SPINAL CORD PATHOLOGY IN CHRONIC EXPERIMENTAL *TOXOPLASMA GONDII* INFECTION

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Received: January 23, 2014; Accepted: January 29, 2014

Infection with the protozoan *Toxoplasma (T.) gondii* causes chronic infection of the central nervous system and can lead to life-threatening encephalomyelitis in immunocompromised patients. While infection with *T. gondii* has long time been considered asymptomatic in immunocompetent hosts, this view is challenged by recent reports describing links between seropositivity and behavioral alterations.

However, past and current researches are mainly focused on the brain during *Toxoplasma* encephalitis, neglecting the spinal cord as a key structure conveying brain signals into motion. Therefore, our study aimed to fill the gap and describes the spinal cord pathology in an experimental murine model of toxoplasmosis.

In the spinal cord, we found distinct histopathological changes, inflammatory foci and *T. gondii* cysts similar to the brain. Furthermore, the recruitment of immune cells from the periphery was detected. Moreover, resident microglia as well as recruited monocytes displayed an increased MHC classes I and II expression. Additionally, the expression of pro- and anti-inflammatory cytokines was enhanced in the brain as well as in the spinal cord. In summary, the pathology observed in the spinal cord was similar to the previously described changes in the brain during the infection.

This study provides the first detailed description of histopathological and immunological alterations due to experimental *T. gondii* induced myelitis in mice. Thus, our comparison raises awareness of the importance of the spinal cord in chronic *T. gondii* infection.

Keywords: *Toxoplasma gondii*, spinal cord, inflammation, infection, myelitis

Introduction

The protozoan *Toxoplasma gondii (T. gondii)* is a significant zoonotic and veterinary pathogen with a global distribution [1, 2]. This obligate intracellular parasite is able to cross all biological barriers and finally invades the host's central nervous system (CNS) [3–5]. The course of toxoplasmosis is well described in two serious health conditions: First, maternal primary infection during pregnancy can lead to various congenital defects and even abortion. Second, infection or re-activation of a pre-existing infection in immunocompromised individuals causes fatal encephalitis if left untreated [2].

Adult acquired toxoplasmosis can also affect the spinal cord. Few reports were published describing myelitis in humans infected with *T. gondii*. Spinal cord pathology

becomes clinically manifested mostly in patients with severe immunosuppressive disorders due to CD4 immune cell deficits, for example, acquired immunodeficiency syndrome (AIDS) or T-cell leukemia-lymphoma [6, 7]. Moreover, *Toxoplasma* myelitis was reported after peripheral stem cell transplantation [8]. Depending on the cyst location, with respect to different segments of the spinal cord, the symptomatology is characterized by motor and sensory loss, urinary sphincter abnormalities and pain [6, 9–12]. *Toxoplasma*-induced myelitis is not only restricted to humans but also some cases were reported in domestic animals [13–15].

While infection with *T. gondii* has long time been considered asymptomatic in immunocompetent hosts, this view is challenged by recent reports describing the link between seropositivity and behavioral, personality and

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neuropsychiatric disorders [16–22]. Interestingly, subtle motor impairment, in the form of prolonged reaction time, was reported in seropositive but otherwise healthy individuals [17, 23–25]. Explained initially as a consequence of impaired long-term concentration ability, the psychomotor deficits might also reflect subclinical spinal cord dysfunctions due to latent toxoplasmosis.

The manipulation of the rodent's behavior in relation to predator–prey interactions is described in recent reports [26–30]. Few studies have also described impaired sensorimotor functions, which manifested in the form of poor performance in grip strength and balance beam tests, diminished exploratory activity and gait alterations, but involvement of the spinal cord was not addressed [31, 32]. All together, these observations suggest that spinal cord pathology should receive increased attention similar to brain pathology, to further elucidate the behavioral changes in mice caused by *T. gondii* infection.

In the last years, increasing progress led to a better understanding of the complex relations between *T. gondii* and its host's neuro-immunological systems. Most of the research is based on animal models, where it was shown that, during *T. gondii* infection, resident microglia and astrocytes were activated and immune cells from the periphery entered the brain. These recruited immune cells comprise of different subpopulations. Initially, cells of the innate immune system for instance dendritic cells, monocytes, macrophages and neutrophils are relevant for fighting against pathogens [33–36]. Further, the recruited dendritic cells function as a mediator between innate and adaptive immunity by recruiting T cells to the brain [37, 38]. CD4⁺ and CD8⁺ T cells control parasite replication to prevent reactivation of the latent infection [39].

