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**The immune response of mice against avian schistosomes:
from local to systemic perspective**

Imunitní odpověď myši proti ptačím schistosomám:
od lokálního k systémovému pohledu

Ph.D. thesis

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Prague, 2023

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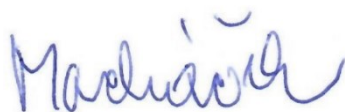
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Abstract

In the host bodies, helminths often undergo complicated migrations that afflict various organs. Defining the immune response is then usually focused only on isolated spatio-temporal fragments but lacks the whole-body context. To broaden the knowledge about the tissue-specific and systemic immune response, this thesis focused on mice infected with two model species of avian schistosomes of the genus *Trichobilharzia*.

T. regenti penetrates the skin and enters the spinal cord within a few days. Most of the parasites die in the skin which is accompanied by an influx of leukocytes. We confirmed that it was partially mirrored also in skin draining lymph nodes by increasing the T helper 2 cells proportion. Interestingly, there were reduced numbers of T cells in the spleen with an increased proportion of the regulatory T cells. Therefore, we conclude that adaptive systemic response differed and the regulatory phenotype dominated. However, the skin phase-induced eosinophilia in blood influenced the milieu of the central nervous system (CNS). Schistosomula which entered the spinal cord triggered inflammation and enhanced expression of eotaxins, which attracted the eosinophils from the blood into the CNS. The antigen-presenting cells contribute to the communication of CNS with the periphery. We employed and optimized light-sheet fluorescence microscopy, an advanced 3D microscopic technique, to detect the cells expressing major histocompatibility complex II (MHC II) and analyzed their amount within the infected spinal cord. Surprisingly, we revealed MHC II⁺ cells localized not only around the destroyed schistosomula but also in the perivascular area of the whole spinal cord.

T. szidati was the second model organism that was studied in this thesis. It is a viscerotropic schistosome migrating to the lungs of mice where it is eliminated. However, it does not trigger the influx of leukocytes into the lungs. Based on these data, we tested the parasite's immunomodulatory potential to attenuate the progression of allergic asthma in mice. We confirmed that *T. szidati* infection diminished the number of eosinophils accessing the lungs of mice with asthma. Intriguingly, the effect seemed to be only local because the infection contrarily boosted the immune reaction of splenocytes against the allergen.

Taken together, the thesis emphasizes the importance of the integrative approach in parasite immunobiology and brings novel data in the field of parasitic neuroinfections and immunomodulatory potential of helminths. Moreover, it points out the knowledge gaps in the immune response against avian schistosomes in mammals.

Key words: *Trichobilharzia*, CNS, lungs, immune response, asthma, mice, 3D imaging

Abstrakt

Migrace helmintů hostiteli často postihuje řadu orgánů, avšak stanovení imunitní odpovědi je většinou zaměřeno pouze na konkrétní místa v daném čase infekce bez širšího kontextu. Tato práce prohlubuje poznání o orgánově specifické a systémové imunitní odpovědi se zaměřením dva modelové druhy ptačích schistosom rodu *Trichobilharzia* v myších.

T. regenti vstupuje do míchy několik dní po penetraci kůže. Kožní reakce je způsobena vstupem leukocytů, jenž souvisí s usmrcením většiny parazitů. Jak jsme prokázali, projevuje se také ve spádových lymfatických uzlinách, kde zvyšuje zastoupení Th2 lymfocytů. Nicméně na systémové úrovni docházelo ke snížení množství T lymfocytů a převažoval regulační fenotyp. Eozinofilie vyvolaná kožní fází infekce se však projevovala i v centrální nervové soustavě (CNS). Schistosomula migrující míchou vyvolala zánět a expresi eotaxinů, jež způsobily příliv eozinofilů i do CNS. Antigen prezentující buňky jsou další možností, jak CNS komunikuje s periferní imunitní odpovědí. Pro detekci hlavního histokompatibilního komplexu II (MHC II, z anglického major histocompatibility complex) jsme zavedli pokročilou 3D mikroskopickou techniku, light-sheet fluorescenční mikroskopii. Ta nám umožnila změřit množství buněk exprimujících MHC II a zejména pozorovat jejich lokalizaci. MHC II+ buňky nebyly překvapivě pouze okolo rozložených parazitů, ale v perivaskulárních oblastech celé míchy.

Druhým modelovým organizmem použitým v této práci byla *T. szidati*. Ta migruje cévami do plic myší, kde jsou schistosomula zničena, aniž by však vyvolala infiltraci leukocytů. Na základě toho jsme se rozhodli otestovat imunomodulační potenciál tohoto parazita na průběh alergického astmatu. Infekce *T. szidati* vedla k potlačení množství eozinofilů v astmatických plicích. Avšak tento efekt se zdá pouze lokální, jelikož infekce naopak zesílila systémovou imunitní reakci splenocytů na alergen.

Předložená práce prokázala význam komplexního přístupu ke stanovení imunitní odpovědi proti parazitům. Navíc rozšířila znalosti o neuropatogenních parazitech a imunomodulačního potenciálu helmintů a poukázala na nedostatky v těchto oborech.

Klíčová slova: *Trichobilharzia*, CNS, plíce, imunitní odpověď, astma, myši, 3D zobrazování

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Abbreviations

APC	antigen-presenting cells
BAL	bronchoalveolar lavage
BBB	blood-brain barrier
BMDC	bone marrow-derived dendritic cells
BSCB	blood-spinal cord barrier
CNS	central nervous system
DC	dendritic cells
dLN	draining lymph nodes
dpi	days post infection
E/S	excretory/secretory
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
LN	lymph nodes
LSFM	light-sheet fluorescence microscopy
MHC	major histocompatibility complex
mLN	mesenteric lymph nodes
NK	natural killer
OVA	ovalbumin
SEA	schistosome egg antigen
SWAP	schistosome worm antigen preparation
Th	T helper
Treg	regulatory T (cell)
wpi	weeks post infection

1. Introduction

1.1. Family Schistosomatidae

Representatives of the family Schistosomatidae are dioecious blood-feeding digenetic trematodes with gastropods as intermediate hosts and mammals or birds as definitive hosts. According to Ebbs et al. (2022) there are 17 genera and more than 130 species (Fig 1). The basal genera (*Austrobilharzia* and *Ornithobilharzia*) are linked with marine snails. Still, the majority of described schistosomes have freshwater intermediate hosts. Miracidia hatched from eggs infect snails and the parasite undergoes several cycles of asexual mating within their body. Cercariae released from the snail find the definitive host and infect it, most often percutaneously. Nevertheless, the experimental infection revealed the possibility of a peroral route (Boulanger et al., 1998; Giver et al., 1999). Cercariae in the vertebrate host transform into schistosomula and migrate through the body. Adults live in the circulatory system, most often in mesenteric venules (Nation et al., 2020). Only a minority of species occupy different locations. Human *Schistosoma haematobium* prefers the vesical plexus of the urinary bladder and there are several bird species (Blair and Islam, 1983; Horák et al., 1998a; Flores et al., 2021) and one mammalian (*S. nasale*) (De Bont et al, 1989) found in veins in the nasal area. The reason for the occupancy of the unusual non-mesenteric habitats is questionable, as it brings worse nutrition supply and speaking of nasal schistosomes also more complicated migration to the egg-laying site. On the other hand, it reduces interspecies competition (Brant et al., 2013).

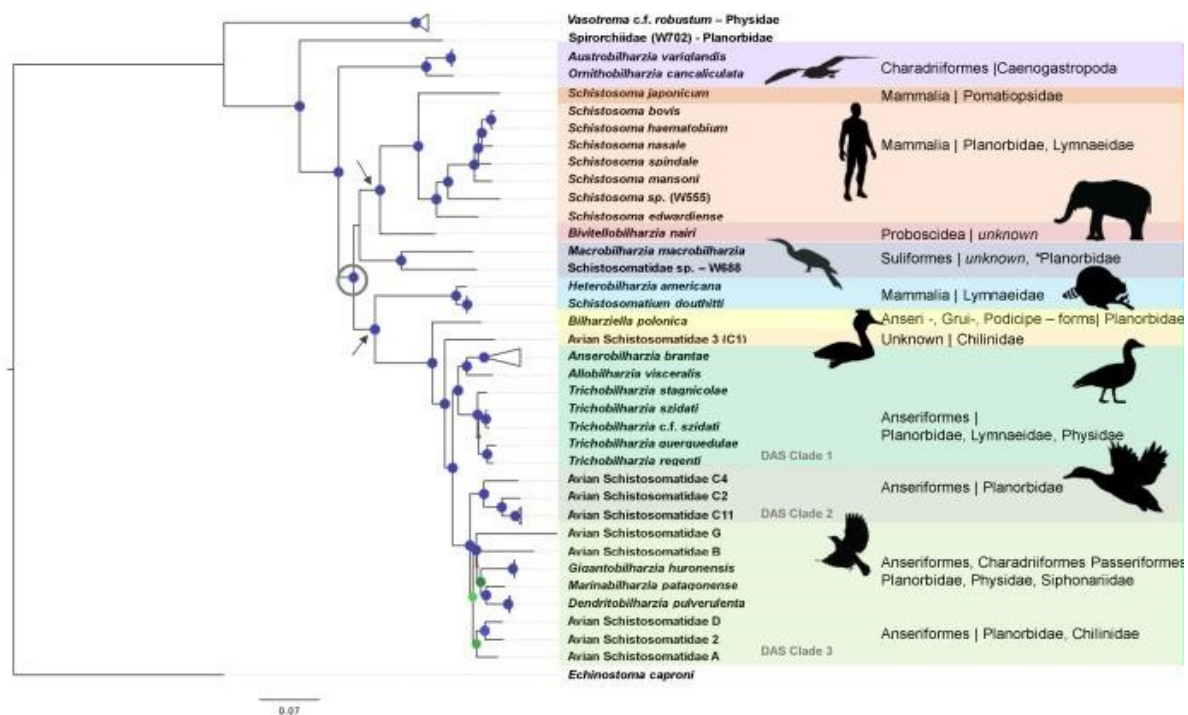


Figure 1: Phylogenetic tree of the genus Schistosomatidae with examples of their definitive host according to Ebbs et al. (2022).

1.2. Human schistosomes

According to the World Health Organization, schistosomes infecting humans are causative agents of schistosomiasis that more than 250 million people suffer from, and over 80% of infected live in Africa (WHO | World Health Organization, n.d.). The disease is typical for low-income countries with inadequate water sanitation and poor hygienic standards. Six well-known species infect humans: *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. intercalatum*, *S. guineensis*, and *S. mekongi* (McManus et al., 2018). Nevertheless, *Schistosoma* species are able to undergo interspecific hybridization. Namely, *S. haematobium* was shown to hybridize with its closest relatives *S. intercalatum* or *S. guineensis* from human species and *S. bovis*, *S. curassoni*, and *S. matthei* from animal species. Therefore, new and unusual human infections can arise and become a diagnostic challenge (Slothard et al., 2020). Eradicating schistosomes is very difficult, considering the abovementioned hybridization potential and plenty of animal reservoirs (Leger and Ebster, 2017). The climate changes and population migration brings new challenges and perils which proves more than 100 new cases of urinal schistosomiasis were described between 2013 and 2018 in Corsica after infection with *S. haematobium*/*S. bovis* hybrids (Rothe et al., 2021).

The cercariae penetration and schistosomula migration within the human host usually have a similar pattern, even though associated pathologies and migration time in the host slightly differ between species. Cercariae penetrate the skin, shed their tails and glycocalyx, and transform into the schistosomula. That can cause, typically in naive individuals like travelers, an itching rash (cercarial dermatitis) (Langenberg et al., 2020). Schistosomula invade the blood vessel after skin penetration and migrate via the circulatory system. Of note, a small proportion of schistosomula enters the lymphatic system instead of the vascular occasionally. Interestingly, schistosomula can escape the lymph node and continue in their migration (Lozzi et al., 1996).

The migratory route for schistosomula typically leads through the lungs and ends in the liver, from where they migrate through the portal vein and mature (Nation et al., 2020). Of note, schistosomula are sometimes accidentally carried into different organs, such as the myocardium or even the brain (Wilson, 2009). Adults live in mesenteric plexus veins (most of the species) or pelvic venous plexus (*S. haematobium*), where they mate and lay eggs. They are primarily passed through the intestinal wall and released with the feces, alternatively to the urinary bladder, and excreted in the urine (*S. haematobium*). However, many eggs are swept to various organs, such as the liver, lungs, and brain, via the blood flow. Taking into consideration that individual *S. mansoni* female produces around 300 eggs daily and *S. japonicum* even 2000 eggs (Moore and Sandground, 1956), they cause harmful pathologies like hepatosplenomegaly, bladder cancer, or cerebral schistosomiasis.

1.3. Immune response against *S. mansoni* in mice

The complex migratory route in the definitive host involves cercariae skin penetration, schistosomula somatic migration via lungs, male or female adults dwelling in the bloodstream, and eggs in the intestinal wall or liver (Fig 2). The invasion of the tissues also causes mechanical damage, and all the stages also produce various immunogenic and immunomodulatory molecules, which are exposed either on their surface or excreted and secreted to the environment. These factors have a direct effect on the tissue environment but also influence the health status of the whole organism.

The following chapters aim to show the effect of parasite migration on the systemic immune response (represented by the spleen and blood) and to compare it with the tissue-specific reactions in the three sites most affected by the parasite: skin, lungs, and hepato-intestinal tract. It focuses on the mouse model of *S. mansoni* infection with a particular emphasis on the most significant differences between local and systemic immune profiles during the parasite migration.

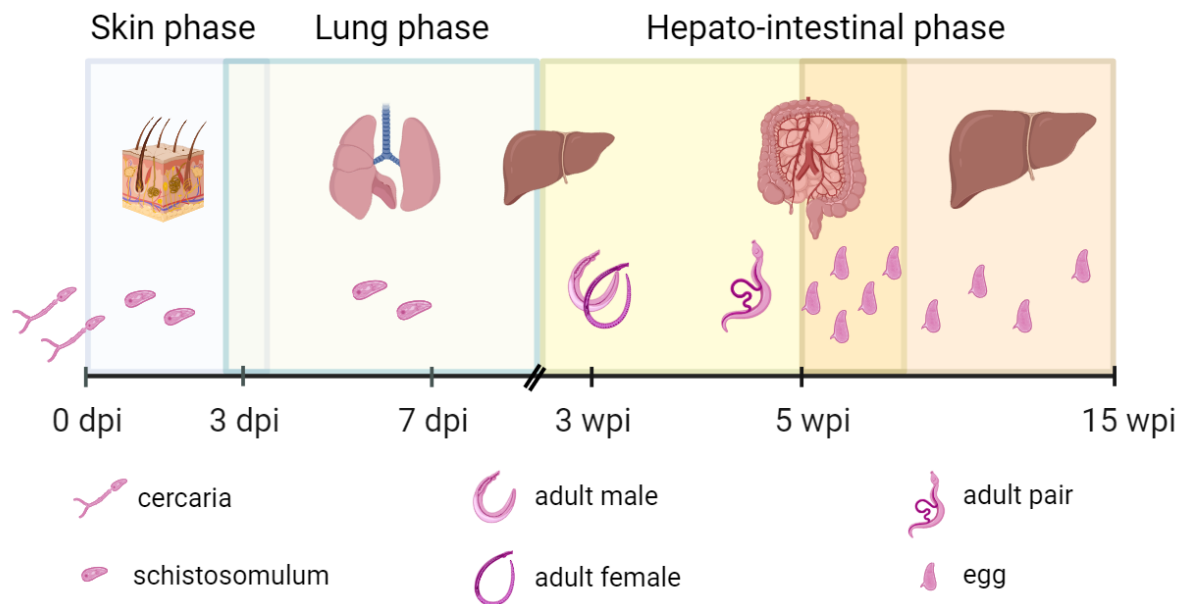


Figure 2: Dynamics of the *S. mansoni* infection of mice. Further description is provided in the main text. (dpi, days post infection; wpi, weeks post infection) Created with BioRender.com

1.3.1. Schistosomes in the skin

Mammalian skin is the mechanical barrier that cercariae must breach. Parasite penetration leads to an accumulation of neutrophils and macrophages in the wound and the thickening of the skin caused by hyperkeratosis within a few hours. The biggest influx of cells consists of neutrophils and some mononuclear cells accumulated around the dead larvae and shaded cercarial tails 2 days post infection (dpi). The successful larvae escape from the skin 3 dpi and enter the blood vessels. The exit of parasites from the skin leads to a gradual decrease in the leukocyte number (Incani and McLaren., 1984; Riengrojpitak et al., 1998).

The infection also induced the expression of major histocompatibility complex (MHC) II (Riengrojpitak et al., 1998) on antigen-presenting cells (APC) in the skin. The typical skin-resident APC are Langerhans cells. Nevertheless, schistosomula excretory/secretory (E/S) products are able to inhibit their migration to the skin-draining lymph nodes (dLN) and limit

the adaptive immune response and activation of a systemic reaction (Angeli et al., 2001). Moreover, the E/S products induce the apoptosis of cells around the schistosomula (Chen et al., 2002). That raises the question of whether they could reduce the lymphocyte population directly in the skin dLN, considering the potential of schistosomula to migrate via the lymphatic system in the early phase (Lozzi et al., 1996). However, the migration of schistosomula into the lymph node (LN) is probably only occasional and does not have such an important impact on the adaptive response. Mountford et al. (1992) and Redpath et al. (2015) described that CD4⁺ cells from skin dLNs are activated and produce both interferon (IFN) γ and interleukin (IL)-4 for a few weeks after the infection, indicating the mixed T helper (Th)1/Th2 polarization of CD4⁺ cells. The same trend was also noticed for splenocytes (Redpath et al., 2015), suggesting that such local and systemic polarization could have a noteworthy role in the recall responses during following reinfections.

The reinfected mice develop a faster response detectable within the first hours after infection. It is represented by the neutrophils and mononuclear cells again, but they are quickly replaced by eosinophils whose numbers culminate between 2 and 3 dpi (Incani and McLaren., 1984). The four-times repeated infection led to massive eosinophilia in the exposed pinnae and increased production of IL-4, IL-10, and IL-13. Also, increased expression of *Arg1*, *Ym1/Chil3* confirms the polarization of macrophages towards the M2. On the other hand, the amount of MHC II cells is reduced, as well as the proliferation of CD4⁺ cells in the skin dLN and their cytokine production after stimulation with schistosomula antigens. Such hyporesponsiveness is also evident in the distal LN, including mesenteric LN (mLN). Interestingly, despite the apparent anergy, the reinfections lead to a reduction in the egg amount. (Cook et al., 2011).

To conclude, the single infection triggered only a weak immune response in the skin and schistosomula were able to escape from the skin fast. Nevertheless, the reinfections proved the activation of the systemic immune response and diminished the infection despite the immunomodulatory potential of the parasite E/S products.

1.3.2. Schistosomes in the lungs

The first schistosomula of *S. mansoni* are detected in mice lungs between 2 and 3 dpi, and their number culminates 7 dpi. Later, it slowly declines until 11 dpi (Wilson, 2009; Nation et al., 2020), but some authors observed schistosomula in the lungs even up to 20 dpi or later (Wheater and Wilson, 1979; Houlder et al., 2021). Schistosomula migrate intravascularly through the circulatory system and sometimes make multiple circuits (Wilson, 2009), which is probably the

reason why they are observed at later time points. Schistosomula in the lungs can disrupt the venules and enter the alveoli, but they are apparently not able to return to the vasculature again, which could trigger tissue damage (Wilson, 2009).

Schistosomula, which enter the alveolar space, induce the infiltration of polymorphonuclear cells (mostly eosinophils and neutrophils) around the worm starting 11 dpi. The volume of cellular foci extends during the infection with a peak 17 dpi. The composition of the infiltrate changes in time with an increasing proportion of mononuclear cells (primarily lymphocytes and macrophages) (Crabtree and Wilson, 1986). The migration of schistosomula also drives the infiltration of CD4⁺ of mixed Th1/Th2 phenotype and CD8⁺ cells into the lungs and even alveolar space as was described in bronchoalveolar lavage (BAL) 21 dpi (Houlder et al., 2023). Interestingly, Redpath et al., (2015) detected only an increased proportion of CD4⁺IL-4⁺ cells and not CD4⁺IFN γ ⁺ in lung dLN. The trend was followed with a little delay in the spleen, which suggests polarization towards the Th2 profile at the systemic level. This novel observation challenges the established view that the Th2 response is initiated after the egg laying.

CD4⁺IL-4⁺ cells from lung dLN and spleen are potent producers of regulatory cytokine IL-10 (Redpath et al. 2015) and substitute the role of regulatory T (Treg) cells, amount of which is not changed in lungs or lymphatic organs in the early time points (Redpath et al., 2015; Houlder et al., 2023). The IL-10 production could be a consequence of the host reduction of the tissue damage, but schistosomula are also able to regulate the immune response actively. The E/S products of schistosomula polarize the bone marrow-derived dendritic cells (BMDC) towards type 2 dendritic cell (DC)/Th2 profile represented by IL-4, IL-5, and IL-10 production (Jenkins and Mountford, 2005). Moreover, recombinant proteins from schistosomula tegument induce the production of IL-10, but also inflammatory IFN γ and TNF by CD4⁺ cells (Araujo et al., 2012).

Considering that elevation of IL-10 is not detected directly in lung tissue but only in lymphatic organs, such as the spleen or lung dLN, we can assume that the IL-10 production is triggered by living schistosomula migrating through the body via the circulatory system. This migration polarized the systemic immune response, which could be essential for a reduction of pathology at the moment but also in the following course of infection.

Schistosomula from primary infection, which did not leave the vessels, seemed intact and without immune cell adhesion (Crabtree and Wilson, 1986). Migrating schistosomula and their E/S products are in close contact with endothelial cells and increase their expression of *I/6*

(Angeli et al., 2001). IL-6 in the lungs participates in decreasing the eotaxins production and eosinophil influx (Wang et al., 2000; Rincon and Irvin, 2012) and therefore increases parasite survival (Angeli et al., 2001). On the other hand, percutaneous vaccination with irradiated cercariae orchestrates an accumulation of cells around the schistosomula in lung vessels in subsequent infection (Crabtree and Wilson, 1986). It suggests the activation of systemic immune response and the development of adaptive immunity with memory cells.

Adults start production of the eggs in the mesenteric plexus veins 5-6 weeks post infection (wpi), which massively affects the systemic immune response but as eggs can be passed via the blood flow into the lungs, the effect can also be local. At the beginning of egg production, there are usually only a few eggs in the lung tissue, but their numbers steadily increase (Crabtree and Wilson, 1986). The number of eggs in the liver correlates with the amount of CD4⁺ and CD8⁺ cells in the lungs, as it increases the potential that eggs will be swept through the body into the lungs. CD4⁺ cells again showed the mixed phenotype represented by the production of IFN γ , but also IL-4, IL-5, IL-13, and even IL-10. Nevertheless, DC in the lungs indicates the progression toward the Th2 direction 7 wpi when the switch towards the Th2 is also evident at the systemic level (Houlder et al., 2023).

To summarize, schistosomula enter the lungs at a time when the skin phase already determines the systemic immune system. The skin phase of the infection seemingly does not change the immune environment of the lungs or their draining LN but polarizes the systemic immunity. CD4⁺ cells in the spleen produce IL-4 at this time and it probably affects and boosts the local immune response against schistosomula in the lungs a few days later. Schistosomula also directly affect the endothelium of blood vessels during their migration by the E/S products and, therefore, attenuate potential inflammation. However, the systemic immunity trained by the vaccination can eliminate parasites directly in the lung blood vessels. In contrast, infection of naive animals develops inflammation in the lungs only when schistosomula penetrate into the parenchyma. The later phase, when schistosomula already settled vasculature of livers and matures, is probably also influenced by the systemic Th2 polarization by eggs in the hepato-intestinal tract but also the lungs.

1.3.3. Schistosomes in the hepato-intestinal tract

Schistosomula, which successfully pass through the lungs, migrate towards the liver. They can circulate via blood vessels through the systemic organs such as the stomach, spleen, small intestine, or kidney with one goal. The successful ones finally migrate through various arteries to

the hepatic portal vein. There, they start feeding on blood, grow, and mature. Later, the adult pairs migrate to the mesenteric vessels and vasculature around the small intestine, colon, or rarely rectum, where they start laying eggs at approximately 5 or 6 wpi. Successful egg passage leads through the blood vessel wall and intestinal wall into the intestinal lumen (Nation et al., 2020). The eggs are passed mainly through the small intestine of mice. This differs from humans, in which the eggs are more common in the colon (Costain et al., 2018). Nevertheless, eggs are frequently passively transported by the blood flow to diverse organs, mainly the liver, where they cause harmful pathology. Adult pairs occasionally change their locations, which supports the eggs' spreading to other systemic organs (Nazione et al., 2020). Therefore, the immune response against the adults and eggs is very complex.

1.3.3.1 Effect of adults

The entry of schistosomula into the liver is accompanied by activation of MHC II-related genes already 3 wpi. Eosinophils enter the liver and create disorganized clusters within the hepatic parenchyma. Similarly, macrophages, DC, natural killer (NK) cells, T, and B cells infiltrate in small amounts the liver at 4 wpi evidently affected by the presence of schistosomula or young adults. (Costain et al., 2022). The initial effect of schistosomula migration and settlement in the liver is accompanied by the enlargement of the spleen 4 wpi (Winkelmann et al., 2022). Nevertheless, the increase in cell numbers is only minor in the spleen, so the changes are rather local than systemic (Costain et al., 2022). The male-only infection shows that adult worms modulate the systemic immune response also at a later time point (8 wpi), represented by B cells and CD8⁺ cells in the spleen. The enhanced CD86 expression on DC indicates that male schistosome has the potential to activate APC even better than conventional bi-sexual infection. In contrast, the female infection did not activate the DC in the spleen at all (Winkelmann et al., 2022).

Shortly after schistosomes start to feed on blood, they have to deal with the presence of toxic haem, which they detoxify into hemozoin (Oliveira et al., 2000). Hemozoin accumulates in the liver where it is phagocytosed by macrophages. That polarizes macrophages into M2 phenotype represented by increased expression of *Ym1/Chil3* and *Arg1* (Truscott et al., 2013), which could contribute to the immune system activation in the liver before the egg deposition.

The potent stimulator of the immune response seemed to be schistosome worm antigen preparation (SWAP), obtained from the adult pairs of *S. mansoni*. Kupfer cells, the resident

liver macrophages, stimulated with the SWAP have the M2 phenotype and produce IL-4 and IL-13 from the very beginning of the liver inhabitation by the parasite (4 wpi). Moreover, they have a much more immense potential for T cell activation than stimulated splenic macrophages (Hayashi et al., 1999; de Oliveira Fraga et al., 2010). SWAP stimulates the liver T cells towards the Th2, but also regulatory phenotype represented by the production of IL-4 and IL-10, respectively, (de Oliveira Fraga et al., 2010) similarly, as was described by Redpath et al. (2015) in lung dLN 3 wpi during schistosomula migration (chapter 1.3.2.). On the other hand, splenic T cells show Th1/Th2 phenotype at the same time (4 wpi), indicating that systemic immune response polarization towards Th2 is delayed (de Oliveira Fraga et al., 2010).

However, SWAP contains, besides the homogenate from adult worms, also eggs, which have strong immunogenic potential (see chapter 1.3.3.2.). The antigen-specific effect of eggs in SWAP could be eliminated in splenocyte restimulation after single-sex infection. Splenocytes from male or female infection produce more IL-4 and especially IFN γ than those from bi-sexual infection. Interestingly, IL-10 is not produced in female-only infections, but after male or bi-sexual infection (Winkelmann et al., 2022). The lyso-phosphatidylserine from the body of adult worms apparently contributes to the stimulation of IL-10 production, as it specifically activates DC, which induce polarization of Treg cells (Van der Kleij et al., 2002).

The progression of the infection obviously enhances the immune responsiveness on both the local and systemic levels. The antigenic potential of the eggs is well known. Nonetheless, the adult worms already modulate the immune response and potentially increase the probability of the successful completion of the life cycle. Adults, especially the male worms, polarize the T cell towards the Th2 phenotype in the liver, which mirrors also in the spleen.

3.3.2. Effect of eggs

For successful completion of the parasite lifecycle, it is necessary to lay eggs in the vessel around the intestine (Fig 3) and enable their passage through the wall of the small intestine or colon. On the other hand, many eggs end up in the liver, where they accumulate and cause detrimental pathology. Interestingly, the cell composition of periovular granuloma differed between the organs (Weinstock and Boros, 1983).

