).

The effects of rTrCB2 might be caused either by its direct immunogenicity or by its enzymatic activity which could be sensed by the protease-activated receptors (Bonnart *et al.* 2017, Cano *et al.* 2019). The latter explanation seems to be more probable as it accords with our report of

the inability to stimulate immune cells by enzymatically inactive rTrCB1.6 (Dvořáková *et al.* 2020 = PUBLICATION #3). Similarly, the proteolytic activity of *S. mansoni* cathepsin B1 is necessary for the induction of T cell response in mice (Soloviova *et al.* 2019). However, the hypothesis needs further experimental validation.

The strong skin inflammation is believed to impede *T. regenti* further migration. Indeed, 90% of schistosomula are estimated to be arrested and eliminated here in mammals (Kouřilová *et al.* 2004a). It seems to be a consequence of the host-parasite evolutionary incompatibility, specifically the inability of *T. regenti*, the avian schistosome, to evade the immune response of mammals. The latter is often seen in human schistosomes which are much better adapted to survive in mammalian hosts (Jenkins *et al.* 2005, Angeles *et al.* 2020). Nevertheless, the particular immune mechanisms responsible for the elimination of the avian schistosomes in the mammalian skin have been unknown. Regarding both historical and recent reports of the harmful impact of NO on human schistosomes (James and Glaven 1989, Ahmed *et al.* 1997, Shen *et al.* 2017), we evaluated its effect on *T. regenti* infection in mice (Macháček *et al.* 2020 = PUBLICATION #5).

We detected iNOS in the epidermal layer adjacent to the penetrating cercariae 8 hpi, but no signal was noticed later either in the epidermis or skin infiltrating leukocytes. We also did not observe any schistosomula damage after their *in vitro* treatment by NO-donors which suggested that NO did not cause acute cytotoxicity to *T. regenti* schistosomula. As the acute cytotoxicity is mostly related to the disruption of aerobic mitochondrial metabolism in human schistosomes (Ahmed *et al.* 1997), our data support the view that early *T. regenti* schistosomula rather rely on anaerobic/microaerobic energy metabolism (Leontovyč *et al.* 2016).

Unexpectedly, further experiments based on *in vivo* inhibition of NO formation suggested ambiguous actions of NO. It likely promoted the parasite growth in the early phase of the infection but prevented it later, which was accompanied by suspended schistosomula migration in the CNS. The latter effects might stem from continuous and chronic debilitation of the parasite, partly related to NO inactivation of its vital peptidases (Macháček *et al.* 2020 = PUBLICATION #5). Specifically, we demonstrated that NO decreased activity of rTrCB1.1 and rTrCB2, the peptidases essential for parasite digestion and migration (Dvořák *et al.* 2005, Dolečková *et al.* 2009). Such NO-related disruption of the proteolytic machinery has already been described for parasitic protists (Colasanti *et al.* 2001, Bocedi *et al.* 2004), but to the best of author's knowledge, this is the first time the phenomenon is reported for parasitic helminths.

Activation of microglia and astrocyte hypertrophy are striking features of the mouse immune reaction in the spinal cord invaded by *T. regenti* schistosomula which were able to escape from

the skin (Lichtenbergová *et al.* 2011). This is a common feature observed also in other helminth neuroinfections, but little is usually known about the function of these glial cells. Astrocytes are generally believed to participate in tissue repair (Sofroniew and Vinters 2010) while microglia are regarded as the major immune players participating in the parasite clearance (Rock *et al.* 2004). However, studies testing these assumptions are mostly lacking for helminth neuroinfections. To better understand the role of astrocytes and microglia in *T. regenti* infected mice, we examined their production of NO and cytokines after treatment by various *T. regenti* antigens (Macháček *et al.* 2016 = PUBLICATION #2, Dvořáková *et al.* 2020 = PUBLICATION #3).