The complex interplay between immune cells is mediated by different molecules, produced at distinct time points after an infection. Among the first cytokines and early activators are IL-1 β and IL-6 [40]. The immune response is continued by dendritic cells, which are the main source of IL-12 in toxoplasmosis and stimulate IFN- γ production by T cells [41, 42]. Rapid production of these key mediators, IL-12 and IFN- γ , results in fewer symptoms [43]. Thus, it is not surprising that IFN- γ plays a critical role in mediating resistance against acute and chronic *T. gondii* infection by subsequent activation of effector cells to control parasite replication [44]. Additionally, parasite control in the CNS relies on *TNF*-dependent nitric oxide (NO) production [45].

The inflammation is counterbalanced by regulatory cytokines, such as IL-10, IL-27, and TGF- β , which help to limit potentially harmful immune responses by NK and T cells [46–48]. This leads to a control of the infection but also creates a hospitable environment assuring the parasite's long-term survival. The chronic phase of infection is characterized by numerous intact *T. gondii* cysts scattered throughout the whole brain being able, thus, to alter directly or indirectly neuronal function and presumably the behavior [49, 50].

Despite the intense research on *Toxoplasma* encephalitis, there is not much acknowledged about the manifestation of the infection in the murine spinal cord. Due to the increasing human relevance complemented with locomotor abnormalities found in murine models, we compared brain and spinal cord pathology in a chronic experimental model of toxoplasmosis. The present study provides the first detailed report of histopathological and immunological alterations due to experimental *T. gondii*-induced myelitis in mice.

Materials and methods

Animals

All animal experiments were approved, according to German and European legislation by the local authorities. Experiments were conducted with adult C57BL/6 female mice (8 weeks old, purchased from Janvier). Five to six animals per group in up to two independent experiments were used.

T. gondii infection

Toxoplasma gondii cysts of the ME49 type II strain were used for this study. Parasites were harvested from the brains of female NMRI mice strain infected intraperitoneally with *T. gondii* cysts 4 to 5 months earlier. Brains obtained from infected mice were mechanically homogenized in 1 mL sterile phosphate-buffered saline (PBS) and the cysts counted using a light microscope. Three cysts were administered intraperitoneally into mice in a total volume of 200 μ L. Control mice were mock infected with sterile PBS.

Brain and spinal cord harvesting

At the chronic stage of infection (8 weeks post infection), mice were deeply anesthetized with isoflurane and then perfused intracardially with sterile ice-cold PBS. Brains and spinal cords were removed and prepared accordingly for histopathological, qRT-PCR and flow cytometry analysis.

Histopathology

Brains and spinal cords were removed and immersed in 4% paraformaldehyde (PFA) for several days. Spinal cord cylinders were cut at different heights and manually arranged in a paraffin tissue microarray (TMA) of 5 $\times$ 7 cylinders per block for parallel processing as previously described [51]. Paraffin-embedded, 4- μ m-thick sections were deparaffinized and conventionally stained with

hematoxylin–eosin (H&E) stain. Immunohistochemical analysis was performed according to our previous publications [52–58] using a BOND-MAX (Leica Microsystems GmbH/Menarini, Germany) with antibodies against ionized calcium-binding adapter molecule 1 (IBA1, 1:2,000, Wako 019-19741, Germany) to label microglia, glial-fibrillary acid protein (GFAP, 1:1,000, DAKO Z033401, Germany) to label astrocytes, myelinbasic protein (MBP, 1:1,600, DAKO A062301, Germany) to label myelin sheets, neurofilament (NF200; 1:160, Sigma-Aldrich N4142-5ML, Germany) to label axons, NeuN (1:1,000, Millipore MAB377, Germany) to label neurons, and anti-Toxo (1:200, Dianova DLN-16734, Germany) to label *T. gondii*. Slides were developed using the Bond™ Polymer Refine Detection kit (Menarini/Leica, Germany). For the evaluation whole tissue sections and TMAs were digitized at 230 nm resolution using a MiraxMidi Slide Scanner (Zeiss MicroImaging GmbH, Germany).

qRT-PCR

After removal, tissue samples from brains and spinal cords were immediately transferred to RNA later (QIAGEN, Hilden, Germany). They were kept at 4 °C for at least 24 h and then stored at –20 °C until RNA isolation. For RNA isolation, the tissue was removed from RNA later and homogenized with 1 mL of TriFast (peqGOLD, Erlangen, Germany) in BashingBeads tubes (Zymo Research, Freiburg, Germany). PeqGOLD HP Total RNA Kit was used for purification and manufacturer's instructions were followed. On-membrane DNase I digestion (peqGOLD, Erlangen, Germany) was performed and RNA purity and concentration was determined by absorbance at 230, 260 and 280 nm in a NanoDrop (Fisher Scientific, Germany).