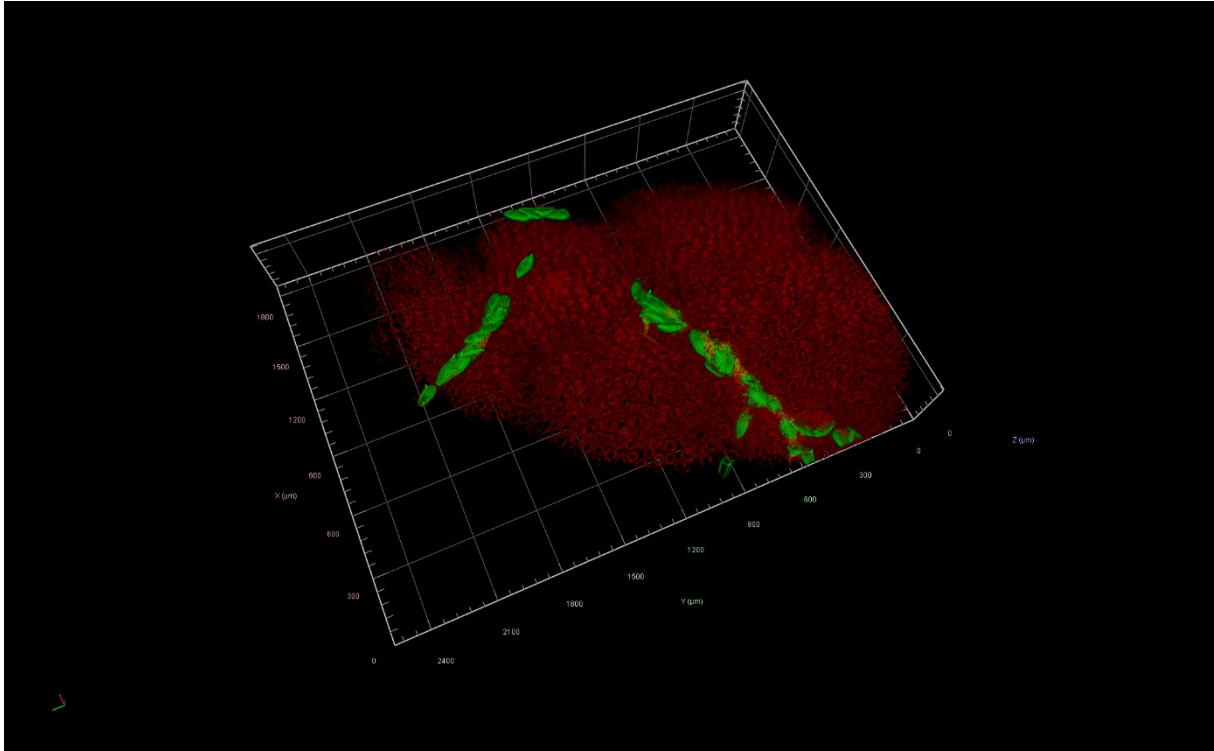


Figure 3: Illustrative picture of *S. mansoni* eggs in blood vessels around the mouse intestine. Data were obtained by light-sheet fluorescence microscopy, green: autofluorescence of eggs; red: nuclei. (unpublished data)

The number of eosinophils, neutrophils, macrophages, DC, CD4⁺ cells, B cells, and other leukocytes in the liver gradually increases in the presence of eggs. The cellularity peaks 8 wpi when the granuloma formation starts. After that, the cell numbers slowly decline, and eosinophils and monocytes are only elevated cell population 15 wpi. The dramatic increase of cellularity at 8 wpi is similar in the spleen but more or less persists for a longer time (Costain et al., 2022). The granuloma in the small intestine and colon differs from the liver. Eosinophils represent the majority of cells infiltrating the liver but in the colon are as abundant as macrophages, which are the dominating population in the small intestine. The lymphocytes were mostly in the liver and rare in the small intestine (Weinstock and Boros, 1983).

The mLN drain the intestine and colon and consists of several smaller nodes that predominantly respond to distinct segments of the small intestine and colon. The cellularity in the mLN increases between 7 and 8 wpi and persists at least up to 15 wpi similarly as in the spleen (Mayer et al., 2020; Costain et al., 2022). The egg production leads to the clear switch to the Th2 phenotype at both local and systemic levels. The schistosome egg antigen (SEA) induces the production of IL-4 by the cells from mLN 8 wpi. Interestingly, cells in the liver and spleen

were prone to produce IL-4 already at 6 wpi (Costain et al., 2022). That could be thanks to the earlier polarization by the adult worms (de Oliveira Fraga et al., 2010; Winkelmann et al., 2022) and the potential of the Kupfer cells in the liver to produce IL-4 as well (Hayashi et al., 1999). On the other hand, the IL-5 amount steadily increases in the liver only, however, in the spleen and mLN declines, after the peak at 12 wpi (Costain et al., 2022), probably as the consequence of eosinophilia dominating in the liver and not the intestine (Weinstock and Boros, 1983).

The later phase of infection is influenced by egg production, which triggers a massive local immune response. Interestingly, the cell infiltration and granuloma formation are faster and more pronounced in the liver than in the intestine. The differences between granuloma formation in the specific organs also mirror in the spleen, representing the systemic immune response, at least at the initial phase of the egg laying. Nevertheless, as the infection progresses, the differences between local and systemic immune profiles are not evident.

1.4. Immune response against avian schistosomes in mice

Avian schistosomes represent the majority of the described species within the family Schistosomatidae. Many of them are able to infect mammals (Loker et al., 2022) and the penetration of human skin and elimination of parasites cause cercarial dermatitis also known as swimmer's itch (Horák et al., 2015). There are no data describing the following migration through the human body and it is generally believed that all parasites are killed in the skin. Nevertheless, avian schistosomes are able to reach the lungs of the rhesus macaques (Olivier, 1953). Because of the physiological similarities, we can speculate that it could be possible also in humans. Immunodeficient mice show better susceptibility to infection (Kouřilová et al., 2004a), so we can hypothesize that immunodeficient humans could also be at risk. The best-described immune response against avian schistosomes in mice is available for the neurotropic *Trichobilharzia regenti* (Horák et al., 1998a) and viscerotropic *T. szidati* (Neuhaus, 1952). Some schistosomula of both species are able to escape from the mouse skin and continue the migration. Nevertheless, *T. regenti* is eliminated in the central nervous system (CNS) and *T. szidati* in the lungs at the latest.

The skin penetrated by *T. regenti* develops edema and vasodilation within the first hours post-infection. The first leukocytes that enter the infected site are neutrophils followed by eosinophils, macrophages, and mast cells. The area of inflammatory foci culminates between first and second dpi and then slowly diminishes (Kouřilová et al., 2004a; Kouřilová et al., 2004b).

The leukocytes together with skin-resident cells produced IL-1 β , IL-6, and IL-10 at the beginning of the infection (Kouřilová et al., 2004b). Successful schistosomula quickly leave the skin and after 2 or 3 dpi enter the peripheral nerves and the CNS (Hrádková and Horák, 2002). Nevertheless, 90% of schistosomula are eliminated in the skin (Hrádková and Horák, 2002) and therefore the immune response is altered by their destroyed bodies, which are a rich source of antigenic material. Production of IL-1 β and IL-6 vanishes, but the amount of IL-4, IL-10, and IL-12 increases 4 and 8 dpi. It indicates a mixed Th1/Th2 response, which was confirmed based on the restimulation of cells from skin dLN with cercarial homogenate (Kouřilová et al., 2004b). Interestingly, an increased level of IgG1 in serum 10 dpi indicates that systemic immune response is more Th2 biased. Moreover, the Th2 shift is even more evident during reinfections in cytokine production capacity by the cells from skin dLN and also antibodies in sera (Kouřilová et al., 2004b). However, more comprehensive data are missing.

The entry of schistosomula into the spinal cord rarely causes leg paresis (Horák et al., 1999). However, schistosomula are usually intact and migration induces only focal edema without any inflammation (Kouřilová et al., 2004a; Lichtenbergová et al., 2011). Most schistosomula are eliminated in the spinal cord between 7 and 14 dpi when the inflammation progresses. According to histological reports, the granuloma around the dying parasites consists mainly of neutrophils, astrocytes, and microglia. Furthermore, eosinophils and lymphocytes can occur in the meningeal space. The cellular immune reaction within the CNS weakens after 21 dpi and there is no granuloma after 28 dpi (Kouřilová et al., 2004a; Lichtenbergová et al., 2011).

The comparison with the systemic immune response is hardly possible because the only data show steadily increasing levels of the antibodies, mainly IgG1 and fewer IgG2a. However, these data are from reinfection when schistosomula did not reach the CNS and therefore mirror only the skin phase of the infection (Kouřilová et al., 2004b).

Compared to *T. regenti*, the immunobiology of *T. szidati* in mammals is only barely known although it represents the species often reported from outbreaks of the human cercarial dermatitis (Horák et al., 2015; Macháček et al., 2018). The skin phase of the mouse infection by *T. szidati* is poorly described and authors generally assume a similar pattern in all avian schistosomes able to infect mammals (Horák et al., 2015). However, our recent preliminary experiments showed significant inter-specific differences in leukocyte infiltration into the skin infected by *T. regenti* and *T. szidati* (Revalová, 2022). This suggests an uneven pathogenic

capacity of the avian schistosome species with possible implications even for human cercarial dermatitis.

Schistosomula of *T. szidati* quickly leave and via the circulatory system enter the lungs. Their number culminates 2 and 3 dpi and after that rapidly drops and only a few dead worms are in the lungs 5 dpi (Horák and Kolářová, 2000; Chanová et al., 2007). Schistosomula often leave the circulatory system and they are mostly in alveolar wall tissue (Chanová et al., 2007). Their migration causes hemorrhage (Olivier, 1953) and edema (Chanová et al., 2007). Interestingly, Chanová et al. (2007) observed no massive leukocyte influx into the infected lungs. They noticed only several lymphocytes on the pleural side. Moreover, the antibodies from the infected mice do not bind on the schistosomula surface and react only with cercariae (Horák et al., 1998b) and therefore we can assume an immunomodulatory potential of schistosomula. However, there are obviously many knowledge gaps in local immune reactions both in the skin and lungs and also in how the parasite migration challenges the systemic immune system.

Taken together, the schistosomula of *T. regenti* and *T. szidati* are in mice infection eliminated by the immune system predominantly in the skin. However, the ones that escape from the skin invade the CNS or lungs, respectively. The CNS infection triggers local inflammation. Surprisingly, there is no strong influx of leukocytes into the lungs. However, there is a lack of data about the systemic immune response and how the local and systemic reactions influence each other.

2. Aims of the thesis

The thesis aimed to compare the local and systemic immune response against avian schistosomes *T. regenti* and *T. szidati* in mice. It broadens the knowledge about the inflammation in the central nervous system and the effect of helminths on allergic diseases. Moreover, introduces a new method for whole organ microscopy in the field of helminthology.

The specific aims were to:

- Compare the local and systemic reaction of the adaptive immune response against *T. regenti* in mice.
- Find out if the inflammatory reaction in the CNS of *T. regenti* infected mice influences the systemic immune response and vice versa.
- Establish the 3D imaging method that would enable to display the immune response in the whole organ context using the model of *T. regenti* infected CNS.
- Examine the immunomodulatory potential of *T. szidati* in the mouse lungs by using the asthma model and test if the effect is local or systemic.

3. List of publications

The thesis contains two publications in chronological order. Moreover, there is added one manuscript in preparation containing a short overview, methods, and results. The discussion covering all topics is listed as a separate chapter 4. My contributions to each publication are stated below.

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

My contribution: Designing the study, performing experiments (sample collection, flow cytometry, cytokine ELISA), data analysis and interpretation, writing the manuscript, and being the corresponding author.

Macháček T., Leontovych R., Šmídová B., Majer M., Vondráček O., Vojtěchová I., Petrásek T., Horák P. (2022): **Mechanisms of the host immune response and helminth-induced pathology during *Trichobilharzia regenti* (Schistosomatidae) neuroinvasion in mice.** *PLoS Pathogens* 18: e1010302.

My contribution: Performing experiments (sample collection, light-sheet fluorescence microscopy, flow cytometry of blood, immunohistochemistry of cytokines), data analysis and interpretation, and writing the associated parts of the manuscript.

Majer M., Šmídová B., Horák P., Schabussova I., Macháček T.: **Pulmonary migration of *Trichobilharzia szidati* (Schistosomatidae) and its potential immunomodulatory effect against allergic asthma in mice.** (manuscript)

My contribution: Designing the study, performing experiments (asthma induction, sample collection, histology, flow cytometry, ELISA, qPCR), data analysis and interpretation, and writing the manuscript

ORIGINAL ARTICLE



WILEY

The peripheral immune response of mice infected with a neuropathogenic schistosome

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Abstract

Trichobilharzia regenti (Schistosomatidae) percutaneously infects birds and mammals and invades their central nervous system (CNS). Here, we characterized the peripheral immune response of infected mice and showed how it was influenced by the parasite-induced inflammation in the skin and the CNS. As revealed by flow cytometry, T cells expanded in the spleen and the CNS-draining lymph nodes 7–14 days post-infection. Both T-bet⁺ and GATA-3⁺ T cells were markedly elevated suggesting a mixed type 1/2 immune response. However, it dropped after 7 dpi most likely being unaffected by the neuroinflammation. Splenocytes from infected mice produced a high amount of IFN- γ and, to a lesser extent, IL-10, IL-4 and IL-17 after in vitro stimulation by cercarial homogenate. Nevertheless, it had only a limited capacity to alter the maturation status of bone marrow-derived dendritic cells (BMDCs), contrary to the recombinant *T. regenti* cathepsin B2, which also strongly augmented expression of *Ccl5*, *Cxcl10*, *Il12a*, *Il33* and *Il10* by BMDCs. Taken together, mice infected with *T. regenti* developed the mixed type 1/2 immune response, which was driven by the early skin inflammation rather than the late neuroinflammation. Parasite peptidases might play an active role in triggering the host immune response.

KEYWORDS

cathepsin B, central nervous system, dendritic cells, lymph nodes, skin, spleen, T lymphocytes

1 | INTRODUCTION

Helminths do often invade the central nervous system (CNS) of vertebrates, including humans, causing severe pathologies, such as in the case of neurocysticercosis (NCC) or neurotoxocariasis.¹ Although the CNS was formerly considered an immune-privileged site with a limited capacity to develop an adaptive immune response, this view has recently been revisited.^{2,3} Inflammatory cells are recruited into the infected CNS as well as pathogen-specific T cells, which were primed in the periphery.⁴

The polarization of helminth-specific T helper (Th) cells largely orchestrates the host immune response. Th1 and Th17 populations are usually associated with host pathology, contrary to Th2 response and expansion of regulatory T cells (Treg) which suggest asymptomatic or mild infection.⁵ For example, symptomatic NCC is linked to increased serum levels of proinflammatory cytokines⁶ while Th2/Treg milieu is related to asymptomatic NCC.^{6–9} However, it is not very clear how the host immune response is influenced by the cysts present directly in the CNS and those disseminated in other body parts. The same applies to *Toxocara* spp. which exhibits a marked

affinity towards the CNS but most larvae are still localized in muscle tissue.^{10,11}

The neurotropic avian schistosome *Trichobilharzia regenti* represents a suitable model to examine how parasite-induced neuroinflammation affects peripheral immunity. Beyond ducks, it is able to infect also mice, employing the initial percutaneous route of infection similar to human schistosomes.¹² Invasion of the host skin is facilitated by cercarial excretory/secretory products (ESP) rich in cysteine peptidases,¹³ such as *T. regenti* cathepsin B2 (TrCB2).¹⁴ It was shown to degrade components of the skin (keratin, collagen, elastin) and also neural myelin basic protein.¹⁴

Despite being equipped with a powerful enzymatic arsenal, most *T. regenti* schistosomula are eliminated in the mouse skin within the first few days after the infection. They are entrapped by the inflammatory infiltrate mostly consisting of neutrophils, eosinophils and macrophages.^{15,16} Increased levels of proinflammatory cytokines, such as interleukin (IL)-1 β , IL-6 or interferon (IFN)- γ , were detected in infected skin biopsies, but the inflammation diminishes within 4–8 days post-infection (dpi).¹⁶ Interestingly, avian schistosomes of the genus *Trichobilharzia* can also penetrate the skin of humans causing cercarial dermatitis, which is considered as a (re)emerging disease.^{17,18} It presents with itching papules, increased eosinophilia and serum IL-4.¹⁹ Nevertheless, the effect on the host immunity of *T. regenti* ESP released during skin penetration is unknown either in mice or humans.

Around 10% of *T. regenti* schistosomula escape the strong immune reaction in the murine skin,¹⁶ migrate along the peripheral nerves and enter the spinal cord as soon as 2–3 dpi.^{12,15,20} Schistosomula preferably move through the white matter of the spinal cord, feed on myelin and injure axons which might lead to neuromotor disorders, especially in immunocompromised mice.^{15,20–22} Neuroinflammation starts to develop 6–7 dpi, which is 4–5 days after schistosomula enter the CNS. They are gradually surrounded by massive clusters of neutrophils, macrophages and microglia^{15,20} which are presumably responsible for killing the parasites. Microglia might also promote the neuroinflammation since they secrete a significant amount of IL-6 and tumour necrosis factor (TNF)- α , but no IL-10 after in vitro stimulation by TrCB2.²³ Neuroinflammation peaks 14 dpi being steadily attenuated until 28 dpi (Macháček, unpublished), but no living schistosomula are found in the CNS after 21 dpi.²⁰ However, effects on peripheral immunity of this neuroinflammation, virtually restricted to the CNS, remain unexplored.

In this study, we monitored dynamics of Th1, Th2, Th17 and Treg populations in spleens and skin- or CNS-draining lymph nodes of mice infected with *T. regenti*. To underpin the findings of T cell polarization during the infection, we also analysed cytokines secreted by splenocytes stimulated by *T. regenti* cercarial homogenate. Moreover, we examined the effect of *T. regenti* antigens on the maturation status of bone marrow-derived dendritic cells (BMDCs) and tested how they influenced the expression of certain cytokines and chemokines by BMDCs. Collectively, these experiments led us to a better understanding of how the neuropathogenic schistosome affects the host peripheral immune response.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

The experimental procedures including animals were reviewed by the Animal welfare committee of Charles University, Faculty of Science and approved by the Czech Ministry of Education, Youth and Sports (ref. no. MSMT-31114/2013-9 and MSMT-33740/2017-2). All experiments were performed by authorized persons in accordance with the European Directive 2010/63/EU and Czech Laws for Biomedical Research (246/1992 and 359/2012).

2.2 | Animals and infection

Female C57BL/6JOLA-Hsd (ENVIGO) mice were housed in the Centre for Experimental Biomodels (Charles University, First Faculty of Medicine) in the specific pathogen-free conditions according to recommendations of the Federation of European Laboratory Animal Science Associations.

Eight-week-old mice were experimentally infected by the avian schistosome *T. regenti*, which is maintained in our laboratory,²¹ according to the routinely used protocol.²² Each animal was exposed to 2000 cercariae, freshly emerged from laboratory-grown *T. regenti*-infected *Radix lagotis* snails, in 50 mL of water in the dark for an hour. After infection, the mice were left in cages with unlimited access to water and feed.

2.3 | Parasite antigen preparation

Homogenate of *T. regenti* cercariae (TrH) and recombinant cathepsin B2 of *T. regenti* (rTrCB2) were prepared to be used in further experiments.

To obtain TrH, cercariae (approximately 15 000 per preparation) were collected from snails and washed three times in sterile 0.1 mol/L phosphate-buffered saline (PBS; pH 7.3) by centrifugation (1600 g, 10 minutes, 4°C). The cercariae were then concentrated in a small volume of PBS, and peptidase inhibitors (Complete Mini EDTA-free, Roche) were added according to the manufacturer's instructions to prevent antigen degradation. The cercarial suspension was then homogenized by a sonicator (3 $\times$ 30 seconds, 30 W, Vibra Cell) and centrifuged (16 000 g, 20 minutes, 4°C). The supernatant (TrH) was filtered through 0.22 μ m strainer.

rTrCB2 was prepared as described elsewhere.¹⁴ Briefly, the recombinant protein was produced in *Pichia pastoris* and purified by Macro-Prep High S Support (Bio-Rad) and fast protein liquid chromatography (BioLogic HR System). Excessive N-linked glycosylation was removed by Native Protein Deglycosylation Kit (Sigma-Aldrich). The lyophilized rTrCB2 was dissolved in sterile PBS and concentrated using Amicon Ultra 0.5 mL centrifugal filters (10 000 MWCO, Merck Millipore). Endotoxin contamination after this preparation is <0.03 EU/ μ g.²³

The protein concentration of both antigens was measured by Quant-iT Protein Assay Kit (Thermo Fisher Scientific). They were used immediately after preparation or stored in -20°C no longer than 2 weeks.

2.4 | Isolation of tissue samples: blood, spleen, lymph nodes, bone marrow

The infected mice were sacrificed 3, 7, 14 and 21 dpi, and healthy mice (referred to as "0 dpi") were used as a control ($n = 5$ for each group). They were deeply anaesthetized by Isoflurine® (Vetpharma Animal Health), bled out and sera were collected and stored in -20°C for no longer than 2 weeks. The serum samples were used for the detection of cytokines or parasite-specific antibodies. Spleen and draining lymph nodes (DLN) (axillary and inguinal for periphery; deep cervical and lumbar for the CNS) were harvested. The tissue was gently squashed in PBS, homogenized by a plastic pestle and filtered through 70 μm cell strainer. The cell suspension was used for flow cytometry characterization (spleen and DLN samples), gene expression analysis using quantitative real-time PCR (qPCR) (spleen samples), and ex vivo stimulation by TrH (spleen samples). Bone marrow progenitor cells were isolated from femurs and tibiae and used for differentiation of BMDCs in vitro (see below).

2.5 | Flow cytometry of splenocytes and lymph node cells

The cell suspensions prepared from the spleen and lymph nodes were washed in PBS and transferred to 96-well U-bottom plates (10^6 cells in 100 μL /well). The cells were treated with anti-CD16/CD32 antibody (clone 93, eBioscience) for 10 minutes and then incubated with a mixture of surface marker antibodies (CD3, CD4, CD8 and CD25) for 30 minutes in the dark at 4°C . The cells were then permeabilized and fixed by True-Nuclear™ Transcription Factor Buffer Set (BioLegend) and incubated with a mixture of antibodies recognizing the transcription factors (T-bet, GATA-3, ROR γ t and Foxp3) for 30 minutes in the dark at 4°C . The samples were acquired by LSRII (Becton Dickinson; BD) using FACSDiva (BD), and data were analysed in FlowJo (BD). Both isotype and fluorescence minus one controls (FMO) were used for CD25 and all transcription factors. Summary of the antibodies is provided in Table S1 and the gating strategy is presented in Figure S1. Briefly, T cell populations were identified as follows: Th1: CD3+CD4+T-bet+; Th2: CD3+CD4+GATA-3+; Th17: CD3+CD4+ROR γ t+; Treg: CD3+CD4+Foxp3+.

2.6 | Splenocyte stimulation

The splenocytes from 0 and 7 dpi mice were stimulated by TrH to assess the antigen-specific immune response. The cell suspension

was prepared as described above, and red blood cells were lysed by ammonium-chloride-potassium (ACK) lysis buffer (157 mmol/L NH_4Cl , 10 mmol/L KHCO_3 and 0.1 mol/L EDTA-Na_2 ; pH 7.2-7.4). After washing with PBS, the cells were resuspended in RPMI 1640 (Lonza) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific), 2 mmol/L L-glutamine (Lonza), 100 U/mL penicillin (Lonza) and 100 $\mu\text{g}/\text{mL}$ streptomycin (Lonza). The cells were cultivated (37°C , 5% CO_2) in 2 mL of the medium in 6-well plates at the density of $2 \times 10^6/\text{mL}$. The cells were stimulated by TrH (10 or 1 $\mu\text{g}/\text{mL}$) or concanavalin A (1.25 $\mu\text{g}/\text{mL}$, Sigma-Aldrich; a positive control) or left unstimulated. After 72 hours, the medium was collected and stored in -20°C for no longer than 2 weeks.

2.7 | Cytokine measurement by ELISA

Concentrations of cytokines in the cell culture supernatants of stimulated splenocytes and in sera of mice were detected using enzyme-linked immunosorbent assay (ELISA). ELISA MAX standard kits (BioLegend) for IL-4, IL-10, IL-17 and IFN- γ were used according to the manufacturer's instructions. The sera were diluted 1:4, while cell culture supernatants were analysed undiluted.

2.8 | Antibody detection using ELISA

Parasite-specific IgG, IgG1 and IgG2a were determined in the serum samples. The 96-well plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated with 100 μL of TrH (6.25 $\mu\text{g}/\text{mL}$) in the carbonate buffer (15 mmol/L Na_2CO_3 and 35 mmol/L NaHCO_3 , pH 9.6) overnight at 4°C . After washing (0.05% Tween 20 in PBS, PBS-T), the wells were blocked with 5% nonfat dry milk (Bio-Rad) in PBS-T for 2 hours. The wells were then incubated with the serum samples diluted 1:80 in PBS-T for 2 hours. Following washing, anti-mouse IgG (Sigma-Aldrich, A2554; diluted 1:8000), IgG1 or IgG2a (Abcam, ab97240 or ab97245, respectively; both diluted 1:2500) peroxidase-conjugated secondary antibodies were added for 1.5 hours. Tetramethylbenzidine liquid substrate (Sigma-Aldrich) was used for visualization of the reaction, which was then stopped with 1 mol/L hydrochloric acid and read at 450 nm (Tecan Infinite M200 reader, Schoeller Instruments). The cut-off value (99.9% confidence level) was calculated according to Ref.²⁴

2.9 | Preparation and stimulation of BMDCs

Bone marrow-derived dendritic cells were prepared as described previously^{25,26} with small modification (instead of GM-CSF produced from cell line J588, recombinant murine (rm) GM-CSF together with rm IL-4 were used). Briefly, BMDCs were generated from bone marrow progenitor cells obtained by flushing femur and tibiae of mice 0 and 3 dpi. After red blood cell lysis (ACK buffer), progenitor cells were seeded at a concentration of 10^5 cells/mL

in supplemented media (RPMI 1640 containing 2 mmol/L L-glutamine, 2 mmol/L HEPES, 40 mg/L gentamicin, 10% FBS, 20 ng/mL rmGM-CSF, and 10 ng/mL rmlL-4) and cultured for 7 days (37°C, 5.5% CO₂). On day 7, the cells were harvested, counted and seeded in 24-well plate for an additional 3 days. On day 10, the cells were stimulated by TrH and rTrCB2 (10 and 1 µg/mL) for 24 hours and lipopolysaccharide (LPS; 1 µg/mL) was used as a positive control. Maturation status of BMDCs was then measured by flow cytometry and gene expression of selected genes was tested using qPCR.

2.10 | Flow cytometry of BMDCs

Bone marrow-derived dendritic cells were harvested by a cell scraper, washed with PBS and stained by antibodies against cell surface markers (CD11c, CD80, CD86, MHCII). Antibody details are provided in Table S1 and the gating strategy is shown in Figure S2. After 15 minutes incubation, the cells were washed twice with PBS and immediately acquired using BD FACS Canto II (BD). Data were analysed using FlowJo (BD).

2.11 | qPCR

Bone marrow-derived dendritic cells or splenocytes were harvested, RNA was extracted and gene expression was quantified as described previously.²⁷ Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen) followed by reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was quantified using TaqMan gene expression assays (see Table S2). Quantification of relative gene expression was analysed as described previously.²⁷ Briefly, the number of the cycle when fluorescence exceeded the threshold in the linear part of the amplification curve (ct) of the gene of housekeeping gene was subtracted from the ct of the gene of interest. This difference (Δ ct) was calculated for all samples. Next, $\Delta\Delta$ ct was calculated as the difference between Δ ct of the particular sample minus the average value of Δ cts of control samples (unstimulated cells). Finally, the results of gene expression are expressed as $2^{-\Delta\Delta$ ct}.

2.12 | Statistical analyses

Differences among groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. In the case of unequal standard deviations, Brown-Forsythe ANOVA and Dunnett's T3 test were applied. If two factors were analysed, two-way ANOVA and Dunnett's or Šidák's test were used. Some data sets (indicated in the text) were log(10)-transformed to meet two-way ANOVA assumptions. The analyses were performed in GraphPad Prism (version 8.2.1), and *P*-values < .05 were considered significant. Data are presented as means with standard deviations.

3 | RESULTS

3.1 | T cells expanded in the spleen and the CNS DLN 7 dpi

T cell immune response was markedly elevated 7 and 14 dpi as demonstrated by increased numbers of CD4+ (*P* < .05, *P* < .01, respectively) and CD8+ T cells (*P* < .05 and *P* < .001, respectively) in the spleen (Figure 1A) and the CNS DLN (Figure 1C; *P* < .001 for both timepoints and populations). The similar trend was noticed also for CD4+ T cells in the peripheral DLN, but their number grew more steadily and not significantly (Figure 1B). In all examined groups, the number of both T cell populations plummeted 21 dpi. Unexpectedly, the decreased number of both CD4+ and CD8+ T cells was noticed 3 dpi in the spleen.

Parasite-specific serum IgG1, IgG2a (Figure 1D) and IgG (Figure S3) were detected as soon as 7 dpi and their levels rose steadily until 21 dpi. Equal levels of IgG1 and IgG2a were detected at each timepoint.

Three days post-infection, two mice (out of five) developed a hind leg paralysis (Video S1). However, it was apparently not associated with any of the examined parameters.