We observed significant differences between the immunogenicity of living schistosomula (LS) and the soluble fraction of their homogenate (HSF). LS did not induce NO production in either astrocytes or microglia which corroborates with our *in vivo* data from the spinal cord, where iNOS was present no more than around 60% of schistosomula 3 dpi (Macháček *et al.* 2020 = PUBLICATION #5). Considering the cytokines, LS induced production of IL-6 in astrocytes which might be associated both with initiating the CNS inflammation nut also nervous tissue repair (Swartz *et al.* 2001). On the contrary, HSF triggered the production of NO, IL-6, and TNF-α which might facilitate the inflammatory processes and nervous tissue damage occurring after the schistosomula death when intrinsic antigens are released (Chao *et al.* 1992, Lichtenbergová *et al.* 2011, di Penta *et al.* 2013).

The similar immunostimulatory properties towards astrocytes and microglia were recorded also for rTrCB1.1 and rTrCB2, but not the inactive isoform rTrCB1.6. It potentially makes the active cysteine peptidases strong PAMPs which trigger the host immune reaction both in the periphery (Majer *et al.* 2020 = PUBLICATION #4) and in the CNS (Macháček *et al.* 2016 = PUBLICATION #2, Dvořáková *et al.* 2020 = PUBLICATION #3). This is generally in agreement with the view that the mammalian immune system can be very sensitive to helminth cysteine peptidases as they are released into the tissues in large amounts contrary to the host own peptidases often tightly stored in intracellular compartments (Sokol *et al.* 2008, Soloviova *et al.* 2019). Of note, none of the tested antigens influenced the secretion of IL-10 or TGF-β which implies their limited capacity to trigger directly the immunoregulatory phenotype in astrocytes or microglia.

The host immune reaction against pathogens varies in different CNS compartments and tissues (Andersson *et al.* 1992, Stevenson *et al.* 1997, Schnell *et al.* 1999) so the accurate data on the parasite migration and distribution within the CNS is required for appropriate interpretation of the experimental outcomes. The conventional imaging methods, such as histology, usually provide a good resolution and overview of a small region of interest. However, they become

laborious and time demanding if a comprehensive screening of bigger tissue samples or even entire organs is necessary. This is why various 3D imaging techniques (e.g. micro-computed tomography (μCT), ultramicroscopy, light sheet microscopy, etc.) have gained emerging popularity not only in parasitology (O'Sullivan *et al.* 2018). We applied some of these techniques to revise the previous histology-based data on *T. regenti* distribution within the vertebrate CNS tissues (Kolářová *et al.* 2001, Kouřilová *et al.* 2004b, Lichtenbergová *et al.* 2011). Additionally, we tested the suitability of fluorescence tracers for staining cercariae/schistosomula to enable better parasite tracking within the CNS.

Imaging of the murine spinal cord by ultramicroscopy and the duck spinal cord by μCT revealed that the schistosomula are predominantly localized in the white matter (Bulantová *et al.* 2016 = PUBLICATION #1). The preference for this tissue, which tends to develop stronger inflammation than the gray matter (Andersson *et al.* 1992), is likely a consequence of the *T. regenti* adaptation to feeding on myelin that is the main constituent of the white matter (Leontovyč *et al.* 2019). If suitable contrasting agents were applied, there was no need to stain the parasite to enable its later identification in the samples imaged by μCT. On the contrary, pre-infection fluorescence staining was required to visualize the schistosomula by ultramicroscopy. For this purpose, we found suitable tracers that retain the fluoresce long enough to study *T. regenti* behavior in the spinal cord (Bulantová *et al.* 2016 = PUBLICATION #1). The major advantage of the latter approach is that more tracers or fluorescently labeled antibodies can be used which would enable more focused spatial characterization of the host immune response (Ertürk *et al.* 2012).

In conclusion, the thesis presents the role of various *T. regenti* antigens in triggering the mouse immune response either in the periphery or within the CNS. Reciprocally, the effects of the anti-parasitic host immune effector molecule, NO, on *T. regenti* schistosomula, and the course of the infection were examined. Finally, the applicability of fluorescence tracers and 3D-imaging techniques for studying *T. regenti* migration in mice and the associated host immune response were tested. Taken together, the thesis markedly extends the knowledge of the host-parasite interactions between the neuropathogenic schistosomes and their accidental mammalian hosts. These novel data set a good starting point for further research on *T. regenti* neuropathogenicity and the impacts of helminth-caused neuroinflammation on the host. Such findings will be valuable not only in the field of parasitic neuroinfections but might also be appreciated in the research of (autoimmune) neurodegenerative diseases.

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