For qRT-PCR, SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Life Technologies, Darmstadt, Germany) was used with 300 ng total RNA in a reaction volume of 10 µL. Triplicate reactions were developed in a LightCycler® 480 Instrument II (Roche, Grenzach-Wyhlen, Germany). Reverse transcription was performed for 15 min at 50 °C followed by 2 min at 95 °C. Subsequently, 45 amplification cycles were run, comprising of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 30 s. TaqMan® Gene Expression Assays (Life Technologies, Darmstadt, Germany) were used for amplification of *HPRT* (Mm01545399_m1), *IFNG* (Mm00801778_m1), *IL1B* (Mm00434228_m1), *IL6* (Mm00446190_m1), *IL10* (Mm00439616_m1), *IL12A* (Mm00434165_m1), *TNF* (Mm00443258_m1). *HPRT*-expression was chosen as reference for normalization, and target/reference ratios were calculated with the LightCycler® 480 Software release 1.5.0 (Roche, Grenzach-Wyhlen, Germany). Resulting data were further normalized on values of control groups and statistically analyzed with GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). All data are shown as mean ± SEM. *p* values ≤ 0.05 were considered statistically significant.

Cell isolation

For mononuclear cell isolation, brains and spinal cords were homogenized in a buffer containing 1M Hepes pH 7.3 and 45% Glucose and then sieved through a 70-µm strainer. The cell suspension was washed and fractionated on 25–75% Percoll gradient (GE Healthcare) for 25 min at 800g without brake. The cells in the interphase comprised of mononuclear cells which were washed and used immediately for further experiments.

Flow cytometry

Single cell suspensions were incubated with an anti-FcγIII/II receptor antibody (clone 93) to block unspecific binding. Thereafter, cells were stained with fluorochrome conjugated antibodies against cell surface markers: CD45 (30-F11), CD11b (M1/70), Ly6G (1A8), Ly6C (HK1.4), CD11c (N418), F4/80 (BM8), MHC I H-2D^b (28-14-8), MHC II I-A/I-E (M5/114.15.2) in FACS buffer (PBS containing 2% FCS) for 30 min on ice and then washed and fixed in 4% paraformaldehyde for 10 min. All antibodies were purchased from eBioscience, Biolegend, or BD. Matched isotype controls were used to set appropriate gates. Cell acquisition was performed on BD FACSCanto™ II flow cytometer. Data were analyzed using FlowJo software (TreeStar). Statistical analysis was performed with GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). Population numbers and median fluorescence intensity values are shown as mean ± SEM. *p* values ≤ 0.05 were considered statistically significant.

Results

Histopathological findings in the brains and spinal cords of mice upon chronic *Toxoplasma* infection

T. gondii-infected animals showed wide-spread cortical and subcortical lesions of different types, intensities and evolutive stages (Fig. 1). Most prominent pathological findings were higher cellularity, due to peripheral inflammatory cells transmigration to the brain, the presence of single or paired cysts, parenchymal hemorrhage, edema and limited degeneration of cortical neurons. The inflammatory cells were organized in foci, not always surrounding the cysts, suggesting the involvement of soluble factors.

In the spinal cord, the histopathological examination of different anatomical segments revealed similar lesions to those observed in the brains, characterized by the presence of high counts of infiltrated inflammatory cells, the presence of the cysts mostly in the grey matter, neuronal degeneration and hemorrhage (Fig. 1). Resident microglia (anti-IBA1) and astrocytes (anti-GFAP) displayed a strong activation in the grey and white matter in the spinal cord.

Fig. 1. Morphological presentation of *T. gondii*-infected brains and spinal cords. A) Cortex of a control brain without infection. B) Infected cortex shows numerous foci of inflammation and higher cellularity due to endogenous microglia and peripheral mononucleated cells. Open arrows highlight cysts with surrounding inflammation. C) Spinal cord cylinder of an uninfected animal. D) Spinal cord cylinder of an infected animal. D' arrows highlight cysts in the spinal cord, D'' vascular proliferations due to chronic infection and accumulation of inflammatory cells, D''' inflammation foci and degenerating spinal cord neurons. E) Immunohistochemistry of a spinal cord cylinder without *T. gondii* infection. F) Immunohistochemical labelling of a *T. gondii* cyst in the vicinity of anterior motor neurons. The open arrow indicates the cyst. Inset: higher magnification of a typical grey matter spinal cord cyst. Scale bars: A–F 500 µm; D'–D''' 125 µm; scale bar in the inset of panel F 20 µm