3.2 | Neuroinflammation did not affect the dynamics of T-bet+ and GATA-3+ T cells in the spleen and DLNs

Dynamics of individual T cell populations throughout the infection was assessed based on their proportion in the spleen and DLN for periphery and CNS. The proportion of CD4+T-bet+ T cells (~Th1) decreased in the spleen (Figure 2A, *P* = .001) and CNS DLN (Figure 2G, *P* = .012) 3 dpi rising back 7 dpi. As for the periphery DLN, CD4+T-bet+ cells reached the top 7 dpi (Figure 2D, *P* = .003) with a slight and insignificant decrease 3 dpi. In all groups, the CD4+T-bet+ cells declined 14 and 21 dpi, even below the levels of healthy mice in the case of the spleen (Figure 2A, *P* < .001 and *P* = .035, respectively).

The percentage of CD4+GATA-3+ T cells (~Th2) rose in the periphery DLN already 3 dpi (Figure 2E, *P* = .011) and reached a clearly defined peak in all examined groups 7 dpi (spleen: Figure 2B, *P* < .001; periphery DLN: Figure 2E, *P* < .001; CNS DLN: Figure 2H, *P* = .009). Afterwards, it steadily decreased to the levels similar to healthy mice. Only in the CNS DLN, the elevated proportion of CD4+GATA-3+ cells persisted up to 14 dpi (Figure 2H, *P* = .008).

The proportion of CD8+T-bet+ T cells in the spleen (Figure 2C) and the periphery DLN (Figure 2F) had the similar trend as CD4+GATA-3+ cells, showing the sharp peak 7 dpi (*P* < .001, resp. *P* = .009) followed by a decrease until 21 dpi. On the contrary, the population was diminished in the CNS DLN 3 dpi (Figure 2I, *P* = .002) with a comeback 7 dpi, which resembled the dynamics of CD4+T-bet+ cells.

The dynamics of CD4+Foxp3+ T cells (~Treg) differed from those mentioned before. In the spleen and the CNS DLN, they were most

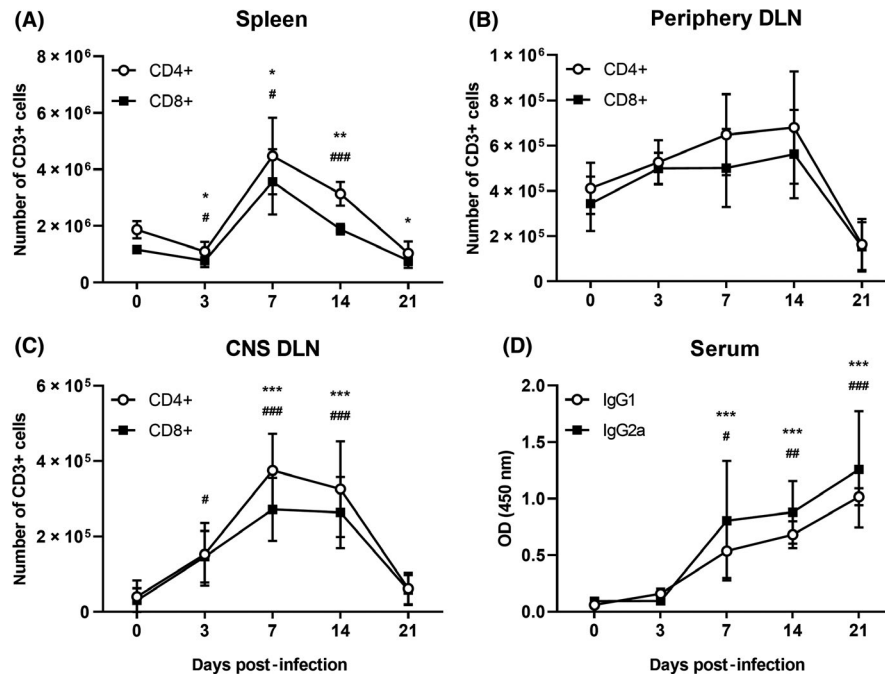


FIGURE 1 *Trichobilharzia regenti*-infected mice developed both humoral and cellular responses 7 days post-infection. Total number of CD3+CD4+ and CD3+CD8+ cells assessed by flow cytometry in the spleen (A), draining lymph nodes (DLN) for periphery (axillary and inguinal) (B) and DLN for the central nervous system (CNS) (deep cervical and lumbar) (C). Serum levels of *T. regenti*-specific IgG1 and IgG2a as measured by ELISA (D). All values are shown as means $\pm$ standard deviation ($n = 5$). Differences between infected and healthy mice (0 days post-infection) were evaluated by Brown-Forsythe analysis of variance (ANOVA) followed by Dunnett's T3 test (A) or ordinary one-way ANOVA followed by Dunnett's test (B-D). Significant differences in number of CD3+CD4+ cells or IgG1 levels are indicated with * $P < .05$, ** $P < .01$, *** $P < .001$. Differences in number of CD3+CD8+ cells or IgG2a levels are indicated with # $P < .05$, ## $P < .01$, ### $P < .001$

prevalent 3 dpi (Figure 3A, $P < .001$ and Figure 3C, $P < .022$, respectively). The same trend was observed for CD25, which appeared on CD4+Foxp3+ T cells most frequently 3 dpi (Figure 3A, $P < .001$). No significant changes in the dynamics of CD4+Foxp3+ cells were noticed in the periphery DLN.

The percentage of CD4+ROR γ t+ (~Th17) cells fluctuated all the time around very low values without any significant changes or trends (data not shown).

Gene expression of the aforementioned transcription factors and cytokines characteristic for the respective T cell subsets (IFN- γ , IL-4, IL-10) was also analysed in the spleen samples. However, no or very little changes were noticed (Figure S4). Briefly, the relative expression of *Tbx21* reached a peak 7 dpi ($P = .026$) after a slight decrease 3 dpi while *Ifng* declined throughout the infection with a significant diminution 14 dpi ($P = .004$). *Gata3* was downregulated 3 dpi ($P = .001$) and increased 21 dpi ($P = .013$) while only a slight decrease was noticed in the case of *Foxp3* 7 dpi ($P = .023$). No trends or significant changes were observed for *Il4*, *Il10* and *Il12a*.

Additionally, we measured the concentrations of IFN- γ , IL-4, IL-10 and IL-17 in the sera of mice. However, we only recognized increased levels of IFN- γ 3 dpi (Figure S5, $P = .009$); the other cytokines did not show significant trends or differences throughout the infection.

3.3 | Cercarial homogenate induced massive production of IFN- γ by splenocytes

Antigen-specific cytokine production by splenocytes from healthy and infected (7 dpi) mice was examined after stimulation by TrH (cercarial homogenate). It induced a massive, dose-dependent production of IFN- γ by splenocytes from infected, but not healthy mice (Figure 4A, $P < .001$). IL-4, IL-17 and IL-10 were produced in the same manner, but in much lower concentrations and only after the stimulation by 10 μ g/mL of TrH (Figure 4B-D, $P < .001$ for all three cytokines). Interestingly, Con A-stimulated splenocytes harvested from the infected mice produced more IL-4 and IL-10, but less IL-17 than those from healthy mice (Figure S6, $P < .001$ for all three cytokines).

3.4 | rTrCB2 markedly altered maturation status of BMDC and boosted the expression of cytokines and chemokines

The maturation status of BMDCs obtained from healthy and infected (3 dpi) mice was evaluated after the stimulation with TrH and rTrCB2 (Figure 5A-C). If compared to the unstimulated group, the proportions of CD80+, CD86+ and MHC II+ cells were

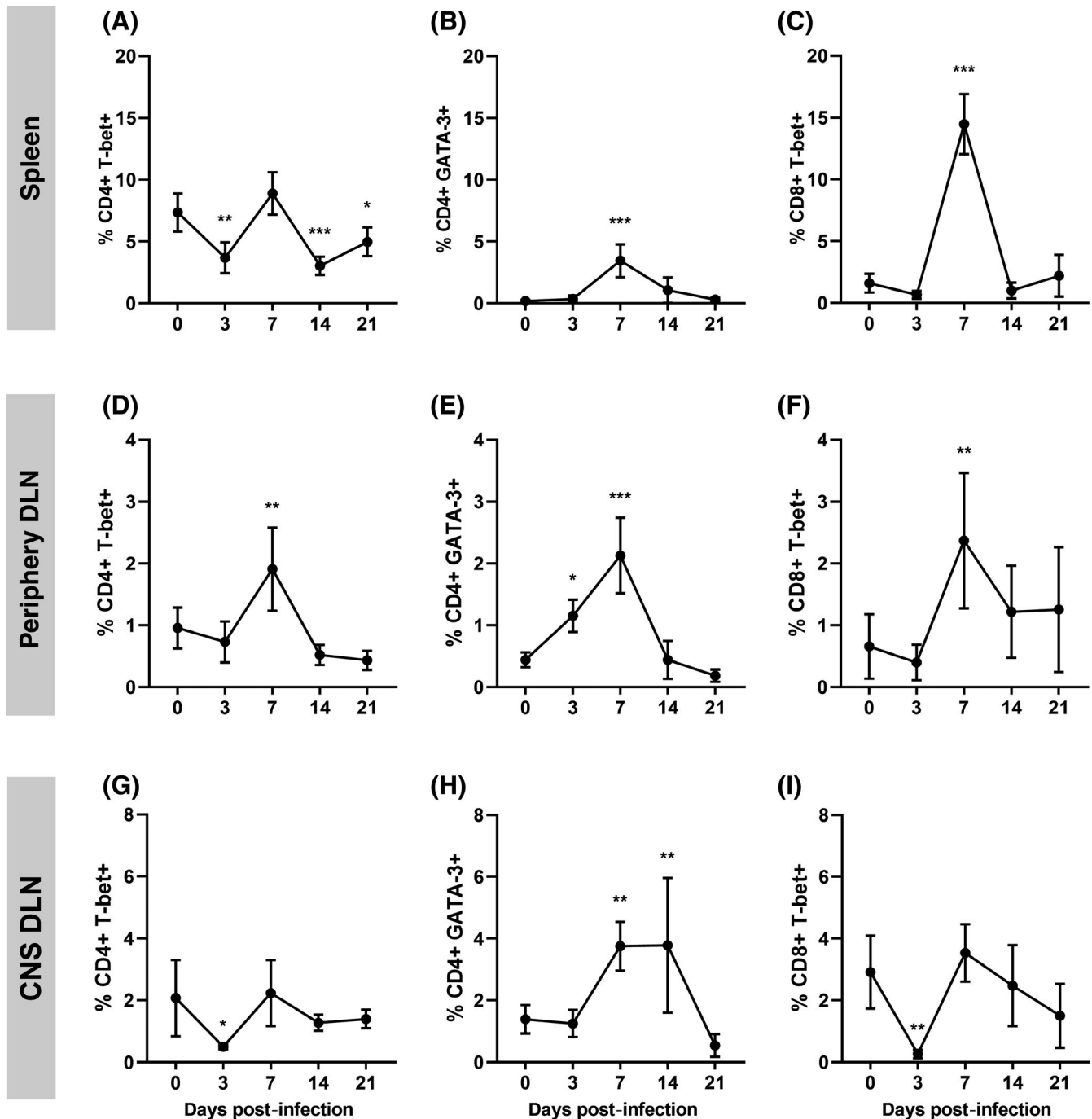


FIGURE 2 Neuroinflammation did not affect the dynamics of T-bet⁺ and GATA-3⁺ T cells in the periphery. Proportion of CD3⁺CD4⁺T-bet⁺ (A, D, G), CD3⁺CD4⁺GATA-3⁺ (B, E, H) and CD3⁺CD8⁺T-bet⁺ (C, F, I) cells in spleen (A, B, C), draining lymph nodes (DLN) for periphery (axillary and inguinal) (D, E, F) and for the central nervous system (deep cervical and lumbar) (G, H, I) during the infection was compared with healthy mice (0 days post-infection). All values are shown as means $\pm$ standard deviation ($n = 5$). Differences between infected and healthy mice (0 days post-infection) were evaluated by ordinary one-way analysis of variance (ANOVA) followed by Dunnett's test. * $P < .05$, ** $P < .01$, *** $P < .001$

significantly increased in BMDCs stimulated by 10 $\mu\text{g/mL}$ of rTrCB2. The effect was dose-dependent and usually stronger in BMDCs from infected mice. As for TrH, it only affected the proportion of MHC II⁺ BMDCs.

Apart from the maturation status, expression of genes coding for chemokines (*Ccl5*, *Cxcl10*; Figure 5D,E) and cytokines (*Tnf*,

Il12a, *Il33*, *Il10*; Figure 5F-I) was also quantified. Similarly to the effects on the BMDCs maturation status, TrH also showed a limited stimulation capacity in terms of boosting cytokine/chemokine expression. In particular, it only triggered *Ccl5* and *Il12* expression in BMDCs derived both from healthy or infected mice. On the contrary, rTrCB2 strongly augmented *Ccl5*, *Cxcl10*, *Il12a*, *Il33* and *Il10*

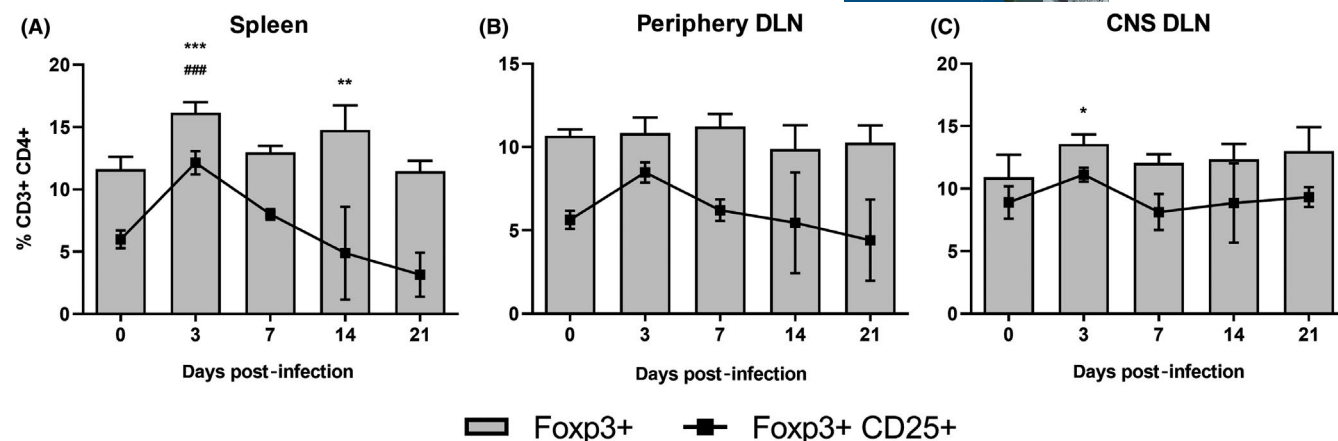


FIGURE 3 The proportion of CD4+CD25+Foxp3+ cells peaked shortly after the infection. Columns represent the proportion of all CD3+ CD4+Foxp3+ cells and lines represent CD3+ CD4+CD25+ Foxp3+ cells in the spleen (A), draining lymph nodes (DLN) for periphery (axillary and inguinal) (B) and for the central nervous system (deep cervical and lumbar) (C). All values are shown as means $\pm$ standard deviation ($n = 5$). Differences between infected and healthy mice (0 days post-infection) were evaluated by ordinary one-way analysis of variance (ANOVA) followed by Dunnett's test. Significantly different results of CD3+CD4+Foxp3+ cells are indicated with * $P < .05$, ** $P < .01$ and *** $P < .001$. Significantly different results of CD3+CD4+CD25+ Foxp3+ cells are indicated with ### $P < .001$

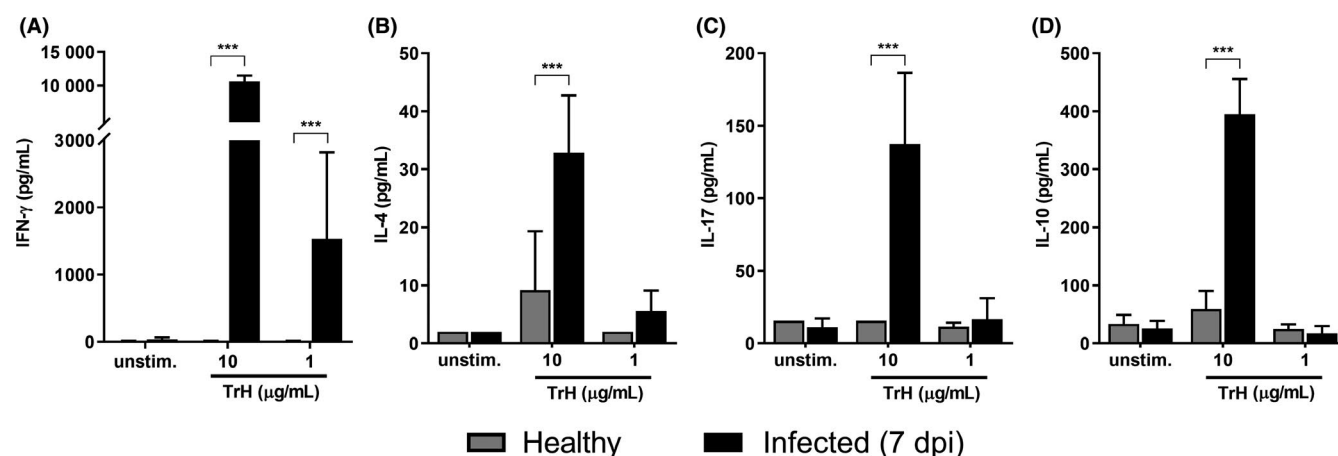


FIGURE 4 Homogenate of *Trichobilharzia regenti* cercariae induced massive production of IFN- γ by splenocytes isolated from infected mice. Production of IFN- γ (A), IL-4 (B), IL-17 (C), IL-10 (D) by splenocytes stimulated by homogenate of *T. regenti* cercariae (TrH). All values are means $\pm$ standard deviation ($n = 5$). Results from healthy mice ("0 dpi") and 7 days post-infection ("7 dpi") were evaluated by two-way analysis of variance (ANOVA) and Šidák's test after log-transformation of data (***) $P < .001$

expression in a dose-dependent manner. The boosting effect was usually more noticeable in BMDCs from infected mice, especially in the case of stimulation by 1 μ g/mL. Neither TrH nor rTrCB2 affected the expression of *Tnf*.

4 | DISCUSSION

In this study, we described the peripheral immune response of mice infected with *T. regenti*. This schistosome exhibits a unique neurotropic behaviour in birds and mammals and the migration through the CNS can cause severe pathologies, such as balance disorder or even leg paralysis.²¹ We observed leg paresis in two mice (out of five) 3 dpi when schistosomula reach the spinal cord.²⁰ Nevertheless, none of the examined immune parameters

was specifically altered in these mice in comparison to other individuals. This corroborates with the previous data which suggested that the paresis is a consequence of direct mechanical damage of the nervous tissue by migrating schistosomula rather than of immunopathological processes.²⁰

The early phase of the infection (represented by "3 dpi" group in our setting) is typical by massive dying of parasites in the skin, which is accompanied by accumulation of neutrophils and other cells with phagocytic capacity. A mixture of cytokines, such as IL-1 β , IL-4, IL-10 and IL-12p40, was detected in biopsies of pinnae at this early stage.¹⁶ Additionally, high amounts of IFN- γ and IL-10 were recorded if the cells isolated from the skin DLN shortly after the infection were stimulated with cercarial homogenate.¹⁶ Although this might indicate early Th1/Treg polarization, the proportion of CD4+T-bet+ (~Th1) cells seemed to be diminished or did not change

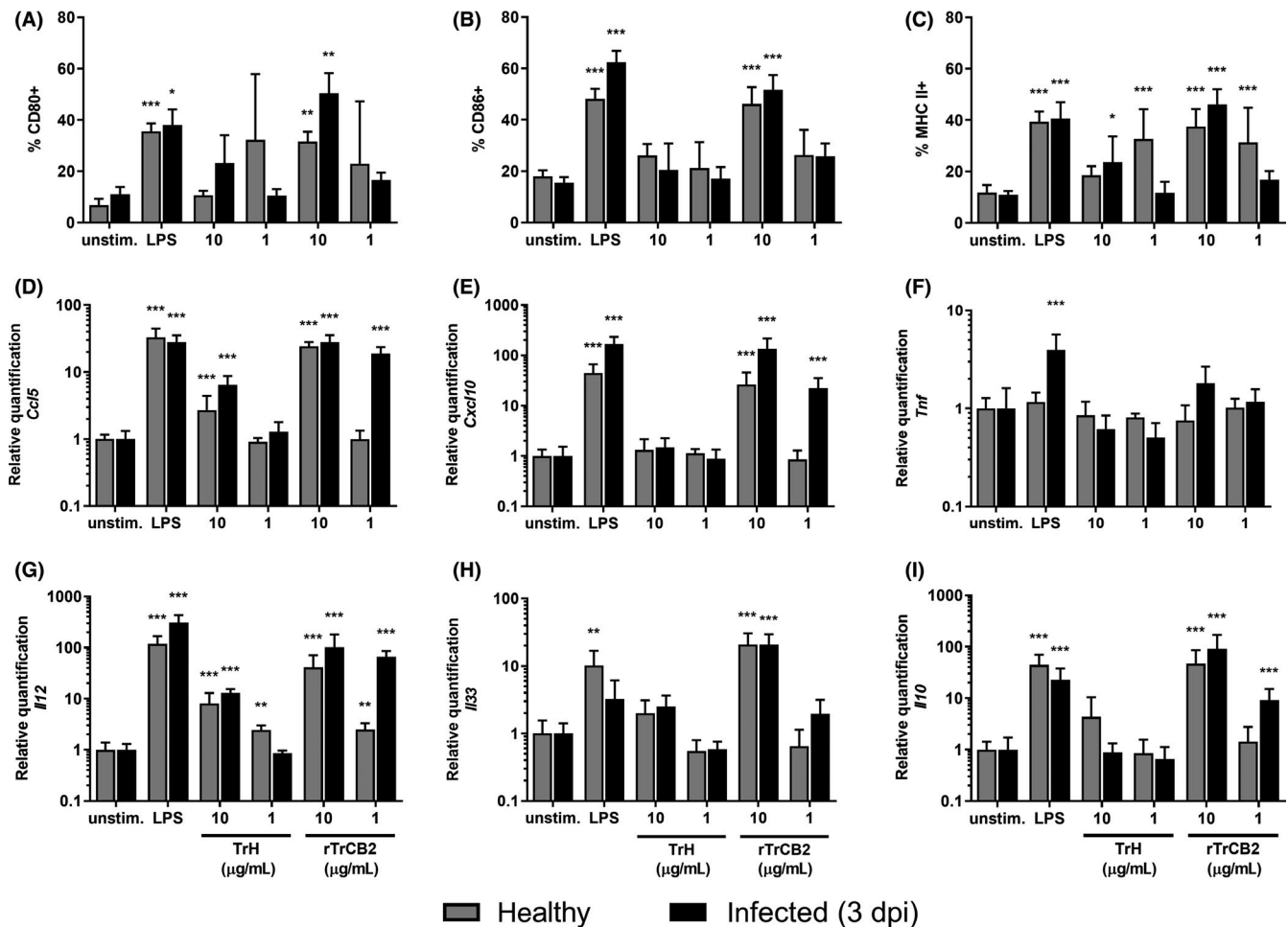


FIGURE 5 *Trichobilharzia regenti* antigens activated bone marrow-derived dendritic cells (BMDCs) in vitro. BMDCs prepared from healthy mice ("0 dpi") or mice 3 days post-infection ("3 dpi") were stimulated by homogenate of cercariae (TrH) or recombinant cathepsin B2 (rTrCB2) of *T. regenti*. Lipopolysaccharide (LPS) was used as a positive control. Proportion of CD80+ (A), CD86+ (B) and MHC II+ (C) BMDCs detected by flow cytometry. Expression of *Ccl5* (D), *Cxcl10* (E), *Tnf* (F), *Il12* (G), *Il33* (H) and *Il10* (I) was evaluated by qPCR. All values are means $\pm$ standard deviation ($n = 5$). Differences between stimulated and unstimulated BMDC were evaluated by two-way analysis of variance (ANOVA) followed by Dunnett's test. Data in A, D, E and G groups were before testing log-transformed. Significantly different results are indicated with * $P < .05$, ** $P < .01$ and *** $P < .001$

in the lymphoid organs examined in our study. On the other hand, CD4+GATA-3+ (~Th2) proportion in the DLN for periphery and especially CD4+Foxp3+ (~Treg) cells in all observed lymphoid organs appeared to be increased suggesting they might contribute to IL-10 production. As for Tregs, it is in contrast with their unchanged presence in the periphery DLN or spleen in early *Schistosoma mansoni* infection.²⁸⁻³⁰ Here, IL-10 is produced by CD4+ T cells,³⁰ specifically Th2²⁸ or dermal dendritic cells.³¹ The regulatory milieu might be induced, among others, by skin commensal bacteria.³⁰ The contribution of dendritic cells cannot be excluded in the case of *T. regenti*-infected mice as well because BMDCs prepared from mice 3 dpi significantly increased *Il10* expression after exposure to rTrCB2. The production of IL-10 seems to be restricted to the infected skin since no changes in IL-10 levels were noticed in serum samples and also *Il10* expression in the spleen remained unchanged. This is in accordance with the data from *S. mansoni*-infected skin, where direct contact with cercariae was necessary to induce regulatory phenotype of

monocyte-derived dendritic cells.³¹ However, deeper exploration of cellular source of IL-10 is warranted.

A mixed type 1/2 response peaked 7 dpi when the percentage of T-bet+ and GATA-3+ cells was highest in all analysed lymphoid organs. However, the most clearly defined peaks were noticed in the spleen and the periphery DLN suggesting that especially here the skin infection was mirrored. Interestingly, only percentage of GATA-3+ cells in DLN for CNS persisted until 14 dpi when the cellular immune response in the spinal cord culminates (Macháček, unpublished). However, a noticeable biological variance was recorded at this timepoint which discourages stronger conclusions. Afterwards, all examined T cell populations returned to the values of healthy controls. It is consistent with recovering of the skin¹⁶ and CNS inflammation and continuous vanishing of living schistosomes.²⁰ The mixed type 1/2 profile was underpinned by equal levels of parasite-specific IgG1 and IgG2a, which are associated with Th2 and Th1, respectively.^{32,33}

Splenocytes isolated from mice 7 dpi, when the cellular immune response reached a peak, were stimulated by cercarial homogenate, which triggered massive antigen-specific production of IFN- γ and IL-10. Considering the proportion of T cells in the spleen 7 dpi, IFN- γ could be produced by CD8+T-bet+ T cells while IL-10 could be associated with a prominent CD4+GATA-3+ T cell population. Interestingly, we detected only a limited antigen-specific production of IL-17 which corroborated with almost unchanged proportions of CD4+ROR γ t+ (~Th17) cells. This was rather unexpected since IL-17 plays an important role in recruitment and activation of neutrophils,³⁴ which quickly infiltrate skin lesions formed around *T. regenti* schistosomula.^{15,16} Transient Th17 response is also induced in acute murine schistosomiasis being mediated by *S. mansoni* cathepsin B1.³⁵ Possibly, IL-17 production might be suppressed by IL-10 in order to prevent excessive tissue pathology.^{36,37}

Although the mixed type 1/2 immunity in the early stage of *T. regenti* infection has been well characterized, the initial steps regarding the activation of dendritic cells by parasite antigens remained unexplored. Thus, we tested the effects of cercarial homogenate on BMDCs. However, it only subtly increased the proportion of MHC II+ cells and slightly prompted the expression of *Ccl5* and *Il12a*. This outcome suggests that cercarial homogenate containing a mixture of somatic antigens only marginally affects BMDCs triggering the inflammation-promoting phenotype. The reason might be that dendritic cells do not normally encounter damaged schistosomula during the early stage of the infection. They should rather recognize ESP released into the host tissues during the skin penetration. Cysteine peptidases, important components of *T. regenti* ESP, are essential for infection of the host and digestion of host components, but they were also suggested to trigger the immediate inflammatory response.¹⁶ To test this assumption, we exposed BMDCs to rTrCB2, the peptidase that is abundant in *T. regenti* ESP being responsible for cleavage of skin proteins.¹⁴ rTrCB2 activated BMDCs much more effectively than cercarial homogenate, and apart from *Il12a* and *Ccl5*, it also induced expression of *Cxcl10*, *Il10* and *Il33*. CCL5 (RANTES) and CXCL10 (IP-10) were observed in the skin after *S. mansoni* infection³⁸ as well and could induce production of IL-6³⁹ which was present in the skin early after *T. regenti* infection¹⁶ or promote Th1 response,⁴⁰⁻⁴² respectively. Furthermore, CXCL10 regulates the proportion of CD4+T-bet+ cells in the circulation and lymph nodes during the infection.⁴³ The cytokine profile expressed by rTrCB2-stimulated BMDCs, including IL-10, IL-12 and IL-33, suggests the mixed type 1/2 response, which corroborates with previous data presented in this study. These effects could be caused either by direct immunogenicity of rTrCB2 or by its enzymatic activity which could trigger protease-activated receptors.^{44,45} The latter explanation is supported by recent observations of inability to stimulate immune cells of inactive *T. regenti* cathepsin B1.6.⁴⁶ Similarly, the proteolytic activity of *S. mansoni* cathepsin B1 is necessary for induction of T cell response.³⁵ Interestingly, although BMDCs were "artificially" prepared from bone marrow progenitors (having never really met the parasite antigens), the cells obtained from mice 3 dpi showed a stronger reaction than those from healthy

ones. It can be explained by epigenetic interactions of the trained immunity⁴⁷ which was observed also after immunization by homogenate of *Fasciola hepatica*.⁴⁸

In conclusion, we are the first to describe the dynamic of the peripheral immunity in mice infected with the neurotropic schistosome *T. regenti*. The study revealed that the inflammation in the skin influenced T-cell-based immune response in the lymphoid organs towards mixed type 1/2 response with a peak at 7 dpi. Later the inflammation in the spinal cord did not probably affect the peripheral immunity so strongly. Last but not least, we proved the capacity of *T. regenti* cathepsin B2 to activate BMDCs much more effectively than cercarial homogenate.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this study.

AUTHOR CONTRIBUTIONS

PH and JH conceived, designed and/or supervised the study. MM, TM and LS conducted the experiments and analysed the data. MM, TM and JH interpreted the findings. MM and TM drafted the manuscript. All authors edited, read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon a reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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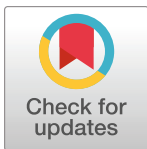
RESEARCH ARTICLE

Mechanisms of the host immune response and helminth-induced pathology during *Trichobilharzia regenti* (Schistosomatidae) neuroinvasion in mice

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Abstract

Helminth neuroinfections represent serious medical conditions, but the diversity of the host-parasite interplay within the nervous tissue often remains poorly understood, partially due to the lack of laboratory models. Here, we investigated the neuroinvasion of the mouse spinal cord by *Trichobilharzia regenti* (Schistosomatidae). Active migration of *T. regenti* schistosomula through the mouse spinal cord induced motor deficits in hindlimbs but did not affect the general locomotion or working memory. Histological examination of the infected spinal cord revealed eosinophilic meningomyelitis with eosinophil-rich infiltrates entrapping the schistosomula. Flow cytometry and transcriptomic analysis of the spinal cord confirmed massive activation of the host immune response. Of note, we recorded striking upregulation of the major histocompatibility complex II pathway and M2-associated markers, such as arginase or chitinase-like 3. Arginase also dominated the proteins found in the microdissected tissue from the close vicinity of the migrating schistosomula, which unselectively fed on the host nervous tissue. Next, we evaluated the pathological sequelae of *T. regenti* neuroinvasion. While no demyelination or blood-brain barrier alterations were noticed, our transcriptomic data revealed a remarkable disruption of neurophysiological functions not yet recorded in helminth neuroinfections. We also detected DNA fragmentation at the host-schistosomulum interface, but schistosomula antigens did not affect the viability of neurons and glial cells *in vitro*. Collectively, altered locomotion, significant disruption of neurophysiological functions, and strong M2 polarization were the most prominent features of *T. regenti* neuroinvasion, making it a promising candidate for further neuroinfection research. Indeed, understanding the diversity of pathogen-related neuroinflammatory processes is a prerequisite for developing better protective measures, treatment strategies, and diagnostic tools.

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Author summary

Infections of the human central nervous system by parasitic worms, such as pork tapeworm or rat lungworm, are a health threat, especially in communities with poor hygiene and sanitation. Understanding the diversity of host-parasite interactions, possibly leading to development of effective treatment or vaccine, requires suitable animal models. Our study deeply characterized the neuroinvasion of the mouse spinal cord by the neuropathogenic flatworm *Trichobilharzia regenti*. Its behavior within the nervous tissue (active migration, eager feeding) and clinical outcome of the infection (weight loss, motor dysfunctions, strong neuroinflammation) resemble those observed in human diseases. Using an integrative approach, we elucidated how the mice fight against the parasites and which mechanisms could be responsible for the pathological sequelae. Moreover, we identified pathways potentially involved in the reparation of the injured nervous tissue. All these features make *T. regenti* in mice a promising candidate for further research in parasitology, and neuroimmunology.

Introduction

Parasitic helminths often invade the central nervous system (CNS) of mammals, including humans. Invasion of the CNS is either a natural part of their somatic migration or represents an unwanted, ectopic localization [1]. The clinical manifestation of helminth neuroinfections ranges from mostly asymptomatic to very severe, leading to sensory or cognitive deficits and seizures or epilepsy [2–4]. Many factors, such as parasite burden or localization within the CNS, influence the course and outcome of the neuroinfection [5]. Moreover, the host immune response affects helminth growth and survival but might also participate in the pathogenesis or behavioral alterations [6,7]. Therefore, a deep understanding of host-parasite immune interactions is required to develop better protective measures, treatment strategies, and diagnostic tools.

The availability of suitable laboratory models dictates the primary orientation of the helminth neuroinfection research. Neurocysticercosis, cerebral angiostrongylosis, and neurotoxocarosis are hence in focus as their models have been established [8–13]. On the contrary, a valid model representing, e.g., the neurological form of schistosomiasis or echinococcosis is lacking [14–16]. Novel model species for studying helminth neuroinfections are thus needed to reveal the diversity of host-parasite interactions and (immuno-)pathological sequelae. Even if they do not fully mirror any specific human disease, investigating their interactions with the host could bring valuable insights. This is also the case of some filarial or intestinal model helminths that only partially reflect the human diseases but are useful after integrating the experimental findings [17,18]. Regarding the neurotropic helminths, the species naturally invading the CNS should be considered as no artificial manipulations, such as injections of parasites into the CNS [12,16], are required.

Trichobilharzia regenti is a schistosome that naturally migrates through the CNS of birds and mammals (birds are the definitive hosts, whereas mammals represent the accidental hosts). After penetration of the host skin, the newly transformed schistosomula find the peripheral nerves *via* which they enter the spinal cord [19,20]. Here they eagerly feed on the nervous tissue, but no significant demyelination is observed [21–23]. However, leg paralysis is often reported in ducks and immunocompromised mice due to a higher schistosomula burden and/or their prolonged persistence within the CNS [21,22]. On the contrary, immunocompetent mice effectively control *T. regenti* neuroinvasion. Indeed, recruited peripheral leukocytes entrap and destroy the schistosomula in the spinal cord 7–14 days post infection (dpi) [22,24].

		<i>T. regenti</i> -infected				Uninfected control			
		7	14	21	28	7	14	21	28
GENERAL	Days post infection								
	Relative body weight	●	●	●	●	●	●	●	●
	Relative spleen weight	●	●	●	●	●	○	○	○
	Behavioral testing	●	○	○	○	●	○	○	○
BLOOD	Anti- <i>T. regenti</i> antibodies	●	●	●	●	●	○	○	●
	Eosinophil counts	●	●	●	●	●	○	○	●
SPINAL CORD	Schistosomula burden	●	●	●	●	(not examined)			
	Parasite DNA load	●	●	●	●	●	○	○	○
	Histology	●	●	●	●	●	○	○	○
	Flow cytometry	●	●	●	●	●	○	○	●
	Transcriptomic analysis	●	●	●	○	●	●	●	○
	Immunohistochemistry	●	●	●	○	●	○	○	○
	Light-sheet microscopy imaging	●	●	○	○	●	○	○	○
	TUNEL assay (apoptosis)	●	●	○	○	●	○	○	○
	Microdissection & proteomics	●	○	○	○	●	○	○	○
HEMISPHERES	Schistosomula burden	●	●	●	●	(not examined)			
	Parasite DNA load	●	●	●	●	●	○	○	○
	Flow cytometry	●	●	●	●	●	○	○	●
BRAIN STEM, CEREBELLUM	Schistosomula burden	●	●	●	●	(not examined)			
	Flow cytometry	●	●	●	●	●	○	○	●

Fig 1. Summary of experimental procedures. C57BL/6J female mice were infected with *T. regenti* cercariae and examined at desired time points (indicated by filled red dots). Uninfected mice were used as controls; particular age-matching is shown for each analysis (filled blue dots). Empty dot means that the analysis was not performed at the respective time point.

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The CNS-resident microglia are also activated [22] but do not seem to harm the living schistosomula, e.g., by the production of nitric oxide [25,26]. Even though living schistosomula are rarely found after 21 dpi [22,24], the host effector immune mechanisms remain unclear.

Here we employed a complex approach, ranging from behavioral testing to ‘omics’ analyses (Fig 1), to explore mechanisms of the host immune response and helminth-induced pathology in the CNS of mice infected with *T. regenti*. Our comprehensive insight allows us to compare *T. regenti*-neuroinvasion with other medically important helminth neuroinfections and uncover the diversity of host-parasite interactions within the nervous tissue. In this regard, the remarkable disruption of neurophysiological functions (not observed in other helminths yet) and a strong M2 polarization in *T. regenti*-infected spinal cords should be of particular interest.

Results

T. regenti triggered peripheral immunity and invaded the CNS, especially the spinal cord

First, we briefly characterized general features of *T. regenti* infection to establish the model and validate its reproducibility in C57BL/6J mice 7, 14, 21, and 28 dpi. The infection did not alter mouse survival, but the infected individuals exhibited a decreased gain in body weight (Fig 2A; $p(7 \text{ dpi}) = 0.0022$; $p(14 \text{ dpi}) = 0.0024$; $p(28 \text{ dpi}) = 0.0009$) despite having the same feed consumption rate as the uninfected group. The infected mice produced both parasite-specific IgG1 and IgG2a as soon as 7 dpi. Levels of Th2-associated IgG1 rose continuously and prevailed over Th1-associated IgG2a (Fig 2B), especially 28 dpi ($p < 0.0001$). These observations of antibody response corroborate with the previous studies [27–29], which demonstrates

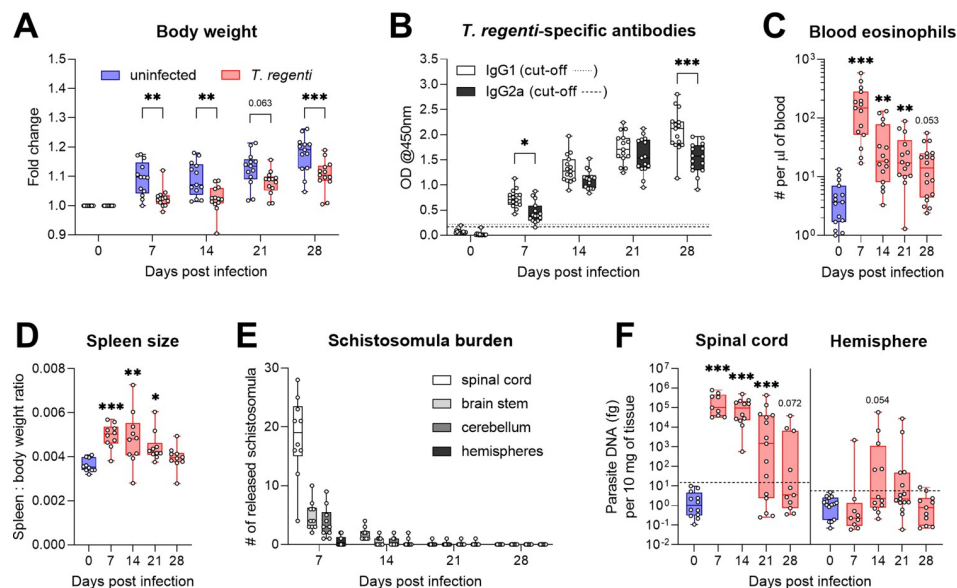


Fig 2. General features of *T. regenti* infection in C57BL/6J mice. (A) Infected mice exhibited a lower gain in body weight than age-matched uninfected controls. (B) The levels of *T. regenti*-specific IgG1 and IgG2a detected in mouse sera rose steadily throughout the infection. Th2-associated IgG1 dominated over Th1-associated IgG2a, especially 28 dpi. (C) Blood eosinophil counts have increased in infected mice 7–21 dpi. (D) Spleen enlargement was observed in infected mice 7–21 dpi. (E) Within the CNS, most viable schistosomula were found 7 dpi being localized predominantly in the spinal cord. The amount of released schistosomula remarkably decreased at later time points. (F) Contrary to those from hemispheres, *T. regenti* DNA was detected in most of the spinal cord tissue samples 7–21 dpi. In each graph, points show data from individual mice. Pooled data from 2–3 independent experiments are shown. Data were evaluated by 2-way ANOVA for repeated measures and Šidák's test (A), ordinary 2-way ANOVA and Šidák's test (B, F), Kruskal-Wallis and Dunn's test (C), or ordinary 1-way ANOVA and Dunnett's test (D); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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reproducibility of the host immune reaction. The number of blood eosinophils peaked 7 dpi and remained elevated 14 and 21 dpi (Fig 2C; $p(7 \text{ dpi}) < 0.0001$; $p(14 \text{ dpi}) = 0.0017$; $p(21 \text{ dpi}) = 0.0053$). A similar trend was observed in the case of spleen enlargement (Fig 2D; $p(7 \text{ dpi}) = 0.0007$; $p(14 \text{ dpi}) = 0.0019$; $p(21 \text{ dpi}) = 0.0456$), which correlates with already reported increased numbers of splenic CD4⁺ and CD8⁺ T cells 7 and 14 dpi [29].

Due to *T. regenti* neurotropism, we further examined the distribution of schistosomula within the CNS, which has not been thoroughly done in C57BL/6J mice. The highest number of viable schistosomula was extracted 7 dpi (27.3 ± 9.8 per mouse, $n = 10$). They predominantly invaded the spinal cord (Fig 2E) but were also localized in the brain stem or the cerebellum. However, only 3 out of 10 mice harbored 1–2 schistosomula in the hemispheres. Markedly fewer schistosomula were released from the entire CNS 14 dpi (3.0 ± 2.1 per mouse, $n = 10$) and 21 dpi (4 schistosomula isolated from 2 mice out of 10); no schistosomula were recorded 28 dpi. Such migration pattern agrees with the historical records (obtained from other mouse strains) showing accumulation of schistosomula in the spinal cord and the highest burden within the CNS around 7 dpi [20,21]. To complement the data on schistosomula distribution, we also analyzed *T. regenti* DNA content in the spinal cord and hemisphere tissue samples. The parasite DNA was present in the spinal cords of all mice 7 and 14 dpi, and most of the mice 21 dpi (Fig 2F; $p(7, 14, \text{ and } 21 \text{ dpi}) < 0.0001$). On the contrary, the hemispheres seldom contained the parasite DNA; less than half of the samples were positive 14 and 21 dpi.

Collectively, our data demonstrated early activation of the peripheral immune response against *T. regenti*, which successfully invaded the CNS, especially the spinal cord, by 7 dpi.

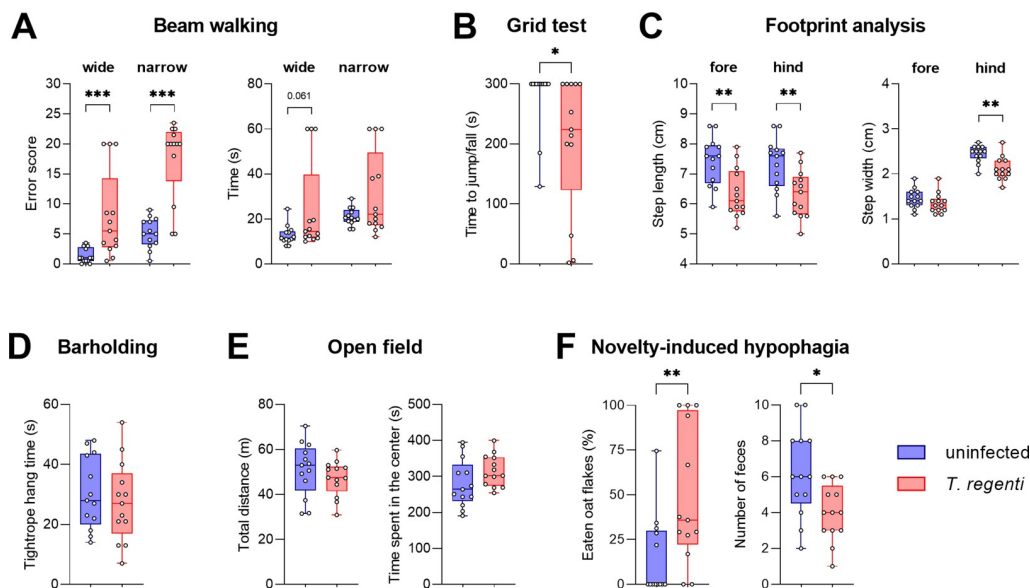


Fig 3. Effects of *T. regenti* infection on mouse behavior 7 dpi. (A) Beam walking on wide and narrow beams, impaired motor coordination in infected mice. (B) Grid test, worse endurance, and strength of the forelimbs and hindlimbs of infected mice. (C) Footprint analysis of forelimbs and hindlimbs, altered posture and gait in infected mice. (D) Bar holding, normal endurance, and strength of the forelimbs in both groups. (E) Open field, unaffected locomotor activity, and anxiety in both groups. (F) Novelty-induced hypophagia, higher amount of eaten oat flakes caused by stronger appetitive motivation, rather than increased anxiety of infected mice. In each graph, points show data from individual mice. Pooled data from 3 independent experiments are presented. Data were evaluated by Mann-Whitney test (A, B, F—eating) or unpaired t-test (C, D, E, F—feces); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Motor function deficits affected the lower body of *T. regenti*-infected mice

The highest schistosomula burden was detected in the spinal cord 7 dpi. At this moment, we assessed the impact of neuroinvasion on various aspects of mouse behavior. Most importantly, the infected mice showed a deficit in motor functions. They made more errors when traversing a wide ($p = 0.0006$) or narrow ($p < 0.0001$) beam (Fig 3A and S1 Video), exhibited lower endurance when hanging upside-down on a grid (Fig 3B; $p = 0.0313$), and made shorter steps (Fig 3C; $p(\text{forelimbs}) = 0.0027$; $p(\text{hindlimbs}) = 0.0059$). Visual observations also showed altered tail posture in the infected mice (tail dragging, reduced tail motility). We suggest that motor functions of the lower body were affected as the performance in the bar holding task, depending predominantly on forelimb strength, was unaltered (Fig 3D), and step width was decreased in hindlimbs only (Fig 3C; $p(\text{forelimbs}) = 0.2545$; $p(\text{hindlimbs}) = 0.001$). However, general locomotor activity in the open field (Fig 3E), plus maze or Y-maze was normal, as well as spatial working memory in the Y-maze (S1 Fig). In the test of novelty-induced hypophagia, the infected mice ate more ($p = 0.0094$) and defecated less ($p = 0.0117$) than controls (Fig 3F). This behavior is more likely related to a stronger appetitive motivation than altered emotionality as we detected no signs of elevated anxiety (Fig 3E) or depression-like behavior in several other tests (S1 Fig). Collectively, our experiments showed that deficits in motor functions were the most evident behavioral sequelae of *T. regenti* neuroinvasion.

Eosinophil-rich infiltrates entrapped and eliminated schistosomula in the spinal cord

After assessing the general impact of *T. regenti* infection on mice, we performed a histological examination of the spinal cord to elucidate the course of the infection in the most affected

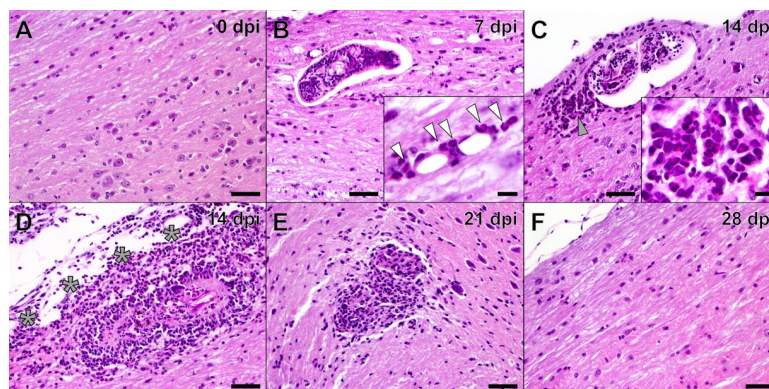


Fig 4. Histological examination of the *T. regenti*-infected spinal cord. (A) In the uninfected spinal cord, no leukocyte infiltration was apparent. (B) The intact schistosomulum in the white matter 7 dpi. No inflammatory cells surrounded the schistosomulum, but eosinophil extravasation was recorded in the adjacent areas (inset, eosinophils marked by white arrowheads). (C) The “rocket-tail” leukocyte cluster behind the schistosomulum 14 dpi. Eosinophils and mononuclear cells prevailed in the cluster (gray arrowhead and inset). (D) The inflammatory lesion around the destroyed schistosomulum 14 dpi. The adjoining leptomeningeal spaces (gray asterisks) were thickened and heavily inflamed. (E) The schistosomulum remnants within the fading inflammatory lesion 21 dpi. (F) The spinal cord tissue 28 dpi, no schistosomula residues or inflammatory foci were evident. Representative images from 2 independent experiments (each with 2–3 mice) per time point are shown. Scale bar (large image) = 50 µm, scale bar (inset) = 10 µm.

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segment of the CNS. Only intact schistosomula were observed 7 dpi being localized predominantly in the white matter (Fig 4B). Flattened cells with squeezed nuclei surrounded some of the schistosomula, while some were followed by “rocket-tail” leukocyte clusters (see below). Extravasation of leukocytes, mainly eosinophils (Fig 4B, inset), was recorded in the areas close to migrating schistosomula. The neuroinflammation culminated 14 dpi when no schistosomula were left unnoticed by the host immune cells. Of note, striking “rocket-tail” leukocyte clusters, consisting mainly of eosinophils and mononuclear cells (Fig 4C, inset), formed behind schistosomula (Fig 4C). Some parasites were still compact but had a thinner body wall and vacuolized internal tissues with condensed nuclei. However, some schistosomula were already damaged, and eosinophils, neutrophils, monocytes, and lymphocytes extensively infiltrated the lesions (Fig 4D). The adjacent leptomeningeal spaces were also thickened and heavily inflamed. The inflammatory foci around hardly distinguishable schistosomula or their remnants were still apparent 21 dpi (Fig 4E), but the leukocyte infiltration was fading away. Neither schistosomula residues nor inflammatory foci were detected 28 dpi (Fig 4F). Taken together, *T. regenti* neuroinvasion caused eosinophilic meningomyelitis with a peak 14 dpi. The cellular infiltrate was presumably essential for parasite elimination.

Eosinophils were the most numerous cells infiltrating the entire CNS during *T. regenti* infection

Based on histological findings, we employed flow cytometry to quantify the immune cells infiltrating the infected nervous tissue to see the dynamics of the cellular immune response. Apart from the spinal cord, we also examined the brain stem, the cerebellum, and the hemispheres, which can also be invaded by schistosomula, however, to a much lesser extent (Fig 2E). The spinal cord and hemispheres were infiltrated mainly by eosinophils, lymphoid cells, and macrophages/monocytes with a peak 14 dpi (Fig 5A and 5D). A similar situation was observed in the brain stem (Fig 5B), where a significant increase was also noticed in microglia and neutrophil counts 7 dpi. In the cerebellum, eosinophil counts spiked 14 dpi (Fig 5C), but neutrophil,

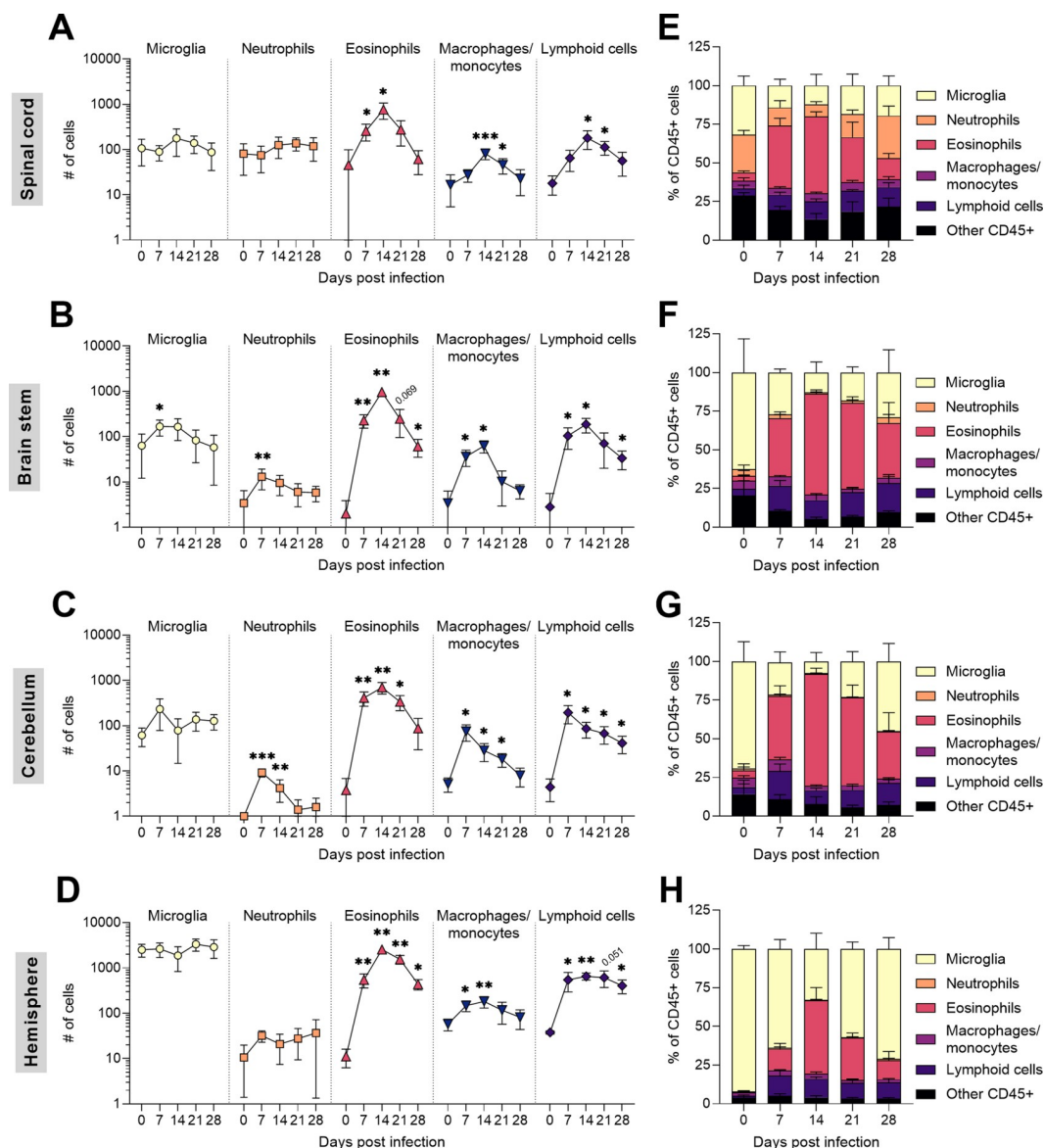


Fig 5. Dynamics of major immune cell populations in the mouse CNS during *T. regenti* infection. (A–D) Microglia, neutrophils, eosinophils, macrophages/monocyte, and lymphoid cells were analyzed by flow cytometry in the spinal cord (A), the brain stem (B), the cerebellum (C), and the hemisphere (D). In all examined segments, the most prominent increase was observed in eosinophil counts 14 dpi. Gating strategies are shown in S2 Text. Data ($n = 5$ per each time point) were evaluated by ordinary 1-way or Welch's ANOVA followed by Dunnett's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E–H) The relative proportion of the examined immune cell populations in the spinal cord (E), the brain stem (F), the cerebellum (G), and the hemisphere (H). Massive infiltration of peripheral leukocytes caused an apparent decrease in the proportion of microglia. Representative data from 1 out of 2 independent experiments are shown.

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macrophage/monocyte, and lymphoid cell counts reached climax already 7 dpi. The microglia counts remained unchanged in all segments, except for the brain stem 7 dpi (see above). However, their relative representation stooped in favor of infiltrating leukocytes, especially eosinophils (Fig 5E–5H). Taken together, *T. regenti* neuroinvasion led to striking infiltration of the CNS with peripheral leukocytes. Eosinophils undoubtedly prevailed in all examined segments having a peak 14 dpi.

Infected spinal cords displayed transcriptional upregulation of immune system pathways and disruption of neurophysiological functions

To get a complex insight into the processes ongoing in the infected spinal cords, transcriptomic analysis of the whole tissue was performed 7, 14, and 21 dpi using respective age-matched uninfected controls. In total, 24 transcriptomes were sequenced with an average number of $43,115 \pm 2,276$ transcripts (Fig 6A). RNA-seq read data are available for download via the NCBI sequence read archive (BioProject ID: PRJNA716607). Differential expression analysis showed the highest number of differentially expressed transcripts (DETs) 7 dpi when more transcripts were downregulated (6,943) than upregulated (4,128). The number of DETs decreased 14 and 21 dpi, and upregulated transcripts prevailed over downregulated ones (Fig 6B and S1 Table).

Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate DETs to get a general overview of the up-/downregulated processes. In total 2,225, 1,536, and 1,248 upregulated KEGG-IDs were identified 7, 14, and 21 dpi, respectively (Fig 6C), and represented 27 (7 dpi), 47 (14 dpi), and 27 (21 dpi) significantly enriched biological pathways (S1 Table). The most enriched pathways were linked to the immune system at all time points (Fig 6D). For example, chemokine signaling and leukocyte transendothelial migration pathways were significantly enriched 7 and 14 dpi when the spinal cord infiltration by peripheral leukocytes was on the rise (Fig 5A). A detailed analysis revealed the upregulation of several chemokines (*Ccl2*, *Ccl5*, *Ccl7*, *Ccl8*, *Ccl24*, *Cxcl9*, *Cxcl16*) and their receptors (Fig 7A and 7B) which facilitate the recruitment of leukocytes into the CNS. Throughout the infection, remarkable enrichment was also noticed in the Toll-like receptor signaling pathway, which participates in recognizing pathogen-associated molecular patterns. Namely, *Tlr8*, *Tlr11*, and *Tlr12* exhibited the highest upregulation (Fig 7C). Pathways associated with antigen processing were also enriched, especially 7 dpi. Indeed, cathepsin S (*Ctss*), a lysosomal enzyme critical for antigen presentation, was found among the top 20 upregulated DETs 7 dpi (Fig 6F) and retained the markedly elevated expression also 14 and 21 dpi ($\log_2$ fold change = 15 and 11, respectively). Accordingly, Th1/Th2/Th17 cell differentiation and B cell receptor signaling pathways were upregulated as soon as 7 dpi, suggesting early adaptive response activation. These transcriptomic data correspond to our experimental results demonstrating the production of parasite-specific antibodies (Fig 2B) or mixed Th1/Th2/Th17 splenic phenotype [29] 7 dpi. Interestingly, complement and coagulation cascades and the C-type lectin receptor pathway were mostly enriched 14 dpi when the highest eosinophil infiltration and first injured schistosomula were found in the spinal cord (Fig 4C and 4D).

The infection of the mouse spinal cord was also linked with significant downregulation of transcription. Overall, 2,492, 827, and 857 downregulated KEGG-IDs were identified 7, 14, and 21 dpi, respectively (Fig 6C), and represented 33 (7 dpi), 18 (14 dpi), and 9 (21 dpi) enriched pathways (S1 Table). They were mostly associated with signal transduction, synaptic transmission, and axonal development (Fig 6E). Accordingly, we observed enormous downregulation of kinesin family members *Kif1a* and *Kif1b*, essential players in the axonal transport of synaptic vesicles and mitochondria [30,31], and cytosolic carboxypeptidase 1 (*Agtbpl1*, also known as *Ccp*) deficiency of which causes coordination deficits [32]. They all were even among the top 20 downregulated DETs at all time points (Fig 6F). On the contrary, the expression of structural neurofilaments remained almost unchanged except for nestin (*Nes*, Fig 7D) required for glial scar formation [33,34]. Of myelin-associated transcripts, only proteolipid protein 1 (*Plp1*) was strikingly upregulated 7 dpi (Fig 7E), which might boost the activation of microglia [35]. Importantly, no transcriptional evidence was found for demyelinating processes (Fig 7E), which accords with previous histopathological studies [22,26]. However,

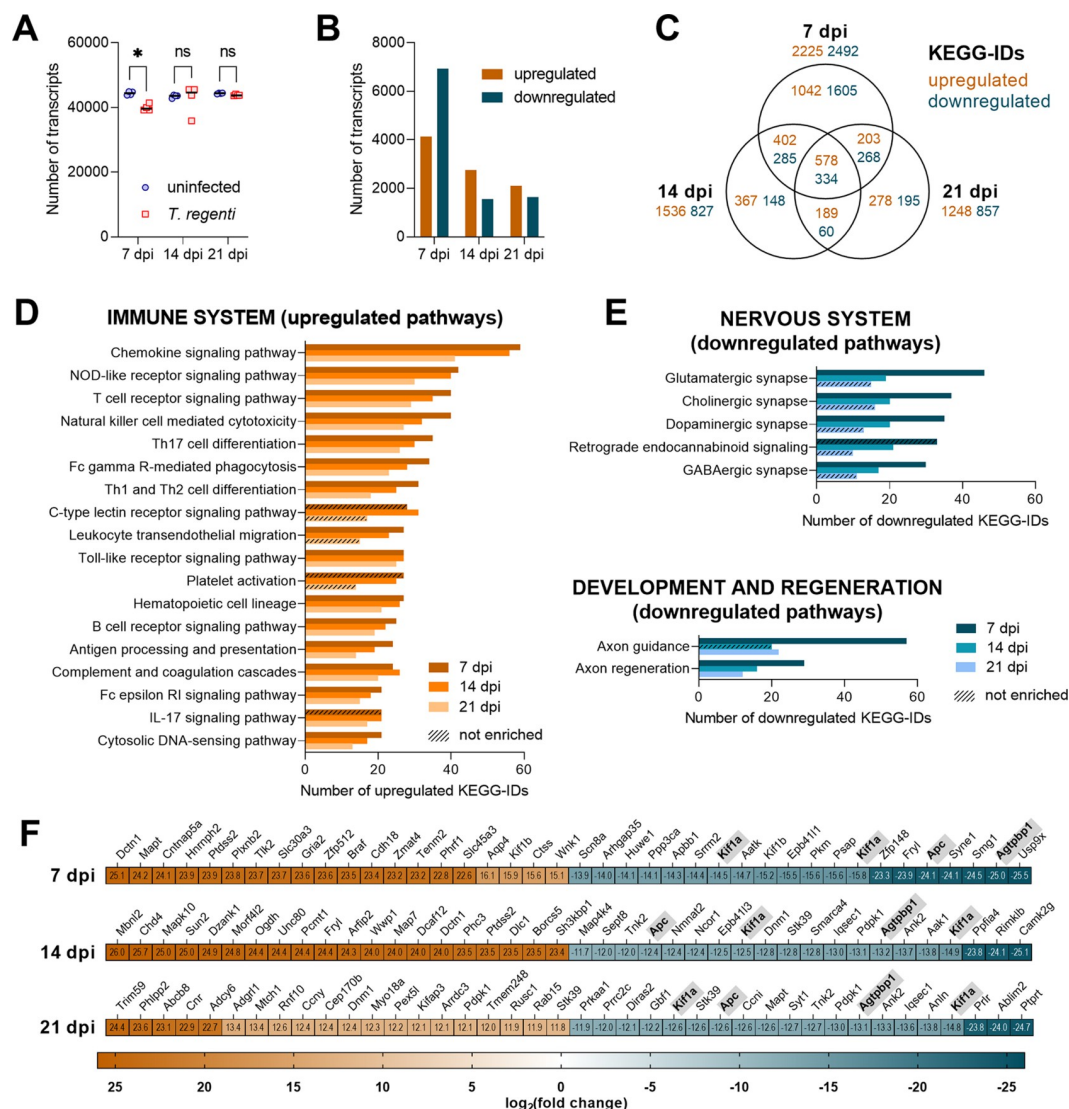


Fig 6. Transcriptomic analysis of the spinal cord of *T. regenti*-infected mice. (A) The number of transcripts identified in the spinal cords of infected or age-matched uninfected (control) mice 7, 14, and 21 dpi. The transcriptomic analysis was performed with a group of mice (n = 4 per each time point) infected independently from other experiments presented herein. Data were evaluated by ordinary 2-way ANOVA and Sidák's test; *p<0.05. (B) The number of differentially expressed transcripts with Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation and their exclusive/shared occurrence in the infected spinal cord 7, 14, and 21 dpi. (C) Differentially expressed transcripts with Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation and their exclusive/shared occurrence in the infected spinal cord 7, 14, and 21 dpi. (D) Enriched immune system pathways of upregulated transcripts according to KEGG annotation. (E) Enriched pathways of downregulated transcripts linked with the nervous system and development and regeneration according to KEGG annotation. (F) Top 20 up-/downregulated transcripts (based on log₂FC) 7, 14, and 21 dpi. Abbreviated gene names are shown, gray background marks genes found among the "top 20" at all time points.

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massive upregulation was observed in the case of the amyloid precursor protein (*App*, Fig 7F) at all time points, which is likely linked to axonal injury caused by migrating schistosomula [22].

Altogether, the transcriptional analysis of *T. regenti*-infected spinal cords substantially elucidated general features of the host-parasite relationship. Most importantly, we demonstrated upregulation of immune processes and downregulation of neurophysiological pathways. Based on the transcriptomic data, we further examined several aspects of *T. regenti* neuroinvasion.

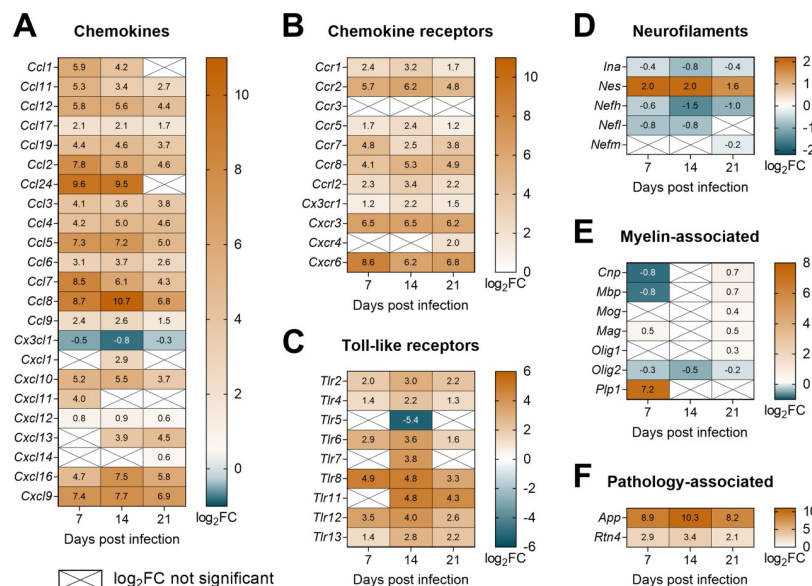


Fig 7. Expression of immune and neural markers in the spinal cord of *T. regenti*-infected mice. (A–F) The heatmaps show log₂fold change (FC) in the expression of chemokines (A), chemokine receptors (B), Toll-like receptors (C), neurofilaments (D), myelin-associated markers (E), and pathology-associated markers (F). Genes/transcripts with log₂FC > 2 or < -2 were considered as upregulated or downregulated, respectively. Only genes with a significant log₂FC are shown unless indicated otherwise (crossed cells). Spinal cords of 4 infected and 4 uninfected age-matched mice were analyzed at each time point.

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Arginase-1 dominated among the proteins found exclusively around the migrating schistosomula, which unselectively fed on the nervous tissue

To better understand the biological processes in the close vicinity of the migrating schistosomula, we microdissected the surrounding tissue within 100 µm from the schistosomula (Fig 8A) and subjected it to the mass spectrometry (MS) analysis. Infected spinal cords were analyzed only 7 dpi as the highest number of schistosomula can be found at this time point and it is possible to properly distinguish the schistosomula-host tissue interface (Figs 6B and 8B). Also, most DETs were found at this time point. Initially, microdissects from three spinal cord segments (sacral, lumbar, thoracic) were analyzed separately. As no significant differences were found in the protein composition of microdissects from different segments (S2 Table, sheets F–H), the subsequent data analysis was performed on merged datasets from all three segments.

Altogether, 1,690 proteins were identified in infected and uninfected (control) spinal cords. In total, 752 proteins were recorded in at least 3 out of 4 replicates and therefore were considered reliably identified. The vast majority (705) of proteins were common to microdissects from infected and control spinal cords, but 15 of them were differentially abundant in infected (8) or control (7) ones (Fig 8C, dashed line outline and S2 Table, sheet C). Fifteen proteins were exclusively found in the microdissects from infected spinal cords (Fig 8C, thick red outline and S2 Table, sheet A), including those presumably involved in the host immune response: arginase (a marker of alternatively activated M2 microglia/macrophages), integrin beta 1 (leukocyte adhesion molecule), or interferon-induced guanylate-binding protein 2 (enigmatic protein with antiparasitic activity; [36]). On the contrary, 35 proteins were exclusively found in uninfected mice (Fig 8C, thick blue lines and S2 Table, sheet B). They were linked mainly with the cytoskeleton and cell signaling, but no specific pathway/pattern was recognized. Corroborating with the proteomic data, most exclusive and differentially abundant proteins were

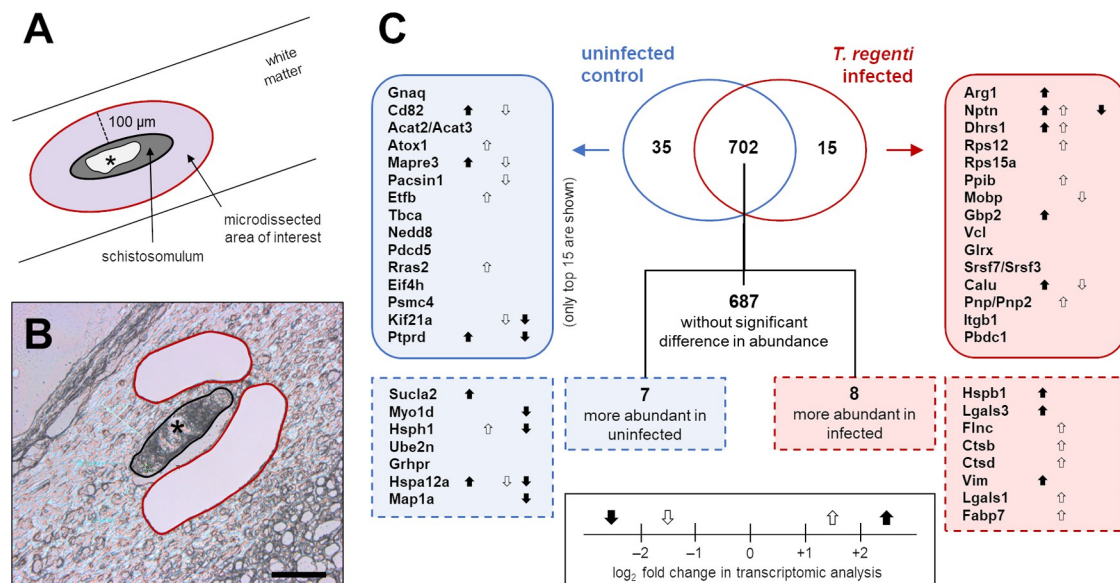


Fig 8. Proteomic analysis of the spinal cord tissue around *T. regenti* schistosomula. (A) A schematic representation of the region of interest. The area within 100 µm from the schistosomulum was microdissected as well as the schistosomulum digestive tract (marked by an asterisk; for intestinal results see the main text). (B) A real image from the microscope, labeling is consistent with (A)—i.e., the already microdissected area is outlined in red. Scale bar = 100 µm. (C) A summary of the proteomic analysis of the microdissected nervous tissue. Proteins exclusively found (thick line) or more abundant (dashed line) in uninfected (blue) or *T. regenti*-infected (red) spinal cords are shown. The up/down arrows indicate a log₂fold change (FC) in the expression of the protein mRNA as revealed by the transcriptomic analysis (see Fig 6). More arrows per one protein mean that more isoforms were detected in the transcriptome. If no arrow is shown, the log₂FC was negligible (−1; +1) or not significant. The proteomic analysis was performed with samples obtained from a group of mice (n = 3–4, see Materials and Methods for details) infected independently from other experiments presented herein.

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identified as differentially expressed (either up- or downregulated) in the “7 dpi” transcriptome. Some of them were represented by multiple transcription variants differing in the level of expression (Fig 8C, up/down arrows and S2 Table, sheet E).

Additionally, the protein composition of the schistosomula digestive tract was analyzed by MS to reveal the parasite diet. A better understanding of *T. regenti* eating habits might help to unveil the host-parasite interactions. Out of 237 reliably identified proteins, 179 were annotated as mouse proteins, 39 as *T. regenti*, and 19 shared homology with both organisms (S2 Table, sheet I). The most abundant among mouse proteins were those associated with myelin, such as myelin basic protein, myelin proteolipid protein, 2,3-cyclic-nucleotide 3-phosphodiesterase, and myelin-oligodendrocyte glycoprotein. Furthermore, neuronal and astrocytic intermediate filaments were present (neurofilament light and medium polypeptides, glial fibrillary acidic protein, vimentin) as well as the CNS extracellular matrix glycoprotein periostin. Interestingly, the schistosomula digestive tract also contained proteins related to the host immune system like lysozyme C-1, arginase, or eosinophil cationic protein 1. Endogenous *T. regenti* proteins were represented mainly by digestive peptidases (cathepsins B1.6, B1.3, B1.4, C, and leucine aminopeptidase), detoxification enzymes (thioredoxin, peroxiredoxin, and glutathione S-transferase), and metabolic enzymes (lactate dehydrogenase A and glyceraldehyde 3-phosphate dehydrogenase). Proteins homologous to both mouse and *T. regenti* included highly conserved proteins such as actin, tubulin, calmodulin, and histones.

Collectively, the proteomic analysis of tissue microdissects demonstrated that markers suggesting activation of the host immune response were present around the schistosomula already 7 dpi. Arginase was the most abundant exclusive protein indicating the presence of microglia/

macrophages with the M2 phenotype. We also found that migrating schistosomula are not selective in the diet as a mixture of proteins associated with the nervous tissue and the host immune system was detected in their digestive tract.

T. regenti infection induced M2 polarization of microglia/macrophages which accumulated around the migrating schistosomula

Polarization of activated microglia/macrophages, either pro-inflammatory M1 or anti-inflammatory M2, largely determines their role in the host immune response. Marked upregulation of *Aif1* (also known as *Iba1*), the marker of microglia/macrophages [37,38], indicated activation of the cells in response to the spinal cord invasion by *T. regenti* (Fig 9A). Initially, Iba-1 was mostly detected in the schistosomula migratory tracks (7 dpi), but later, it accumulated around schistosomula (14 dpi) or even within their damaged bodies (Fig 9D). As this observation suggested the active participation of microglia/macrophages in the schistosomula elimination, we further examined the expression of markers related to M1/M2 polarization. Despite upregulation of M1-promoting cytokines (*Ifng*, *Il1b*) throughout the infection, inducible NO synthase (*Nos2*), the effector molecule of M1 polarization, exhibited none or negligible changes in expression (Fig 9B). It corroborates our previous immunohistochemical data demonstrating the lack of inducible NO synthase in the infected spinal cord [26].

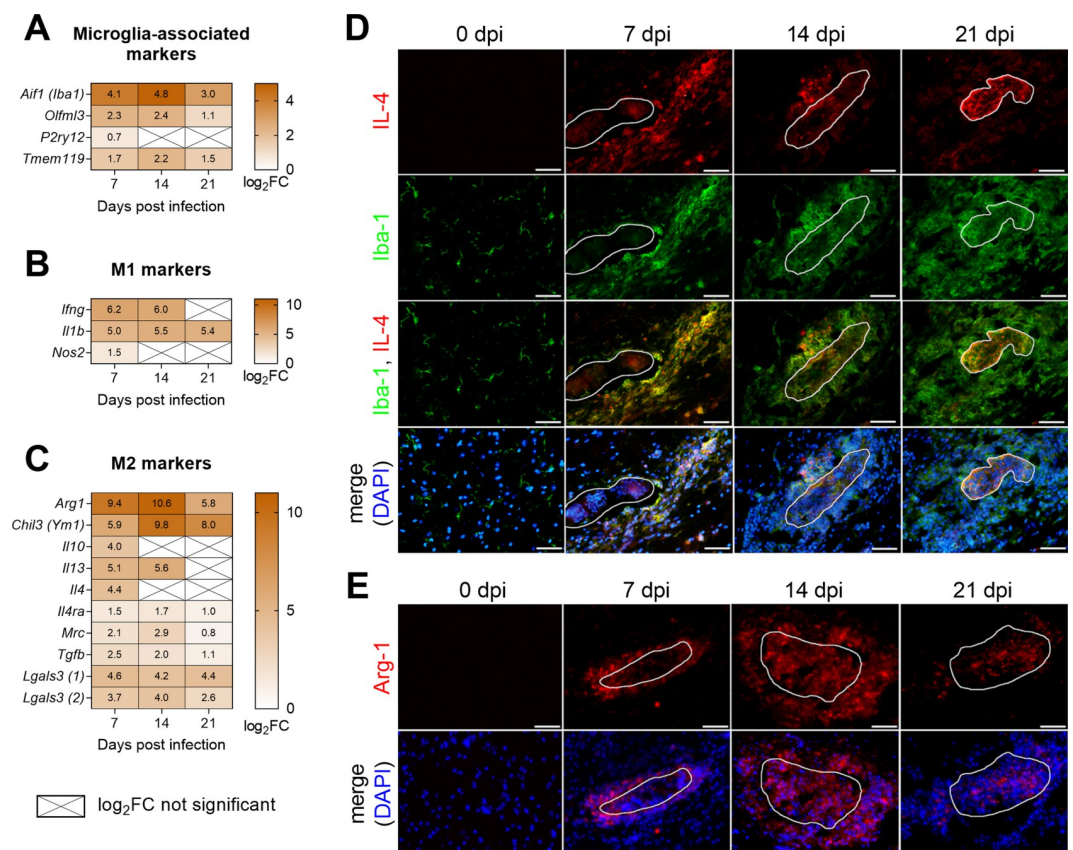


Fig 9. M1/M2 polarization in the spinal cord of *T. regenti*-infected mice. (A–C) The heatmaps show $\log_2$ fold change (FC) in the expression of markers associated with microglia (A), M1 (B), and M2 (C) polarization. Genes/transcripts with $\log_2FC > 2$ were considered upregulated, and only genes with a significant $\log_2FC$ are shown unless indicated otherwise (crossed cells). Spinal cords of 4 infected and 4 uninfected age-matched mice were analyzed at each time point. (D–E) Immunolocalization of IL-4+Iba-1 (D), and Arg-1 (E). Representative images are shown, the white line indicates the space occupied by the schistosomulum. Scale bar = 50 μ m.

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On the other hand, we recorded striking upregulation of M2-associated markers, such as arginase (*Arg1*) and chitinase-like 3 (*Chil3*, also known as *Ym1*). Accordingly, M2-triggering cytokines (*Il4*, *Il10*, *Il13*, *Tgfb*) were upregulated 7 and/or 14 dpi along with M2-promoting galectin-3 (*Lgals3*) [39] (Fig 9C). To validate the transcriptomic data, we proceeded with the immunohistochemical detection of IL-4, which drives M2 polarization and the production of arginase [40]. IL-4 colocalized with Iba-1 in the schistosomula migratory tracks 7 dpi but diminished 14 dpi (Fig 9D). However, a strong IL-4 signal was detected in the area of the destroyed schistosomula 21 dpi. On the contrary, arginase (Arg-1) closely surrounded the schistosomula 7 dpi (Fig 9E), which agreed with the previously shown microdissection data (Fig 8C). At 14 dpi, the Arg-1 signal spread further into the tissue but accumulated mostly within the damaged schistosomula 21 dpi.

Collectively, transcriptomic and immunohistochemical data confirmed a strong M2 polarization of microglia/macrophages in the infected spinal cord, especially in the close vicinity of the schistosomula.

3D-imaging revealed the expansion of MHC II⁺ cells during *T. regenti* neuroinvasion

Expression of major histocompatibility complex (MHC) II, either by professional antigen presenting or other cells, mirrors the activation status of the host immune response. In *T. regenti*-infected spinal cords, we recorded striking upregulation of *Cd74*, *H2-Aa*, *H2-Ab1*, and other members of the MHC II pathway throughout the infection (Fig 10A). Immunohistochemistry revealed accumulation of MHC II⁺ cells around the schistosomula and partial colocalization of MHC II with Iba-1, the marker of microglia/macrophages (Fig 10B). Thanks to the availability of a suitable model (MHC II-EGFP mice), we employed light-sheet fluorescence microscopy (LSFM), which allowed us to display a 3D tissue context and visualize MHC II distribution *in toto* 7 and 14 dpi. High levels of background autofluorescence impeded analysis of the gray matter, so we examined white matter regions of the thoracic spinal cord, which is preferred by the schistosomula anyway [41].

Seven dpi, the MHC II⁺ cells were scattered in the parenchyma, but some of them already contacted the schistosomula (Fig 10C and S2 and S3 Videos). Massive MHC II⁺ clusters formed around the schistosomula 14 dpi when the MHC II signal often copied the shape of parenchymal blood vessels and anterior or posterior median sulci (Fig 10C and S4 Video). These observations correlated with data from the volumetric analysis of the MHC II signal, which rocketed 14 dpi ($p < 0.0001$). Indeed, a 1000-fold increase was recorded in comparison to uninfected mice (Fig 10D). We also attempted to count the exact amount of MHC II⁺ cells, but the clusters were so dense that single nuclei were hardly recognizable, which hampered the analysis. Nevertheless, the large-scale optical sectioning provided us with a robust insight into the intensity of the immune reaction (seen as MHC II accumulation) around individual schistosomula. We established three categories of the immune reaction (Fig 10E): “mild” (+) with very few MHC II⁺ cells around/behind (“rocket tail”) the intact schistosomula; “moderate” (++) with noticeable infiltration of MHC II⁺ cells enclosing the intact schistosomula (but no MHC II signal within the schistosomula); “severe” (+++) with massive clusters enclosing the damaged schistosomula (MHC II signal present also inside the schistosomula). While mild and moderate reactions prevailed 7 dpi, severe reactions dominated 14 dpi (Fig 10F; $p = 0.0009$).

Altogether, transcriptomic analysis and 2D/3D imaging demonstrated a strong upregulation of the MHC II pathway in *T. regenti*-infected spinal cords. Interestingly, the MHC II occurred not only around the migrating schistosomula but also along the blood vessels and within the meningeal areas indicating extensive neuroinflammation.

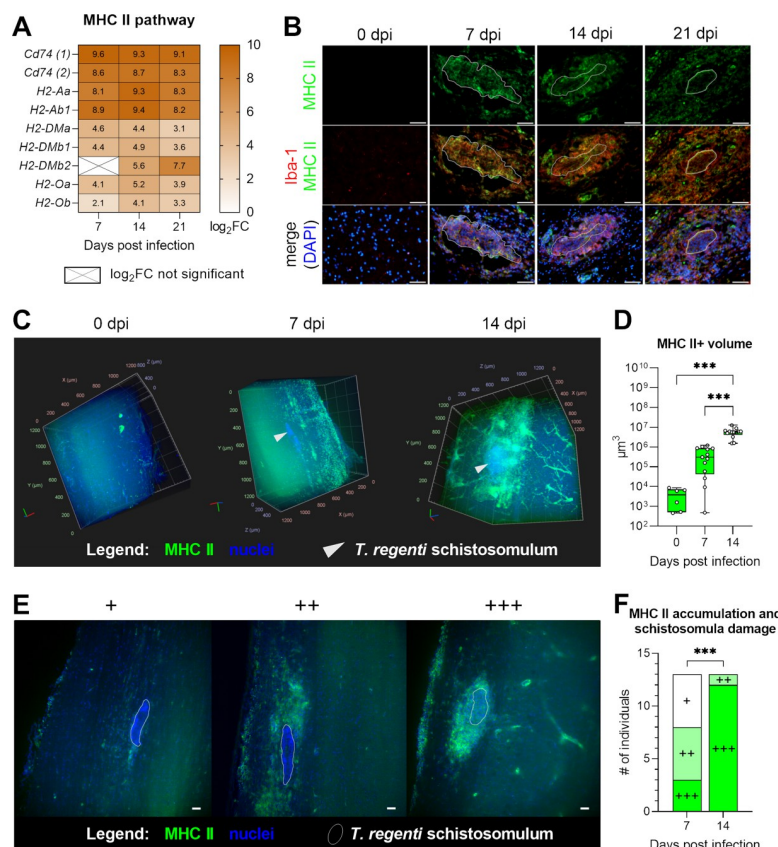


Fig 10. Major histocompatibility complex (MHC) II in the spinal cord of *T. regenti*-infected mice. (A) The heatmaps show log₂fold change (FC) in the expression of markers associated with the MHC II pathway. Genes/transcripts with log₂FC >2 were considered upregulated, and only genes with a significant log₂FC are shown unless indicated otherwise (crossed cells). Spinal cords of 4 infected and 4 uninfected age-matched mice were analyzed at each time point. (B) Immunolocalization of MHC II and Iba-1. Representative images are shown, the white line indicates the space occupied by the schistosomulum. Scale bar = 50 μ m. (C) 3D images obtained by light-sheet fluorescence microscopy (LSFM) showing MHC II+ cells in the spinal cord. Schistosomula are marked with grey arrowheads. (D) Quantification of MHC II+ volume around schistosomula. It was computed in the virtual box around the schistosomula (400 $\times$ 800 $\times$ 110 μ m, S5 Video) or six randomly chosen areas in healthy mice. (E) Patterns of MHC II accumulation and its relation to schistosomula damage. Three categories were recognized: (+) very few MHC II+ cells around/behind intact schistosomula, (++) noticeable infiltration of MHC II+ cells enclosing intact schistosomula (but no MHC II signal within the schistosomula), (+++) massive clusters enclosing damaged schistosomula (MHC II signal present also inside the schistosomula). 2D images were obtained by LSFM and created from five stitched planes in the z-axis (1.4 μ m each). Scale bar = 50 μ m. (F) Quantification of MHC II accumulation and schistosomula damage using the categories from (E). Thirteen schistosomula out of two mice were examined at each time point. Data were evaluated by Kruskal-Wallis and Dunn's test (D) and Fisher's exact test (F); ***p<0.001.

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T. regenti infection affected vascular permeability, blood-brain barrier integrity and triggered reactive astrogliosis

Schistosomula migration within the CNS might cause severe pathology. First, we focused on changes in vascular permeability and integrity of the blood-brain barrier, which are critical for maintaining CNS homeostasis. Among markers of vascular permeability, aquaporin-4 (*Aqp4*), the water channel localized to astrocyte endfeet, was strikingly upregulated 7 dpi (Fig 11A). Expression of E-cadherin (*Cdh1*) and occludin (*Ocln*), proteins responsible for tightness of the blood-brain barrier [42], was also increased, especially 7 dpi (Fig 11B). On the contrary, remarkable downregulation was noticed in tight junction protein 1 (*Tjp1*, also known as *Zo1*),

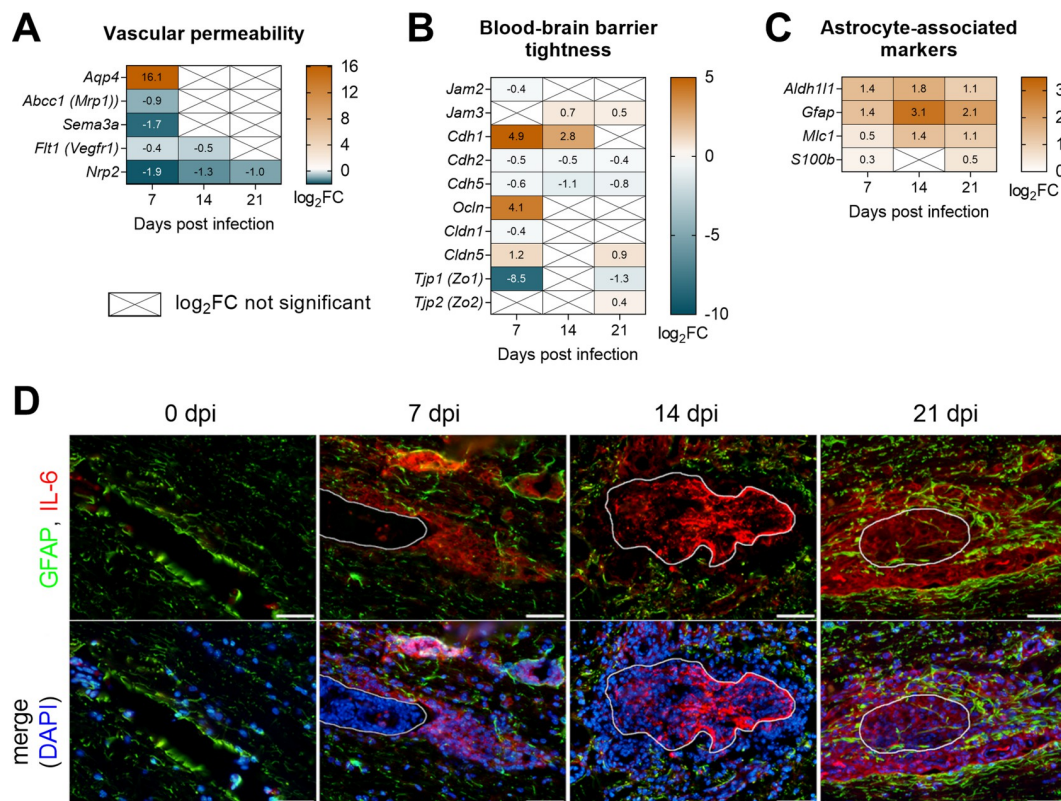


Fig 11. Pathology associated with *T. regenti* migration within the mouse spinal cord. (A–C) The heatmaps show log₂ fold change (FC) in the expression of markers associated with vascular permeability (A), blood-brain barrier tightness (B), and astrocytes (C). Genes/transcripts with log₂FC > 2 or < -2 were considered upregulated or downregulated, respectively. Only genes with a significant log₂FC are shown unless indicated otherwise (crossed cells). Spinal cords of 4 infected and 4 uninfected age-matched mice were analyzed at each time point. (D) Immunolocalization of astrocytic glial fibrillary acidic protein (GFAP) and astrogliosis-promoting IL-6 demonstrated the most prominent astrocyte hypertrophy 21 dpi. Representative images are shown, the white line indicates the space occupied by the schistosomulum. Scale bar = 50 μ m.

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a scaffold protein anchoring tight junctions to the actin fibers [42]. Although our transcriptomic data suggested that vascular permeability and blood-brain barrier integrity were mildly altered 7 dpi, we did not observe any significant increase in extravasation of Evans blue into the nervous tissue (S2 Fig).

Second, we examined the tissue injury associated with schistosomula migration. Astrocytic glial acidic fibrillary protein (*Gfap*) was upregulated 14 and 21 dpi (Fig 11C), which pointed to the initiation of reactive astrogliosis and formation of glial scar [43]. While GFAP showed normal distribution within the nervous tissue 7 dpi, it accumulated around schistosomula lesions and in their migratory tracks at later time points (Fig 11D). Astrocyte hypertrophy was most prominent 21 dpi when astrocyte processes enclosed the schistosomula or even grew through the space occupied by them. To support the observation of astrocyte activation, we also immunolocalized IL-6, which promotes astrogliosis and glial scar formation [44]. IL-6 was detected in the schistosomula migratory tracks 7 dpi and in the area of the damaged schistosomula and their surroundings 14 and 21 dpi (Fig 11D). These data indicated that reactive astrogliosis and glial scar, presumably driven by IL-6, developed to repair the tissue injured by schistosomula migration.

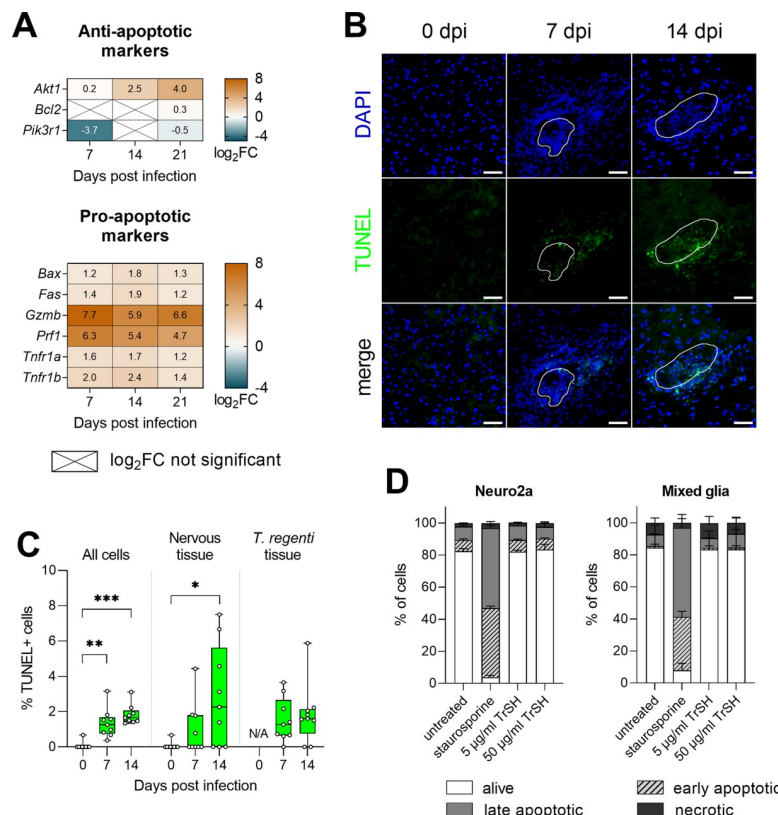


Fig 12. Apoptosis in the spinal cord of *T. regenti*-infected mice. (A) The heatmaps show log₂fold change (FC) in the expression of pro- and anti-apoptotic markers. Genes/transcripts with log₂FC > 2 or < -2 were considered as upregulated or downregulated, respectively. Only genes with a significant log₂FC are shown if not indicated otherwise (crossed cells). Spinal cords of 4 infected and 4 uninfected age-matched mice were analyzed at each time point. (B) Detection of DNA fragmentation (TUNEL+ cells) in the spinal cord cryosections. Representative images are shown, the white line indicates the space occupied by the schistosomulum. Scale bar = 50 μ m. (C) Quantification of TUNEL+ cells in the spinal cord cryosections shown in (B). The frequency of TUNEL+ cells is shown among all cells or separately for the host nervous tissue and *T. regenti* tissue. (D) Detection of apoptotic populations in Neuro2a and mixed glial cultures treated by *T. regenti* schistosomula homogenate (TrSH) for 48 hours; staurosporine was used as a positive control. Pooled data from 4 experiments are shown. Data (C, D) were evaluated by Kruskal-Wallis and Dunn's test; *p<0.05, **p<0.01, ***p<0.001.

<https://doi.org/10.1371/journal.ppat.1010302.g012>

DNA fragmentation occurred at the host-schistosomulum interface, but schistosomula antigens did not trigger apoptosis in neurons or glial cells

Apoptosis plays an important role in host-parasite interactions as it can be responsible for tissue pathology or help parasites evade the host immune system. In *T. regenti*-infected spinal cords, we observed upregulation of pro-apoptotic genes *Gzmb* and *Prf* coding for granzyme B and perforin-1, respectively (Fig 12A). They are expressed by cytotoxic T lymphocytes and natural killer cells which employ them to induce apoptosis in target cells [45]. Therefore, we stained fragmented DNA, a hallmark of apoptosis, by the TUNEL assay to localize the apoptotic cells within the infected spinal cord. While no TUNEL+ cells were found in the uninfected spinal cord, their frequency increased 7 and 14 dpi, both in host and schistosomula tissues (Fig 12B and 12C). The TUNEL+ cells were localized especially at the host-parasite interface, but some were also recorded in the schistosomula migratory tracks. To assess the possibility that schistosomula-derived molecules directly induce apoptosis of the host neural cells, we stimulated mouse Neuro2a and primary mixed glial cultures with a soluble fraction of schistosomula

homogenate. However, the treatment did not alter the frequency of apoptotic populations (Fig 12D). These data suggested that apoptosis was not a major feature of *T. regenti* neuroinvasion and was not responsible for tissue pathogenesis.

Discussion

Apart from being a causative agent of human cercarial dermatitis [46,47], *T. regenti* invades the CNS of experimentally infected mice [19,20]. However, neuroinvasion by this species has not been reported from humans. Here we thoroughly investigated the invasion of the mouse CNS by *T. regenti* to better understand the diversity of host-parasite interactions during neuroinfections. Although *T. regenti* does not fully recapitulate any human neuroinfection, it represents a well-characterized and easily trackable system, providing novel insights into the diversity of pathogen-related neuroinflammation.

Of the mouse CNS, *T. regenti* predominantly affects the spinal cord to where it migrates from the skin *via* peripheral nerves [19–22,41]. Such a route is extraordinary among neurotropic helminths, which mostly rely on hematogenous spread into the CNS [48]. Indeed, *T. regenti* intraneural pathway rather resembles that of the neuropathogenic amoeba *Naegleria fowleri* [49]. Corroborating with the previous studies [19–22], we observed the highest schistosomula burden in the spinal cord 7 dpi. In addition, we detected *T. regenti* DNA in some of the hemisphere samples 14–21 dpi, but we assume that it originated from a few and most likely already damaged schistosomula since viable parasites were only rarely isolated even 7 dpi. Largely restricted access of schistosomula to the brain could explain the lack of working memory deficit, anxiety, or depression-like behavior, which stem from dysfunction of hemisphere centers [50–52].

By affecting mostly the spinal cord, *T. regenti* differs from other neurotropic helminths, such as *Angiostrongylus cantonensis*, *Toxocara* spp., or *Taenia solium*, which primarily invade the brain and impair cognitive functions or cause seizures [9,53–56]. Nevertheless, *T. regenti* resembles the neurotropic larvae of *A. cantonensis* and *Toxocara* spp. regarding size and active migration within the nervous tissue [9,54]. On the contrary, passive dissemination of schistosome eggs into the spinal cord induces human neuroschistosomosis, which often manifests as myelitis [57]. Despite sharing some histopathological or clinical features, such as myelitis and motor dysfunction of lower/hind limbs [58], *T. regenti* neuroinvasion cannot be directly compared to neuroschistosomosis, mainly due to the different nature of the causative agents (migrating schistosomula vs. disseminated eggs). As a whole, *T. regenti* neuroinvasion represents a unique biological phenomenon, but in particular regards, it can be compared to other neurotropic helminths. Regarding the general features, such as size and behavior, *T. regenti* represents a relevant comparative model especially for studying the pathology related to the migration of neurotropic helminths such as *A. cantonensis* or *Toxocara* spp.

Our complex approach revealed a serious impact of *T. regenti* neuroinvasion on the well-being of mice. They manifested with the reduced gain in body weight despite unchanged feed consumption or locomotion. We speculate that this could be explained by increased energy demands related to fighting the infection [59–61]. Of note, a decreased body weight was observed at all time points. It indicates that the peripheral immune response, with a peak 7 dpi [29], and the later neuroinflammation could have affected the weight gain. Reduction of body weight accompanies mouse infections with *A. cantonensis* and *Toxocara* spp. as well and often correlates with impaired neurological scores [55,62,63]. These observations highlight the systemic impacts of helminth neuroinfections, however, direct evidence linking the weight loss with presumably increased energy consumption related to the host immune reaction is missing.

Extensive behavioral testing revealed deficits in lower body motor functions in *T. regenti*-infected mice. Up to now, mainly ducks (definitive hosts) and immunocompromised mice were reported to suffer from neuromotor disorders related to *T. regenti* neuroinvasion [19]. This fact was explained by the higher schistosomula burden in such hosts and hence more frequent axonal injury [21,22]. Similar neuromotor abnormalities are also often present in mice infected with *A. cantonensis* and *Toxocara* spp. [53,62,64,65]. Dysregulated expression of myelin-associated genes, extensive tissue demyelination, and neuronal apoptosis are believed to be the pathological basis of such behavioral changes [63,65–68]. However, we detected no remarkable apoptosis or downregulation of myelin-associated genes in *T. regenti*-infected spinal cords. Also, no loss of myelin was seen in previous histological studies [22,26]. On the contrary, our transcriptomic data unveiled a massive downregulation of neuronal signal transduction, synaptic transmission, and axonal development. For example, we noticed striking downregulation of kinesin superfamily motor proteins *Kif1a* and *Kif1b*, which are essential for anterograde axonal transport [30,31]. Their effect is evident in *Kif1a*- and *Kif1b*-knockout mice, which exhibit reduced motor activity, abnormal limb movements, and impaired balance [69,70]. Therefore, we suggest that the disruption of neurophysiological functions in the spinal cord was responsible for the observed motor deficits. Such wide-range downregulation of neuronal functions has so far not been noticed in other transcriptomic studies focusing on helminth neuroinfection [65,66] but was recently reported from mouse brains infected with *Toxoplasma gondii* [7]. Specifically, Boillat *et al.* propose that behavioral alterations caused by *T. gondii* reflect side effects of neuroinflammation, i.e., the neuro-immune response plays a major role in modulating host behavior [7]. Therefore, we suggest that impairment of neurophysiological functions, potentially triggered by neuroinflammation, might be a novel pathological mechanism also in helminth neuroinfections.

Contrary to ducks, mice as accidental (dead-end) hosts efficiently control *T. regenti* neuroinvasion. This makes them apt species for studying the protective immune response. Moreover, the lack of well-defined duck strains and a suitable toolbox (mainly antibodies) markedly limits the immunological studies on *T. regenti* in ducks anyway. Our complex data strongly support the view that rapid activation of the mouse immune response leads to the elimination of schistosomula within 2–3 weeks post infection. It agrees with historical records of schistosomula suspended growth and reduced survival in immunocompetent mice [21,22,71]. Such immune-mediated host incompatibility is also observed in the case of *A. cantonensis*—it flourishes in rats but is retarded in mice. The latter develop much stronger neuroinflammation than rats, which is pronounced after the death of migrating larvae [9,11,72,73]. On the contrary, mice are suitable paratenic hosts for *Toxocara* spp., which are well adapted to survival in their tissues, including the CNS [74]. Similarly, viable cysticerci of *T. solium* actively evade the human immune response and cause no or mild inflammation [56]. Hence, *T. regenti* in mice is a perfect model enabling us to study natural mechanisms leading to helminth elimination within the CNS.

A strong eosinophilic meningomyelitis was the most prominent histopathological hallmark of *T. regenti* neuroinvasion. Comparison of peaks in eosinophil counts in the peripheral blood (7 dpi) and the spinal cord (14 dpi) demonstrated spatiotemporal separation of the eosinophil-based immune response in the periphery and within the CNS. A similar distinction was also observed in mice infected with *A. cantonensis* [73]. Recruitment of eosinophils into the *T. regenti*-infected spinal cords, found in our study, was presumably mediated mainly by eotaxin-2 coded by *Ccl24* [75], which displayed 180- and 700-fold increased expression 7 and 14 dpi, respectively. However, participation of other eosinophil-attracting chemokines upregulated at these time points (*Ccl5*, *Ccl7*, *Ccl8*) cannot be excluded and is even likely. Moreover, elevated expression of *Chil3* could have boosted maintenance of the tissue eosinophilia [76], especially

14 dpi. It was suggested for *A. cantonensis*-infected mice that Chil3 aggravates eosinophilic meningitis *via* a positive feedback loop mediated by IL-13 [73] which was also markedly upregulated in our samples. Our data collectively demonstrate a significant infiltration of eosinophils into the *T. regenti*-infected spinal cords, suggesting their prospective role in eliminating schistosomula.

Eosinophils harm helminths by antibody- and/or complement-induced secretion of toxic granule proteins and reactive oxygen species [77]. The role of eosinophils in limiting the parasite burden was demonstrated in murine neurocysticercosis [78], but specific effector mechanisms employed to combat helminths have not been defined. As our dissection and histological data indicated elimination of schistosomula around 14 dpi, we searched the transcriptomic datasets for pathways related to eosinophil effector functions upregulated/enriched mostly at this time point. Interestingly, we found that the complement and coagulation cascades were enriched. Specifically, components of C1 complex and C2, C3, C4, and C5 complement components were significantly upregulated ($\log_2$ fold change >2), suggesting activation of the classical complement pathway initiated by antigen-antibody complexes. This mechanism is known to trigger eosinophil degranulation leading to killing schistosomula of *S. mansoni* [79] and we propose that it harms *T. regenti* schistosomula within the CNS in the same manner.

Eosinophil extracellular DNA traps (EETs) represent another component of the antimicrobial arsenal. Being released in the C-lectin-dependent manner, the EETs can immobilize helminths and facilitate their further clearance, as recently demonstrated in the case of nematode microfilariae [80]. In *T. regenti*-infected spinal cords, the C-lectin receptor signaling pathway was mostly enriched 14 dpi and, e.g., dectin-1 was upregulated ($\log_2$ fold change >5) even at all time points. Of note, dectin-1 is a C-type lectin functioning as a transmembrane pattern-recognition receptor [81] mediating the release of eosinophil extracellular DNA traps [80]. Our observation raises an exciting question to be further tested of whether EETs are released in *T. regenti*-infected spinal cords and if they can halt the actively moving helminths in the CNS.

Microglia, the CNS-resident macrophage-like cells, are vital players in the CNS innate immunity as they sense and eliminate invading pathogens [82]. Therefore, they were implicated as the cells initiating the neuroinflammation in *A. cantonensis*- or *T. regenti*-infected mice [25,83]. Our transcriptomic and 2D/3D imaging data confirmed upregulation of microglia/macrophage markers in the infected spinal cord and continuous accumulation, even very intimate, of Iba-1+ MHC II+ cells around the schistosomula as soon as 7 dpi. At this time point, we noticed upregulation of *Tlr2*, *Tlr6*, *Tlr8*, and *Tlr12*, which can be expressed by microglia [84]. Of them, the heterodimer of TLR2/TLR6 might participate in recognition of the *T. regenti* infection similarly to murine neurocysticercosis [85]. However, the role of TLR8 and TLR12 in the initial schistosomula recognition is expected to be relatively minor as they both are intracellular receptors, and mouse TLR8 is even nonfunctional in terms of immune recognition [86]. Moreover, a C-type lectin-dependent pathway, including dectin-1 as a receptor [87,88], might be involved in detecting glycans, which are abundant on the surface and in the excretory-secretory products of the parasites [89,90]. Phagocytosis by mononuclear cells of glycan-rich material was shown in murine neurocysticercosis and promoted the infiltration of immune cells into the CNS [8,91]. Thus, we conclude that microglia might serve as sensors for *T. regenti* in the spinal cord using diverse and complementary recognition pathways.

Beyond recognition, Lichtenbergová *et al.* proposed that activated microglia/macrophages play the main role in destroying *T. regenti* schistosomula in the nervous tissue [22]. However, our data show that a strong type 2 cytokine milieu, driven by upregulated *Il4* and *Il13*, was established in the infected spinal cords suggesting induction of the alternatively activated M2 microglia/macrophage [40,92–94]. The transcriptomic M2 signature was confirmed by the

detection of arginase, a hallmark of alternatively activated microglia/macrophages, on the protein level. Induction of such environment is typical also for other helminth neuroinfections [95–97] and can be interpreted as helminth-induced prevention of M1 polarization, which is associated with the production of detrimental nitric oxide. Corroborating with this assumption, *T. regenti* schistosomula were shown not to trigger nitric oxide production *in vitro* or the spinal cord [25,26]. The role of microglia/macrophages in killing *T. regenti* schistosomula thus needs to be significantly reconsidered. We propose that activated microglia/macrophages phagocytose the tissue debris in the schistosomula migratory tracks—similarly to the gitter cells in *Toxocara*-infected brains [54,68]. Later, they might also clean the opsonized remnants of the already damaged schistosomula. Localization of Iba-1+ and/or MHC II+ cells, revealed by 2D/3D imaging techniques, and enriched FcγR-mediated phagocytosis pathway underpins the novel hypothesis on microglia/macrophage functions in *T. regenti*-infected spinal cords.

Finally, we suggest that the M2 polarization promotes repair of the injured nervous tissue. Astrocyte-mediated IL-6-driven formation of the glial scar, which we observed 14–21 dpi, is commonly considered the primary mechanism of the nervous tissue repair, also known from other helminth neuroinfections [22,98,99]. However, M2 microglia/macrophages promote tissue regeneration and remyelination by driving differentiation of oligodendrocytes [100,101]. *Chil3*, expressed and secreted by M2 cells, promotes oligodendrogenesis [102] and arginase helps axon regeneration by synthesizing polyamines, such as spermidine [103,104]. Both *Chil3* and *Arg1* were massively upregulated in *T. regenti*-infected spinal cords, and arginase was even the most abundant protein found in the close proximity to the schistosomula. At the same time, no signs of demyelination or remarkable tissue pathology were noticed. Thus, we conclude that induction of M2 polarization within the infected spinal cord limits extensive pathology potentially associated with *T. regenti* neuroinvasion. Identification of the parasite molecules responsible for this effect is warranted as they might represent suitable, tissue-specific immunomodulators applicable against autoimmune or neurodegenerative diseases.

In summary, we present a comprehensive, transcriptome-driven study of the schistosome invasion into the mouse spinal cord. We revealed a remarkable downregulation of neurophysiological functions, which contribute to motor deficits observed in infected mice. The host immune response is dominated by eosinophilic inflammation, which eliminates the infection. A strong M2 polarization evidenced, i.e., by substantial production of arginase, presumably prevents extensive tissue damage. Due to parasitological, immunological, or pathological similarities with human neuropathogens, *T. regenti* represents a suitable model for further research of neuroinfections.

Materials and methods

Ethics statement

Animal experiments were conducted in compliance with the European and Czech legislation (EU Directive 2010/63/EU, Act No 246/1992). Animal welfare committees of the Faculty of Science, Charles University, and the Ministry of Education, Youth and Sports of the Czech Republic approved all experimental procedures (MSMT-33740/2017-2, MSMT-37946/2020-3). Isoflurane anesthesia followed by transcardial perfusion (exsanguination) were employed to sacrifice the mice as indicated below.

Animals

C57BL/6J OlaHsd mice (Envigo, Venray, Netherlands) were housed in the Centre for Experimental Biomodels (First Faculty of Medicine, Charles University) or the animal housing facility (Faculty of Science, Charles University). The mice were kept in groups of 4–10 individuals/

cage (if not stated otherwise) and provided with food and water *ad libitum*; food consumption was monitored on a weekly basis. Infection with *T. regenti* cercariae was performed at the age of 8 weeks. Mixed glial cultures were prepared from newborn pups of both sexes, no older than 48 hours. Light-sheet fluorescence microscopy imaging was performed on organ samples from MHC II-EGFP C57BL/6J knock-in mice provided by Prof. Jan Černý (Department of Cell Biology, Faculty of Science, Charles University).

Parasites and infection of mice

A complete life cycle of *T. regenti* is kept at the Department of Parasitology, Faculty of Science, Charles University. Freshwater snails (*Radix lagotis*) and domestic ducks (*Anas platyrhynchos* f. *domestica*) are used as intermediate and definitive hosts, respectively [19]. Percutaneous infection of female mice was done according to the well-established “water bath” protocol using the infection dose of 2000 freshly collected cercariae [22,26,29,41].

Experimental design

A comprehensive investigation was carried out to examine the effects of *T. regenti* infection on mice, emphasizing the host immune response and pathology in the CNS (Fig 1). To characterize the general/systemic impact of the infection, body and spleen weights were monitored as well as levels of *T. regenti*-specific serum antibodies, blood eosinophil counts, and behavioral alterations of infected mice. Host-parasite interactions in the CNS, especially in the spinal cord, were then broadly explored by several approaches summarized in Fig 1. Uninfected mice were always used as a control group, and specific age-matching is shown in Fig 1. The time points at which the analyses were performed represent distinct phases of the infection [22].

ELISA

T. regenti-specific IgG1 and IgG2a were analyzed in the serum samples (diluted 1:80) of mice according to [29]. The cut-off value was calculated according to [105] for a 99.9% confidence level.

Parasite burden in the CNS

Two complementary methods were used to assess *T. regenti* burden in the CNS. First, we directly quantified *T. regenti* schistosomula actively released from the spinal cord, brain stem, cerebellum, and hemispheres after the organs were torn into 1–2 mm pieces by sharp forceps in phosphate-buffered saline (pH 7.4; PBS) [26]. The organ suspensions were independently inspected by three people within 6 hours which is enough for viable schistosomula to leave the tissue.

Second, we detected schistosomula DNA by a quantitative real-time polymerase chain reaction (PCR) in the spinal cord and hemisphere samples intended for flow cytometry. Specifically, we used the material remaining in the cell strainers after filtration of the cell suspensions (~10 mg, see “Flow cytometry” for details). Total DNA was isolated by Exgene Tissue SV (plus!) (GeneAll; Lisbon, Portugal) using 100 µl of the elution buffer and stored at –20°C. qPCR was then carried out in iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) employing iQ SYBR Green Supermix (Bio-Rad; Hercules, California, USA). *Trichobilharzia*-specific *Sau3A* repetitive sequence was selected as the molecular target, 5'-GTGACTTGCTA-CAGGTTGG-3' (forward) and 5'-GGCAAGCTCGTATACCATTC-3' (reverse) primers were used [106] to amplify the PCR product of the expected size of 200 bp. Five µl of eluted DNA were added into an individual reaction, and each sample was run in a triplicate. UltraPure

DNase/RNase-free dH₂O (Invitrogen; Waltham, Massachusetts, USA) was included as a negative control, while 10 ng of DNA isolated from *T. regenti* cercariae (corresponding to the highest standard, see below) served as a positive control in each run. qPCR amplification was performed as follows: 3 min at 95°C, 40 cycles of 15 s at 95°C, 15 s at 57°C, 15 s at 72°C, and 1 min at 72°C. Quantitative cycle values were obtained, and the final parasite DNA calculation was done according to the standard curve ranging from 10 ng to 1 fg (a series of 10-fold dilution). Melting curves were always checked to ensure the specificity of the reaction. The cut-off value was calculated as the mean plus three standard deviations of samples from uninfected mice.

Behavioral tests

Several tests were performed and evaluated by blinded researchers to explore various aspects of mice behavior (see Table 1 for details). A detailed description of task procedures can be found in S1 Text. Mice intended for behavioral tests were housed individually. In all the tests, the apparatuses were cleaned with 70% ethanol and dried after each session.

Histology

Spinal cords were carefully extracted from isoflurane-anesthetized mice perfused by heparinized (10 IU/ml) PBS and 4% paraformaldehyde (PFA). The tissue was post-fixed in 4% PFA overnight at 4°C, dehydrated, and embedded in paraffin blocks. Five µm thick sections were prepared and routinely stained with hematoxylin-eosin.

Flow cytometry

Flow cytometry was employed to characterize peripheral and CNS-infiltrating leukocytes in infected mice. Uninfected mice age-matched to 7 and 28 dpi were used as controls to prevent a potential age-related bias, especially in the infiltration of the CNS. As no differences were found (S2 Text), only uninfected mice age-matched to 7 dpi are shown in the main text. The blood samples were processed individually, but three mice were pooled to obtain adequate and robust amounts of leukocytes in the CNS samples.

First, the blood was collected from the right atrium of isoflurane-anesthetized mice and mixed with an equal volume of 10 mM EDTA in PBS on ice. Erythrocytes were lysed with

Table 1. Behavioral testing battery. The tests are listed by their temporal order.

Test	Behavior assessed	Parameters analyzed	Ref.
Elevated plus maze	anxiety; activity	time spent in the open arms; risk assessment behavior; total number of arm visits; total distance walked; looking down behavior	[107,108]
Open field	locomotor activity; anxiety	total distance walked; time spent in the center of the arena	[108–112]
Beam walking	motor coordination	time to traverse the beam; error score	[109,110,112]
Bar holding	endurance and strength of the forelimbs	tightrope hang time	[111]
Marble burying	anxiety	proportion of marbles buried to 2/3	[113]
Novelty-induced hypophagia	anxiety	amount of eaten oat flakes; number of feces; latency to first tasting	[108]
Y-maze	spatial working memory; activity	proportion of correct triads to all triads (alternation); total number of arm entrances; total distance walked	[114]
Grid test	endurance and strength of the forelimbs and hindlimbs	time to jump/fall	[112,115,116]
Tail suspension	depressive-like behavior	immobility duration	[112,117]
Forced swimming	depressive-like behavior	immobility duration	[117]
Footprint analysis	posture and gait characteristics	forelimb and hindlimb step length and step width	[116]

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ACK buffer [29] and the remaining cells were processed for flow cytometry. Following the blood collection, the mice were transcardially perfused with PBS, and the spinal cord, the brain stem, the cerebellum, and the left hemisphere were extracted and further processed on ice. The tissues were gently mechanically homogenized, filtered through a 70 μ m cell strainer, and the leukocytes were separated from myelin using 30/70% Percoll (GE Healthcare; Chicago, Illinois, USA) gradient centrifugation [118]. Specifically, the cells were carefully collected from the 30/70% Percoll interphase and processed for flow cytometry.

After cell suspensions were prepared, anti-CD16/CD32 antibody (1:100; eBioscience; San Diego, California, USA; clone 93) was added for 10 minutes, being followed by a mixture of surface marker antibodies: APC-eFluor 780 anti-CD45 (1:100; eBioscience; clone 30F11); PE-Cy7 anti-CD11b (1:120; BioLegend; San Diego, California, USA; clone M1/70); FITC anti-F4/80 (1:100; BioLegend; clone BM8); PE anti-SiglecF (1:80; BioLegend; clone S17007L); APC anti-Ly6G (1:150; BioLegend; clone 1A8). The cells were stained for 30 minutes at 4°C in the dark, and Hoechst 33258 (1:22,500; Sigma Aldrich; St. Louis, Missouri, USA) was added 10 minutes before measurement to exclude dead cells. The samples were measured by CytoFLEX S (Beckman Coulter; Brea, California, USA; BC) using CytExpert (BC) and analyzed in FlowJo (v. 10.7.1). FMO controls were used for all markers; in the case of F4/80, isotype control was also included. The gating strategy for each tissue is shown in S2 Text. Cell populations of interest were characterized as follows: microglia: CD45^{med} CD11b⁺, macrophages/monocytes: CD45⁺ CD11b⁺ F4/80⁺ Ly6G⁻ SiglecF^{med}, eosinophils: CD45⁺ CD11b⁺ F4/80⁺ Ly6G⁻ SiglecF⁺, neutrophils: CD45⁺ CD11b⁺ F4/80⁺ Ly6G⁺ SiglecF^{low/med}, lymphoid cells: CD45⁺ CD11b⁻.

Transcriptomic analysis

RNA isolation, library preparation, and sequencing. Total RNA was isolated from the whole dissected spinal cords of infected and age-matched control mice using TRIzol (Invitrogen). Four biological replicates per group were processed for each time point. Residual DNA was removed by TURBO DNA-free Kit (Invitrogen), and the quality and integrity of RNA were inspected by Bioanalyzer 2100 (Agilent; Santa Clara, California, USA). Pair-end (2x100 bp) sequencing was performed on the BGISEQ-500 platform.

Bioinformatic analysis. Raw reads were inspected for sequencing quality by FastQC (v. 0.11.5) [119], and low-quality nucleotides (Phred quality score <20) were removed using Trimmomatic (v. 0.39) [120]. Trimmed reads were mapped to transcriptome inferred from the reference genome (v. GRM38) using RSEM (v. 1.3.3) [121], and transcripts with mapping rate >10 expected counts were considered as transcribed. Differential gene expression between the infected samples and the respective age-matched controls was calculated using DESeq2--package (v. 1.12.3) [122] in R (v. 3.5.2) at each time point. Transcripts with log₂fold change (log₂FC) >2 or <-2 and false discovery rate (FDR) of <= 0.05 were recorded as the differentially expressed. For a global overview of protein classes and metabolic pathways enriched at each time point, the reference transcriptome was translated using Transdecoder (v. 3.0.1) [123] and annotated by GhostKOALA (v. 2.2) [124] to KEGG database [125]. Upregulated (log₂FC >2) and downregulated (log₂FC <-2) transcripts with KEGG annotation were submitted to KEGG Mapper online tool to reconstruct metabolic pathways. Enriched metabolic pathways for each time point were identified by Fisher's exact test (adjusted p-value <0.05) in R (v. 3.5.2).

Laser capture microdissection and proteomic analysis

Embedding, cryosectioning, and laser microdissection. Cervical, thoracic, and lumbar spinal cord segments were extracted from mice previously perfused by heparinized (10 IU/ml)

PBS and 4% PFA. The segments were post-fixed in 4% PFA for 5 hours at room temperature (RT), washed in PBS for 3×5 min, immersed in Tissue Freezing Medium (Leica; Wetzlar, Germany) for 30 min at RT, and frozen at -80°C . Ten-micrometer sections (CM3050 S Research Cryostat, Leica) were mounted onto membrane frame slides (MMI; Ecching, Germany) and stored at -80°C until use.

Laser microdissection was carried out using the MMI SmartCut system (Olympus CKX41 inverted microscope; Olympus SmartCut Plus software). Areas of interest were isolated from each spinal cord segment with a final volume of $7.5 \times 10^6 \mu\text{m}^3$ [126]. Specifically, the area of interest was the white matter of each spinal cord segment within up to 100 μm around the schistosomula. The randomly selected white matter of appropriate segments from uninfected mice was taken as a control sample. The microdissects were stored at -80°C until MS analysis. It was performed on either merged (cervical, thoracic, and lumbar all together, $7.5 \times 10^6 \mu\text{m}^3$ of each, $n = 4$, i.e., $22.5 \times 10^6 \mu\text{m}^3$ of microdissects per a replicate) or separated ($n = 3$, $7.5 \times 10^6 \mu\text{m}^3$ of microdissects per an individual segment replicate) spinal cord segments.

Additionally, the intestinal content of *T. regenti* schistosomula was also microdissected and analyzed. The intestinal content of schistosomula from 4 mice was pooled for one sample, and the experiment was performed in a triplicate. Due to a low yield of the microdissected material, only $1.3 \times 10^6 \mu\text{m}^3$ of the intestinal content was microdissected per sample.

MS analysis and data evaluation. Microdissects were processed for nanoflow liquid chromatography-MS analysis according to [126]. Briefly, proteins were denatured, reduced, and alkylated in one step using the In-StageTips method [127]. Raw data were processed and quantified with the MaxQuant software (v. 1.6.10.43) with a built-in Andromeda search engine against the protein databases of *M. musculus* (downloaded in June 2020 from www.uniprot.org) and *T. regenti* (trichobilharzia_regenti.PRJEB4662.WBPS14.protein) downloaded from the WormBase (parasite.wormbase.org) with parameter settings according to [126]. Quantification was performed using the label-free algorithm [128]. Further data analysis was done by Perseus software (v. 1.6.14.0) [129], where Student's t-tests with permutation-based FDR calculation (250 randomizations) ($\text{FDR} = 0.05$, $S_0 = 0.1$) were performed. Proteins considered to be reliably identifiable were further annotated and classified into protein groups according to the KEGG database [125].

Immunohistochemistry

Ten-micrometer cryosections of spinal cords were prepared as already published [26] and stored at -80°C before use. After tempering, they were washed with PBS, permeabilized with 0.1% (v/v) Triton X-100 in PBS, blocked with 0.1% Triton X-100 1% (w/v) bovine serum albumin (BSA) in PBS, and incubated overnight at 4°C with primary antibodies: monoclonal rat anti-IL-4 (1:40; Abcam; Cambridge, UK; clone BVD4-1D11), monoclonal rat anti-IL-6 (1:200; Acris antibodies; Herford, Germany; clone MP5-20F3), monoclonal rat anti-MHC II (1:150; Abcam; clone NIMR-4), polyclonal rabbit anti-arginase-1 (Arg-1) (1:1000; Thermo Fisher Scientific; Waltham, Massachusetts, USA), polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:1000; Dako; Santa Clara, California, USA), polyclonal rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) (1:1000; Synaptic Systems; Göttingen, Germany). Negative rabbit serum was used as a control for Arg-1, GFAP, and Iba-1. The following day, the slides were washed with PBS, incubated with corresponding secondary antibodies (goat anti-rabbit F(ab')₂ AlexaFluor 594, goat anti-rabbit F(ab')₂ AlexaFluor 488, goat anti-rabbit Alexa Fluor 488 - all 1:1000; Cell Signaling Technology; Danvers, Massachusetts, USA; and goat anti-rat Alexa Fluor 568; 1:1000; Abcam) for 60 minutes at RT, washed with PBS and mounted in Fluoroshield with DAPI (Sigma Aldrich). Slides with detection of Arg-1 were captured by

NIKON TiE 2 and processed in ImageJ (v. 1.8). Other slides were captured by Olympus BX51 equipped with Olympus DP-2 camera and processed in Quick Photo Micro (v. 3.0) and Gimp (v. 2.10.22). Per each time point, 4 mice were used, and at least 3 schistosomula-positive slides per mouse per antibody combination were analyzed. The mice used for this analysis were infected independently from other experiments presented herein.

Light-sheet fluorescence microscopy

MHC II-EGFP C57BL/6J knock-in mice were perfused by ice-cold heparinized PBS and 4% PFA (pH 7.4), and their spinal cords were post-fixed in the PFA overnight at 4°C. The tissue was cleared in CUBIC-1 solution [25% (w/v) urea, 25% (w/v) Quadrol, 15% (v/v) Triton X-100 (all Sigma-Aldrich) in dH₂O] for at least 5 days (or until the tissue was transparent) in the dark, orbital shaking at 37°C. Afterwards, the tissue was washed [0.5% (w/v) BSA, 0.01% (w/v) sodium azide, 0.1% (v/v) Triton X-100 in PBS] in the dark, orbital shaking at 37°C for 5 hours. Washed samples were stained with DRAQ5 (Thermo Fisher Scientific) (1:7500) for 2 days in the dark, rocking at 4°C. Finally, the tissue was incubated in CUBIC-2 solution [22.5% (w/v) urea, 9% (v/v) triethanolamine (Sigma-Aldrich), 45% (w/v) sucrose, 0.1% (v/v) Triton X-100 in dH₂O; refractive index = 1.47] at least for 5 days in the dark, orbital shaking at 37°C. The tissue clearing protocol was adapted from [130] and modified by Jaromír Novák and Jan Pačes (Department of Cell Biology, Faculty of Science, Charles University). Samples were mounted in 1% (w/v) low-melt agarose and processed on Zeiss Lightsheet Z.1 using 10× illumination and 20× detection objectives. Data were analyzed and processed in ZEN (black edition) (v. 9.2.8.54; Zeiss), Arivis (v. 3.1.3; Arivis), and DaVinci Resolve (v. 16.2.7.010; Blackmagic design). The mice used for this analysis were infected independently from other experiments presented herein.

Evaluation of the blood-brain barrier integrity

The integrity of the blood-brain barrier was evaluated by the extravasation of Evans blue into the nervous tissue [131]. The mice were subtly anesthetized (100 mg/kg ketamine, 10 mg/kg xylazine), and 200 µl of 2% (w/v) Evans blue were injected into a lateral tail vein. After 1 h, the mouse was transcardially perfused by 40 ml of heparinized (10 IU/ml) PBS and the spinal cord, brain stem, cerebellum, and hemispheres were isolated and weighed. The tissue was then mechanically homogenized in 50% of trichloroacetic acid (spinal cords, brain stems, and cerebella in 100 µl, hemispheres in 300 µl), centrifuged (20 min, 10,000×g) and the supernatant was diluted 4-fold with ethanol. The fluorescence intensity was measured at 620/680 nm and converted to the amount of Evans blue using a standard curve. The mice used for this analysis were infected independently from other experiments presented herein.

Apoptosis assays

DNA fragmentation, a hallmark of apoptotic cells, was detected on cryosections of the spinal cords (see “Immunohistochemistry” for preparation) by Click-iT Plus TUNEL Assay with Alexa Fluor 488 dye (Thermo Fisher Scientific) according to the manufacturer’s instructions. Then the slides were mounted in VectaShield with DAPI to stain all nuclei and examined by a fluorescence microscope (Olympus BX51). The images were processed in ImageJ, by which the proportion of TUNEL+ nuclei around and within the schistosomula was calculated and compared to uninfected mice. Per each time point, 4 mice were used, and at least 2 schistosomula-positive slides per mouse were analyzed. The mice used for this analysis were infected independently from other experiments presented herein.

Apoptotic effects of *T. regenti* antigens were tested *in vitro* on Neuro2a cell line and primary mixed glial cultures. Neuro2a were obtained from Ondřej Honc (Department of Physiology, Faculty of Science, Charles University) and grown in Dulbecco's modified Eagle's medium supplemented by 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (all from Lonza; Basel, Switzerland). Mixed glial cultures were prepared from newborn mouse pups and grown as already described [25]. The cells were seeded into 12-well plates and treated with a soluble fraction of schistosomula homogenate (5 and 50 µg/ml; see [25] for a detailed preparation) for 48 hours. Staurosporine (final concentration 0.1 µM) was used as a positive control. Next, the cells were washed by PBS, trypsinized, and stained by Dead Cell Apoptosis Kit with annexin V-Alexa Fluor 488 and propidium iodide (Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were measured by LSR II flow cytometer using Diva (Becton Dickinson; Franklin Lakes, New Jersey, USA) and analyzed in FlowJo (v. 10.7.1).

Statistical analyses

Statistical analyses and data visualization were conducted in GraphPad Prism (v. 9) if not already stated otherwise. Particular tests applied are indicated in the figure legends, p-values <0.05 were considered significant and are indicated as follows: *p<0.05, **p<0.01, ***p<0.001. The exact p-value is also shown if it was 0.05–0.10. Individual data are presented within the graphs, or “n” is clearly stated if summary statistics (mean ± standard deviation) were used for better graphical representation.

Supporting information

S1 Table. Spinal cord transcriptomic data.
(XLSX)

S2 Table. Microdissection proteomic data.
(XLSX)

S1 Text. Detailed description of behavioral tests.
(DOCX)

S2 Text. Gating strategies and flow cytometry data not presented in the main text.
(DOCX)

S1 Fig. Behavioral data not presented in the main text.
(TIF)

S2 Fig. Evaluation of the blood-brain barrier integrity by Evans blue in uninfected (0 dpi) and infected (7 dpi) mice. (A) Representative images of extracted spinal cords and brains. **(B)** Quantification of Evans blue in the nervous tissue. Data were evaluated by unpaired t-test, p values are shown.
(TIF)

S1 Video. Beam walking, uninfected and infected (7 dpi) mice.
(MP4)

S2 Video. Light-sheet fluorescence microscopy, the uninfected mouse (0 dpi).
(MP4)

S3 Video. Light-sheet fluorescence microscopy, the infected mouse (7 dpi).
(MP4)

S4 Video. Light-sheet fluorescence microscopy, the infected mouse (14 dpi).
(MP4)

S5 Video. Visualization of the virtual box used for quantification of MHC II+ volume around schistosomula.
(MP4)

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Pulmonary migration of *Trichobilharzia szidati* (Schistosomatidae) and its potential immunomodulatory effect against allergic asthma in mice

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Background

The concept of immunomodulation by helminth and preventing asthma has been known for a long time since the old friend hypothesis (Rook et al., 2004). Nevertheless, the majority of the studies use antigens and molecules from parasites not migrating through the lungs (*Heligmosomoides polygyrus*, *Fasciola hepatica*) or developmental stages which are not associated with the lungs (adults of *Nippostrongylus brasiliensis* or adults and eggs of *Schistosoma* sp.) (Finlay et al., 2014). Avian schistosome *Trichobilharzia szidati* migrates through the lungs of mice but does not induce an influx of leukocytes despite damaging of the tissue (Chanová et al., 2007). Therefore, we hypothesize that schistosomula have immunomodulatory potential which could be beneficial in the prevention or therapy of asthma attacks. We tested the effect of the *Trichobilharzia szidati* infection on the course of ovalbumin (OVA)-induced asthma. Mice were infected 3 days before the intranasal (i.n.) challenge to reach a maximal number of living schistosomula in the lungs before inducing an allergic reaction and leukocyte infiltration.

Methods

Animals and ethics statement

All experiments with animals were approved by the animal welfare committees of the Faculty of Science, Charles University, and the Ministry of Education, Youth and Sports of the Czech Republic (MSMT-31963/2020-5). The BALB/c female mice (Charles River) were housed in the Centre for Experimental Biomodels (Charles University, First Faculty of Medicine) and provided with food and water *ad libitum*.

Experimental design

Mice (8-10 weeks old) were randomly divided into several groups: naive controls (further referred to as the “PBS” group), mice infected with *T. szidati* (the “Ts” group), mice with induced asthma (the “OVA” group), and mice with induced asthma and infected with *T. szidati* (the “OVA+Ts” group)

Asthma induction

The mice were immunized intraperitoneally with 10 µg of ovalbumin (OVA) grade V (Sigma Aldrich) in 100 µl of alum (Serva) and 50 µl of 0.1 M phosphate buffered saline (PBS) on days 0 and 14. On days 21, 22, and 23, the mice were anesthetized with 5% (v/v) isoflurane (airflow rate of 2.5 l/min) and challenged intranasally (i.n.) with 100 µg OVA in 30 µl of PBS. In the control groups (“PBS” and “Ts”), OVA was replaced by an equal volume of PBS only in both i.p. or i.n. treatment. The mice were sacrificed on day 24 under ketamine/xylazine anesthesia. After blood collection, the lungs were lavaged with 1 ml of cold PBS and transcardially perfused with 10 ml of PBS.

Parasite infection

The life cycle of *Trichobilharzia szidati*, using *Lymnea stagnalis* (the snail intermediate host) and *Anas platyrhynchos* f. *domestica* (the avian definitive host), has been well established at our department (Charles University, Faculty of Science). The mice were exposed to 1500 freshly emerged cercariae adopting the “water bath” protocol (1-hour exposure in the dark) (Majer et al. 2020, Macháček et al. 2022). In the case of the “OVA+Ts” group, the mice were infected on day 18.

Parasite homogenate preparation

T. szidati schistosomula homogenate (TsH) for cell stimulations was prepared according to Machacek et al. (2016). Freshly emerged cercariae were passed through the 23G syringe 20 times and washed with schistosome culture medium 169 (Basch, 1981) supplemented with 100 U/ml penicillin (Lonza), 100 µg/ml streptomycin (Lonza), and 0.25 µg/ml amphotericin B (Lonza) and cultivated (37°C, 5% CO₂) 72 hours. Then the schistosomula were collected, washed with PBS, and sonicated (3 × 30 s, amplitude 30 W; Vibra Cell) in PBS with cOmplete Mini protease inhibitors (Roche). The supernatant from homogenate was filtered through 0.22 µm strainer and the protein concentration was measured with Quant-iT Protein Assay Kit (Invitrogen).

Histology

The left lung was fixed in 4% neutral buffered formaldehyde and embedded in a paraffin block. Five µm thick sections were stained with hematoxylin-eosin (H&E). The stained sections were scanned with Axioscan Z.1 (Zeiss). The histopathology scoring was adapted from Park et al. (2020). Specifically, the presence of polymorphonuclear cells in peribronchial and perivascular areas was assessed according to the following grading system: 0 – no cell; 1 – few cells; 2 – ring of cells one layer deep; 3 – ring of cells 2–4 layers deep; 4 – ring of cells more than 4 layers deep. For each mouse, 4 areas in 10 sections were analyzed and the average score was calculated.

Splenocyte stimulation

Splenocytes were harvested according to the established protocol (Majer 2020). Briefly, the spleen was passed through the 70 µm strainer, erythrocytes were lysed with ACK buffer, and the splenocytes washed with PBS were resuspended in the complete RPMI 1640 medium (supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 2 mM L-glutamine (Lonza), 100 U/ml penicillin, and 100 µg/ml streptomycin).

The splenocytes (2x10⁶ cells in 800 µl) were cultivated (37°C, 5% CO₂) with TsH (10 µg/ml) or sterile PBS for one hour and then endotoxin-free OVA (50 µg/ml; InvivoGen) was added for 72 hours. Treatment with concanavalin A (1.25 µg/ml, Sigma-Aldrich) was used as a positive control, or the cells were left unstimulated.

ELISA

The concentration of cytokines (IL-4, IL-5, IL-10, IL-17 and IFN γ) in the supernatants obtained from stimulated splenocytes was measured using ELISA MAX Standard Set Mouse (BioLegend).

Flow cytometry

The superior, inferior, and post-caval lung lobes were minced into small pieces and incubated in 3 ml of the digest solution (0.26 U/ml of Liberase TL (Roche), 166 U/ml of DNase I (Sigma-Aldrich) in complete RPMI 1640) at 37°C for 45 minutes. The digested tissue was mashed through the 70 μ m strainer, washed with PBS, and erythrocytes were lysed with ACK buffer. Cells were counted by the Bürker counting chamber and one million living cells from each mouse were used for flow cytometry analysis. The dead cells were stained with Zombie aqua (1/600; BioLegend) for 20 minutes at room temperature. The samples were washed with FACS buffer (3% FBS in PBS) and stained with the following antibody mix for 20 minutes at 4°C: anti-16/32 (93, 1:100, eBioscience), APC-eFluor 780 anti-CD45 (30-F11, 1:100, eBioscience), PE-Cy7 anti-CD11b (M1/70, 1:120, BioLegend), PE-Dazzle 594 anti-CD11c (N41, 1:100, BioLegend), FITC anti-CD64 (X54-5/7.1, 1:80, BioLegend), APC anti-Ly6G (1A8, 1:100, BioLegend); PerCP-Cy5.5 anti-CD4 (GK1.5, 1:150, BioLegend); PE anti-CD170 (SiglecF) (S17007L, 1:100, BioLegend). Unstained control and fluorescent minus one (FMO) controls for CD11c, CD64, and SiglecF markers were performed. All samples were acquired by LSR II (BD) with FACSDiva (BD) and analyzed with FlowJo (BD). Simplified gating strategy is stated below in Fig 1.

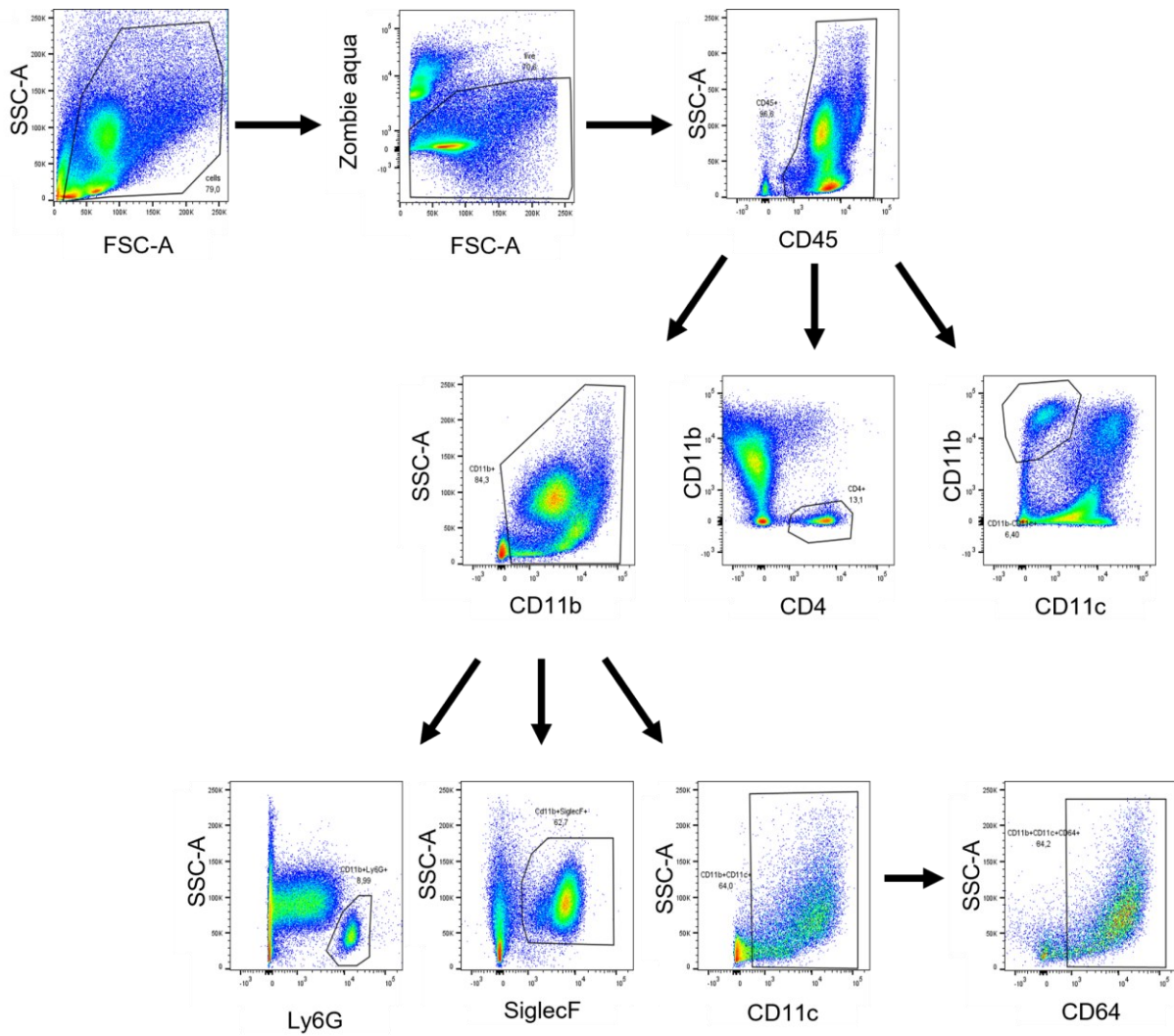


Figure 1: Simplified gating strategy of lungs

Cytokine bead array

Cytokine (IL-4, IL-5, IL-10, and IFN γ) concentration in BAL fluid was measured using Cytokine Bead Array (CBA) (BD) according to the manufacturer's instructions. All samples were acquired by LSR II (BD) with FACSDiva (BD) and analyzed with FlowJo (BD).

RNA isolation and qPCR

The middle lung lobe and lung draining lymph nodes (dLN) were snap frozen in liquid nitrogen. Up to 20 mg of the lobe or thoracic LNs (tLN) (pooled from all mice in the group) were homogenized by Precellys® CKMix Tissue Homogenizing Kit (Bertin) using Precellys® 24 tissue homogenizer (Bertin) at 5500 rpm for 20 s.

RNA was isolated by RNeasy Mini Kit (Qiagen) and quantified by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). The samples were treated with DNase I,

RNase-free (Thermo Scientific) and transcribed into cDNA by High-Capacity cDNA reverse transcription kit (Applied Biosystems).

The amplification was performed using CFX Duet Real-Time PCR System (Bio-Rad) with LightCycler 480 SYBR Green I Master (Roche) and the following primers: *Il1b* (fwd: GAT CCA CAC TCT CCA GCT GCA; rev: CAA CCA ACA AGT GAT ATT CTC CAT G), *Il5* (fwd: AGC ACA GTG GTG AAA GAG ACC TT; rev: TCC AAT GCA TAG CTG GTG ATT T), *Il10* (fwd: GCA TGG CCC AGA AAT CAA GG; rev: AGA AAT CGA TGA CAG CGC CTC), *Arg1* (fwd: AAG ACA GGG CTC CTT TCA GG; rev: GCC AAG GTT AAA GCC ACT GC), *Chil3* (fwd: ACT CCT CAG AAC CGT CAG AT; rev: CGC ATT TCC TTC ACC AGA ACA), *Gapdh* (fwd: AAC TTT GGC ATT GTG GAA GG; rev: ACA CAT TGG GGG TAG GAA CA), *Actb*: (fwd: GCT CTT TTC CAG CCT TCC TT; rev: CTT CTG CAT CCT GTC AGC AA). The relative gene expression was calculated by $2^{-\Delta\Delta C_t}$ method after normalization to *Gapdh/Actb* and is shown relative to control ("PBS") mice.

Statistical analysis

One-way ANOVA followed by Dunnett's test was used for the comparison of PBS and OVA+*Ts* with the OVA group for histopathology score and flow cytometry including most of the the CBA results. Kruskal-Wallis followed by Dunn's test was used for IL-4 results from CBA. One-way ANOVA followed by Sidak's test was used for the comparison "OVA" and "OVA+*Ts*" obtained from qPCR. The analyses were performed in GraphPad Prism (v 9.5.0). Two-way ANOVA followed by Sidak's test was used to compare OVA and OVA+*Ts* groups in ELISA.

Results

***Trichobilharzia szidati* infection tendentially reduced asthma-associated pathology.**

Asthma induction initiates massive infiltration of polymorphonuclear cells into the lung tissue and perivascular spaces. Our experiments showed that mice infected with *T. szidati* prior to the i.n. challenge with the allergen (Fig 1A) showed a reduced number of cells around the blood vessels, bronchioli, and alveolar space (Fig 1B, C) and decreased blood content in the BAL (visual inspection, data not shown). The *T. szidati* infection itself worsened the histopathology score only marginally.

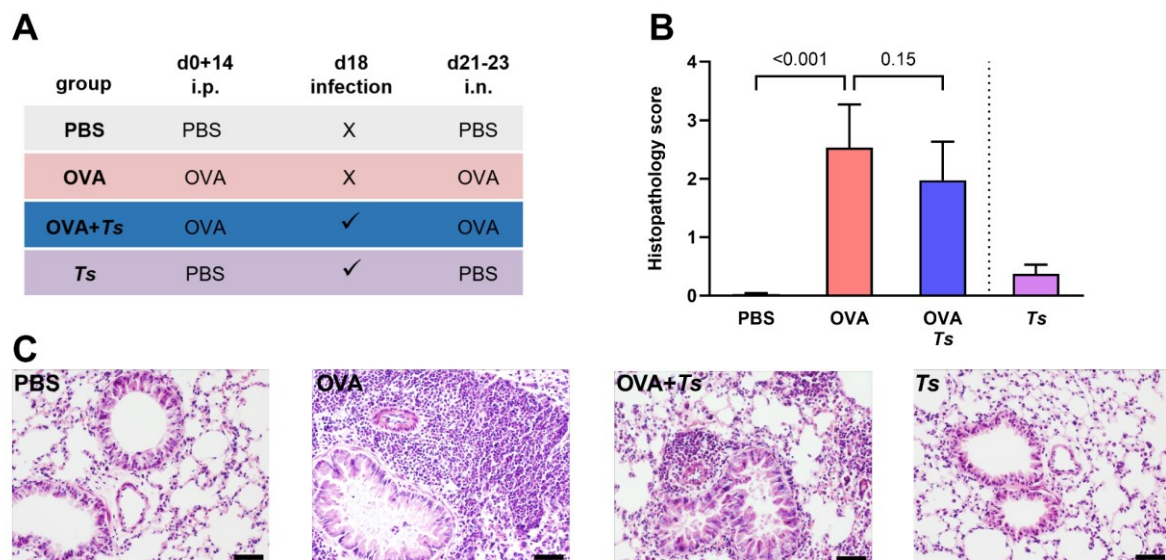


Figure 1: Effect of *Trichobilharzia szidati* infection on asthma-associated pathology.

(A) Experimental design: PBS (phosphate buffered saline) group was treated with PBS only, OVA (ovalbumin) group was treated with chicken egg OVA, OVA+Ts was treated with OVA and infected with *T. szidati*, Ts group was treated with PBS and infected with *T. szidati*; (B) Histopathology score of lungs. The average score for each mouse (n=7) was calculated based on 40 randomly selected areas per lung and compared using one-way ANOVA followed by Dunnett's test for comparison of PBS and OVA+Ts with the OVA group and P values are shown; (C) Representative pictures of hematoxylin-eosin stained lungs (scale bare = 50 μ m).

***T. szidati* infection reduced granulocytes in asthmatic lungs.**

Eosinophils are the dominant cell population migrating into the lungs during asthma. The effect which was indicated by histology was confirmed by flow cytometry. The total amount of leukocytes (CD45+ cells) was reduced in the OVA+Ts group (data not shown). The number of the major cell population, eosinophils (SSC^{high}CD11b+CD11c-SiglecF+), decreased by more

than one-third. Neutrophil influx (CD11b+Ly6G+) was also suppressed as well as a population of macrophages characterized by CD11b+CD11c+CD64+ (“interstitial macrophages”) (Fig 3A). Interestingly, the alveolar macrophage numbers (FSC^{high}SSC^{high}CD11b-CD11c++) were not changed except the *Ts* group (data not shown). The infection did not influence the total number of CD4+ T cells (CD11b-CD4+). The same trend of mitigated inflammation was confirmed in BAL but did not reach statistical significance (Fig 3B).

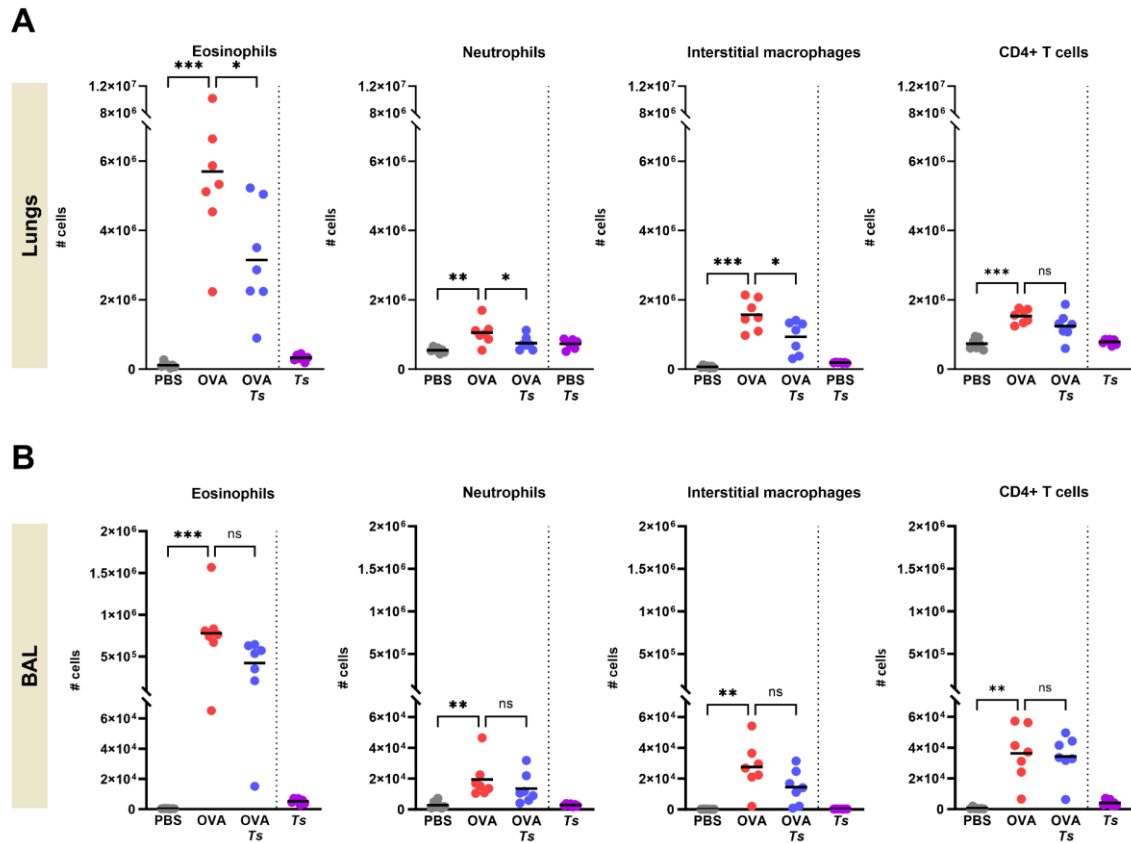


Figure 3: Immunophenotyping of cells in lungs and bronchoalveolar lavage

(A) Flow cytometry analysis of lung tissue and (B) cells obtained from bronchoalveolar lavage. Each dot represents an individual mouse. Comparison of OVA+*Ts* and PBS group with OVA group was performed by one-way ANOVA followed by Dunnett’s test. Significant differences are indicated with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

***T. szidati* infection helped to diminish the Th2/M2 milieu in asthmatic lungs.**

Asthma is associated with Th2 and M2 polarization of lymphocytes and macrophages, respectively. *T. szidati* infection downregulated the expression of M2 markers (*Chil3*, *Arg1*), which were elevated after asthma induction. Moreover, migration of schistosomula through the lungs upregulated the expression of regulatory cytokine *Il10* (Fig 4A). Surprisingly, we hardly detected *Il5* in OVA group (data not shown), although this cytokine is important for eosinophil activation. This was in contrast with IL-5 level in BAL fluid measured by CBA (Fig 4B) which reflected the high eosinophil numbers in OVA group (Fig 3). Furthermore, *T. szidati* infection reduced the IL-5 amount similarly, although the effect was statistically marginal. Preliminary analysis of the expression of *Il5* in lung dLN indicated high expression only in OVA group. Interestingly, we did not detect increased expression of this cytokine directly in lung tissue (data not shown). IL-5 was probably produced by cells from tLN, where we detected high expression of *Il5* in OVA group only (data not shown). The Th2/M2 downregulation was also connected with the regulation of the IL-4 and increase of IFN γ levels in BAL (Fig 4B).

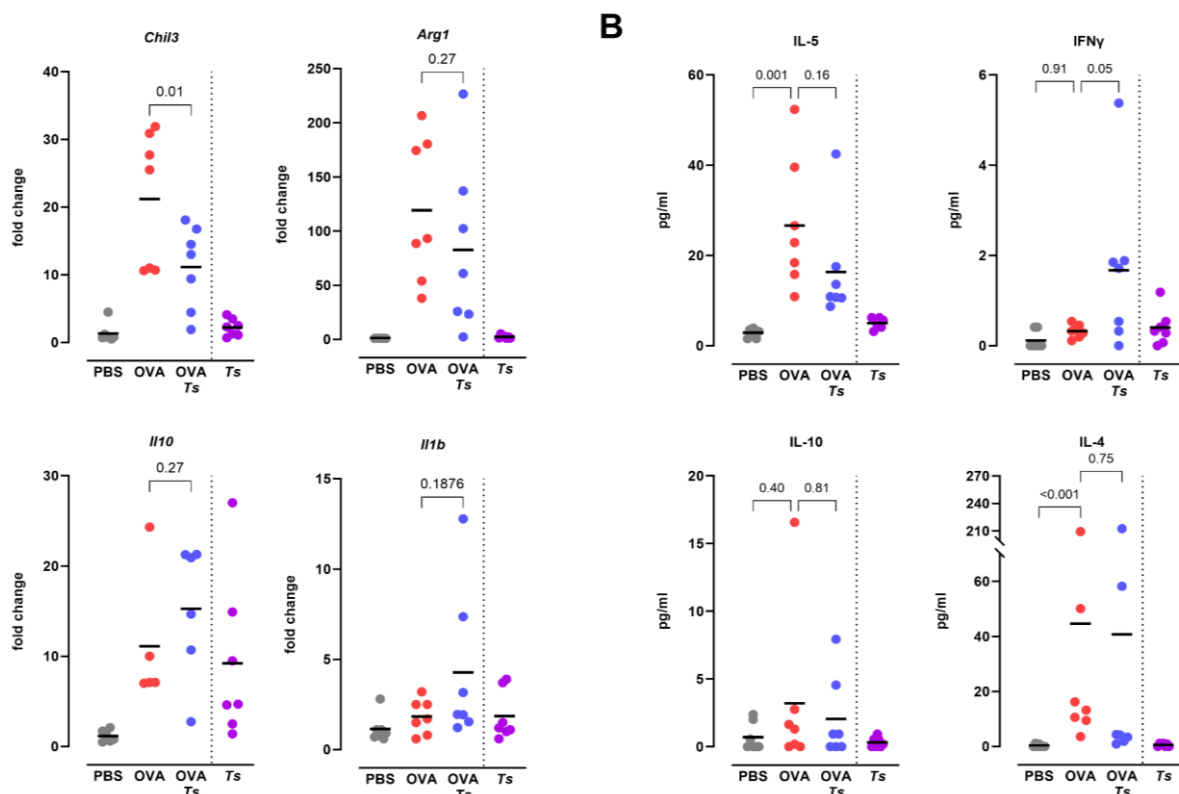


Figure 4: The Th2/M2 milieu in lungs and BAL.

(A) Relative expression of *Chil3*, *Arg1*, *Il10*, and *Il1b* in lung tissue to PBS control. The fold change was calculated as the $2^{-\Delta\Delta C_t}$. (B) IL-5, IFN γ , IL-10, and IL-4 concentrations in bronchoalveolar lavage fluid measured by Cytokine Bead Array. Data were evaluated by one-way ANOVA followed by Sidak's

test for comparison OVA with OVA+*Ts* group (A) and Dunnet's test (IL-5, IFN γ , IL-10) or Kruskal-Wallis followed by Dunn's test for comparison of PBS or OVA+*Ts* with OVA group (B).

***T. szidati* schistosomula homogenate synergistically enhanced the production of cytokines by splenocytes after OVA restimulation.**

Antigen-specific splenocyte restimulation was performed to reveal if the effect of *T. szidati* infection on asthma was only local or systemic. Cells harvested from OVA and OVA+*Ts* groups were cultivated for 1 h with schistosomula homogenate (TsH), and then OVA was added. TsH alone showed as a potent trigger for IFN γ production. Surprisingly, TsH massively boosted the cytokine production when used with OVA (Fig 5). The production of cytokines after OVA-only stimulation was low probably due to a low OVA concentration (50 μ g/ml).

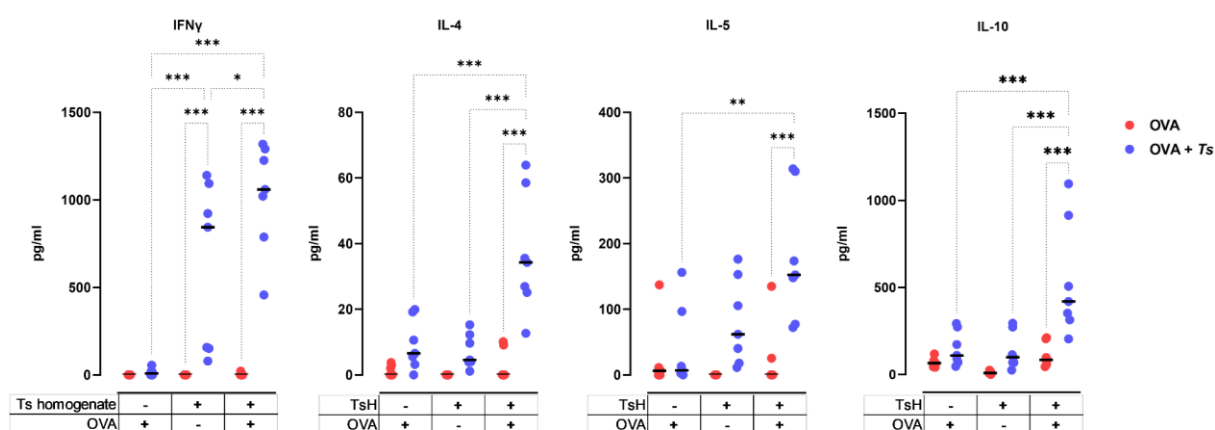


Figure 5: Antigen-specific cytokine production by splenocytes.

Splenocytes from OVA (red) and OVA+*Ts* (blue) groups were stimulated with *T. szidati* schistosomula homogenate (TsH) and OVA. IFN γ , IL-4, IL-5, and IL-10 were measured by ELISA. Data were tested using two-way ANOVA followed by Sidak's test. Significant differences are indicated with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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4. Discussion and conclusions

The thesis aims to broaden the knowledge about the immune response against helminths migrating in mice, which can control and eliminate the infection. Many studies focus only on the particular site, and stage of the infection and omit the infection dynamics and different affected organs. However, these “battlefields” influence each other. The lymphatic system is an interconnected network of the tissue-specific dLN and spleen, which more or less mirrors the overall (systemic) immune status. The publications from the presented thesis (Majer et al, 2020; Macháček et al., 2022), and the unpublished manuscript help to understand the crosstalk between the local and systemic immune response during the infection of mice by two avian schistosomes with unequal migration strategies and pathogenicity: the neurotropic *T. regenti* and the viscerotropic *T. szidati*. In the next sections, the novel data are commented and discussed along with the novel questions that emerged from my research.

4.1. From local to systemic perspective of *T. regenti* infection

T. regenti has a neurotropic affinity and leaves the skin within a few days after infection (Hrádková and Horák, 2002; Kouřilová et al., 2004a). Therefore, it brings a perfect opportunity to compare the local immune reactions in the skin or CNS and how they influence each other. To dissect this multi-level communication, we employed histology and immunohistochemistry (IHC) to depict the local reaction around the schistosomula in the spinal cord. Transcriptomic analysis and flow cytometry helped to see the complexity of the immune state in the whole CNS, and light-sheet fluorescence microscopy (LSFM) allowed us to discover the complexity in the spatial context of the large piece of organ. Specific CNS dLN and skin dLN were used to depict the reaction of the adaptive immune response in the specific site of the parasite migration. Analysis of the spleen and peripheral blood represented the systemic immune response.

The early immune response in the skin peaks between 1 and 2 dpi and is represented by the neutrophils, macrophages, monocytes, and eosinophils (Kouřilová et al., 2004a; Kouřilová et al., 2004b). Schistosomula leave the skin quickly and enter the peripheral nerves and the CNS after 2 or 3 dpi (Hrádková and Horák, 2002). However, no further immune profile has not been available for this early stage of the infection, making the proper understanding of the host response impossible. Therefore, we tested the reaction of the adaptive immune response by the transcription factor-based phenotyping of the T cell in skin dLN (Majer et al., 2020). We

observed an increased proportion of the Th2 cells and a similar trend also for Treg cells. Surprisingly, the Th1 cell proportion did not increase, in contrast with the observation of Kouřilová et al. (2004b), who described mixed Th1/Th2 response in the skin dLN. However, they used cercarial homogenate, which is a potent inducer of IFN γ secretion, as we confirmed (Majer et al., 2020), and therefore even a low number of Th1 cells produced a significant amount of the cytokine. Additionally, the stagnation of Th1 amount in the skin dLN can be influenced not only by the presence of dying cercariae but also by the further migration of schistosomula and polarization of systemic immune response.

We observed a reduction of T cell numbers in the spleen 3 dpi, mainly the Th1 and CD8⁺ cells. On the other hand, the proportion of Treg cells significantly increased (Majer et al., 2020). The same trend was evident in the CNS dLN and, therefore, could be caused by the invasion of the peripheral nerves and spinal cord by the parasite and may be the consequence of the blood-spinal cord barrier (BSCB) leakage. In fact, the leg paresis was observed at this time point so we can assume the nervous tissue damage (Horák et al., 1999; Majer et al., 2020). Treg cell numbers increase within a few days in the CNS dLN after ischemic stroke (Ito et al., 2019) or West Nile virus brain infection (Graham et al., 2014). Considering the vulnerability of the CNS, we can speculate that the Treg cells-driven reduction of Th1 and CD8⁺ cells could prevent the possible detrimental effect of inflammation and autoimmune reactions as was described in spinal cord injuries (Li et al., 2022).

We tested the integrity of the BSCB and blood-brain barrier (BBB) only 7 dpi and without positive results. Nevertheless, the transcriptomic analysis of the spinal cord at the same time point revealed the upregulation of the proteins responsible for the tight and adherens junction formation (*Cdh1* and *Ocln*) (Macháček, 2022). It could orchestrate the BSCB reparation and resealing process (Lacaz-Vieira et al., 1999; Zhao et al., 2022). Unfortunately, there is a lack of detailed data about the milieu of the CNS 3 dpi.

The inflammatory cells around the schistosomula in the spinal cord are observed from the 7 dpi. Their numbers are steadily increasing, and after 14 dpi only granulomas with no intact worms are in the CNS (Kolářová et al. 2001; Lichtenbergová et al, 2011). Lichtenbergová et al. (2011) described that the granulomas contained mainly microglia and macrophages, and Kouřilová et al (2004a) observed few eosinophils in perivascular and meningeal space. Nevertheless, we showed the massive infiltration of eosinophils into the whole CNS, demonstrating that

eosinophils represented the most numerous leukocytes infiltrating the CNS. Their numbers culminated 14 dpi, and we detected them in granuloma around the destroyed parasite as well as in meningeal spaces (Macháček et al., 2022).

We expected that for the attraction of the eosinophils into the CNS was responsible eotaxin-1 and -2 (Jose et al., 1994; White et al., 1997), whose genes (*Ccl11*, *Ccl24*) were up-regulated in the infected spinal cord already at 7 dpi (Macháček et al., 2022). Here, we described an interesting concomitant of the peak blood eosinophilia triggered by the skin phase of the infection 7 dpi and the subsequent spread of cells into the CNS - presumably following the rising gradient of the eotaxins produced within the nervous tissue (Macháček et al., 2022). A similar trend of eosinophilia in the blood, followed by eosinophilic meningitis orchestrated by the chemokine expression in the CNS, was described in the neurotropic nematode *Angiostrongylus cantonensis* infected mice (Li et al., 2015).

Antigen-presenting cells contribute to the activation of the lymphocytes and their migration into the site of infection. Therefore, we focused on the MHC II expression as a canonical marker of the antigen presentation pathway in the infected spinal cord. The transcriptomic analysis revealed upregulation of the MHC II pathway during the infection and IHC confirmed the accumulation of the MHC II⁺ cells around the schistosomula, which was expressed apparently by microglia (Macháček et al., 2022). To avoid the limitation of the IHC of the tissue sections, we employed the 3D LSFM technique. It is a novel imaging technique that allows to study large pieces of organs, or even the whole body (Mai et al., 2023), and not only isolated foci, e.g., around the parasite. Moreover, it brings more informative 3D views and possibilities for advanced image analysis. We facilitated the quantification of the MHC II⁺ accumulation around the parasite and confirmed massive clusters of the MHC II⁺ cells around the dying schistosomula 14 dpi. Moreover, the LSFM allows visualization of whole spinal cord segments, which led to the finding that the MHC II⁺ expression increased not only around the parasites but in the whole spinal cord 14 dpi, apparently also in the perivascular spaces of the spinal cord parenchyma and anterior and posterior sulci (Macháček et al., 2022). This observation challenged the view that the *T. regenti*-associated neuroinflammation is restricted to only several foci, not affecting the whole organ. These signs of such a massive host reaction are likely mirrored in the systemic motor dysfunction, which we detected in infected mice (Macháček et al., 2022).

The MHC II⁺ cells in the spinal cord perivascular area were probably also microglia, which participated in the communication with the systemic immune system and mediated the

entry of lymphocytes into the CNS from the periphery (Aguirre and Miller, 2002). However, the entry of T cells into the CNS is usually associated with detrimental pathology, such as in the case of multiple sclerosis (Dendrou et al., 2015), and therefore must be strictly controlled (Tabet et al., 2022). We detected lymphoid cells in the CNS with a peak 14 dpi (Macháček et al., 2022) and confirmed the histological observation of small numbers of T cells in perivascular and meningeal space and also in spinal cord parenchyma (Kouřilová et al., 2004a; Lichtenbergová et al., 2011). Unfortunately, the specific lymphoid cell populations were not analyzed. However, we observed an evident increase in the Th2 population in the CNS dLN 7 and 14 dpi (Majer et al., 2020). Therefore, we can speculate that peripherally-triggered T cells entering the spinal cord participated in the polarization towards type 2 milieu, which we confirmed by the detection of markers alternatively activated microglia and macrophages (M2) in the spinal cord 7 and 14 dpi (Macháček et al., 2022).

The systemic immune response differed from the CNS. We observed the mixed Th1/Th2 cell populations in the spleen with a clear peak 7 dpi (Majer et al., 2020), when also the spleen was biggest (Macháček et al., 2022). In contrast with the splenomegaly caused by schistosomes (Winkelmann et al., 2022), the spleen enlargement did not continue due to the slow vanishing of the inflammation in the absence of further development of the parasite. Our serological study (Majer et al., 2020) also confirmed parasite-specific antibodies IgG2a and IgG1 typical for Th1 and Th2 cell types, respectively (Finkelman et al., 1988). The cercarial homogenate even stimulated the splenocytes isolated 7 dpi mainly the production of the IFN γ . The systemic immune response was apparently influenced by the skin phase of the infection at this time point (Majer, 2020) and the latter neuroinvasion did not markedly affect it.

To sum up, the initial infiltration of the CNS is probably influenced by the systemic immune response and regulates the potential danger of autoimmune reaction after BBB leakage. However, the acute phase of the immune response against migrating schistosomula through the spinal cord and brain is apparently restricted to the CNS and CNS dLN. It culminates between 7 and 21 dpi and it is polarized towards the Th2/M2 phenotype. In contrast, systemic immune response in the spleen and blood at this time is no longer affected by the regulatory reaction and reacts mainly on the skin phase and the consequences of the cercaria infection. This thesis brings new insights into the communication between CNS and systemic immune response. Although the concept of immune privilege of the CNS was disproved (Louveau et al., 2015), keeping homeostasis is of course necessary. Interestingly, the regulation of the immune response in

the spinal cord was evident at the beginning of the infection. Later on, however, the expression of eotaxins and MHC II facilitated the infiltration of leukocytes into the CNS from the periphery. Therefore, further studying of these phenomena will be interesting not only for a better understanding of the immune response in the CNS but also for potential reparative processes that must follow after the granuloma disappearance.

4.2. *T. szidati* immunomodulation on local and systemic levels

T. szidati schistosomula cause mechanical damage of the lungs, however, without apparent influx of the leukocytes (Chanová et al., 2007). Therefore, we tested their immunomodulatory potential against ovalbumin (OVA)-induced asthma in mice. To verify the local effect of schistosomula in the lungs, we performed histological analysis and flow cytometry to analyze the cellular composition of the lungs. The local polarization of the immune response was described by the cytokine bead array of the BAL and qPCR of the lung tissue and lung dLN. The effect on the systemic immune response was tested by the antigen-specific restimulation of the splenocytes.

Our histopathological analysis indicated a better prognosis for the group with OVA + *T. szidati* (OVA+*Ts*) than the OVA only. This trend was supported by the flow cytometry, which revealed a significant, one-third reduction of the eosinophils in the lungs. A similar trend in the reduction of eosinophils during asthma was observed after *S. japonicum* infection (Mo et al., 2008). Nevertheless, it was probably caused by the Treg upregulation on the systemic level induced by the E/S products from *S. japonicum* eggs deposited in the liver and intestine (Li et al., 2023). Another helminth migrating through the lungs, *Nippostrongylus brasiliensis*, also reduced the asthma progression, but only after reaching its final destination in the intestine, which shows the remote immunomodulatory effect. The lung phase of the migration did not have a positive impact (Wohlleben et al., 2004).

The *T. szidati* migration induced the expression of *Il10* in the infected lungs, which could reduce the amount of IL-5 in BAL and *Il5* expression in lungs dLN. IL-5 is essential for eosinophil activation, and the reduction of this cytokine via the IL-10 regulatory pathway was observed in *N. brasiliensis* (Wohlleben et al., 2004) or *S. mansoni* (Mangan et al., 2006) facilitated amelioration of asthma. The source of IL-10 could be Th2 cells, which produced the regulatory cytokine in lungs dLN during *S. mansoni* schistosomula migration through the lungs (Redpath et al., 2015). On the other hand, the downregulation of M2 markers (*Chil3* and *Arg1*) and Th2-associated cytokines IL-5 and IL-4 in the lungs did not fully support this hypothesis.

The alternative source of the IL-10 could be B cells, which regulate the asthma progression in *S. mansoni* adult male worm infection (Mangan et al., 2006) or in chronic schistosomiasis with the presence of eggs (van der Vlugt et al., 2012). Nevertheless, revealing the cells producing IL-10 during *T. szidati* lung infection needs to be done in the future.

Interestingly, the restimulation of the splenocytes with the OVA indicated that *T. szidati* infection of mice with asthma increased the responsiveness of the splenocytes by production of IL-4, IL-5, IL-10, and IFN γ . Moreover, the addition of schistosomula homogenate even boosted the cytokine production. A similar trend was observed after *S. mansoni* infection, which increased OVA-specific cytokine production by splenocytes and while declined the asthma pathology in the lungs (Mangan et al., 2006). We assume that the schistosomula modulates the immune response in their close vicinity only, which can bring exciting opportunities for lung-targeted asthma therapies.

Although the concentration of OVA for restimulation was too small, and there is a need to repeat this experiment, it clearly showed that the immunomodulatory potential of *T. szidati* is rather local than systemic. The whole body homogenate of schistosomula did not show beneficial potential, and it will be necessary to focus on their E/S products.

5. Summary

The presented thesis broadens the knowledge of the immune response of mice against avian schistosomes and emphasizes the importance of the immune response characterization in the whole-body context.

The most significant findings:

- Tissue specific response against migrating *T. regenti* through the CNS is different than the polarization of the systemic immune system.
- Light-sheet fluorescence microscopy technique brings an excellent opportunity to depict immune response against helminths *in toto*.
- *T. szidati* shows the potential of immune response modulation and alleviates the progression of asthma.

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