Fig. 2. Immunohistochemical representation of uninfected (A–E) and infected (A'–E') spinal cords. A, A') anti-IBA1 stain shows marked and widespread activation of microglia in the infected animals, B, B') anti-GFAP stain shows general activation of astrocytes, C, C') myelination was not effected as highlighted by normal anti-MBP stain without differences between the groups, D, D') anti-NeuN stain shows signs of degenerating neurons in the spinal cord grey matter in infected animals, E, E') anti-NF200 stain shows normal configuration of axons. Magnification of white and grey matter insets: 80×

Control animals showed only very small foci of astrocytes whereas in infected animals the background is filled with processes of activated astrocytes (Fig. 2). However, the inflammatory response did not lead to a reduction of myelin stain in the white matter tracts (anti-MBP), nor could we recognize a marked neuronal loss (anti-NeuN). Axonal processes were not involved in any destruction as highlighted by a normal neurofilament stain (anti-NF200).

*Ly6C^{hi} inflammatory monocytes and neutrophil granulocytes were recruited to the central nervous system during chronic *T. gondii* infection*

Next, we focused on the characterization of the resident and recruited immune cells in the central nervous system during the chronic infection. We detected that following infection with *T. gondii*, high numbers of myeloid cells were recruited from the periphery to the brain, comprising of CD45^{hi}CD11b⁺ (control: $0.3 \pm 0.1\%$, *T. gondii*: $42.2 \pm 6.2\%$) and CD45^{hi}CD11b⁺ (control: $0.1 \pm 0.05\%$, *T. gondii*: $7.7 \pm 1.3\%$) cells (Fig. 3B, C). Control brains predominantly consisted of microglia cells (CD45^{lo}CD11b⁺) as measured by flowcytometric analysis (Fig. 3A).

Whereas control spinal cords contained higher rates of CD45^{hi}CD11b⁺ recruited myeloid cells (control: $1.3 \pm 0.3\%$) compared to those in the brains (Fig. 3D), after infection, we detected similar cell compositions in the spinal cords and in the brains (*T. gondii*: CD45^{hi}CD11b⁺: $29.6 \pm 4.7\%$, CD45^{hi}CD11b⁺: $11.5 \pm 1.5\%$) (Fig. 3E, F). Furthermore, we identified two distinct populations among the CD45^{hi}CD11b⁺ recruited cells: neutrophil granulocytes (Ly6G⁺) and inflammatory monocytes (Ly6G⁺Ly6C^{hi}). Percentages from the total cells isolated from the brain were significantly increased in the chronic phase of *T. gondii* infection for Ly6C^{hi} inflammatory monocytes (control: $0.02 \pm 0.009\%$, *T. gondii*: $2.7 \pm 0.6\%$) as well as neutrophil granulocytes (control: $0.05 \pm 0.03\%$, *T. gondii*: $0.3 \pm 0.07\%$) compared to non-infected controls (Fig. 4B, C).

Analysis of the CD45^{hi}CD11b⁺-recruited cells in the spinal cord revealed that mainly neutrophil granulocytes invaded the control spinal cords (control: $1.0 \pm 0.2\%$) (Fig. 4D). During chronic infection, the proportion of neutrophils did not undergo significant changes (*T. gondii*: $1.8 \pm 0.9\%$). However, it is important to mention that here we present the percentage of total cells, while the absolute numbers were definitely altered. Contrasting, the population of Ly6C^{hi} inflammatory monocytes became predominant (control: $0.2 \pm 0.02\%$, *T. gondii*: $2.9 \pm 0.8\%$) (Fig. 4E, F).

Fig. 3. CD45^{hi} cells were recruited to the brain and spinal cord during chronic *T. gondii* infection. Invasion of the central nervous system by recruited cells (CD45^{hi}) was demonstrated by flow cytometry. Cells isolated from brains or spinal cords were stained for CD45 and CD11b and three distinct populations were found: resident microglia were CD45^{lo}CD11b⁺, recruited T, B and plasmacytoid dendritic cells were CD45^{hi}CD11b⁻ and recruited myeloid cells were CD45^{hi}CD11b⁺. Data are shown as dot plots of control (A) and *T. gondii*-infected (B) brain cells, (C) displays the percentages of respective gates as bars (White bars – Control, Black bars – *T. Gondii*). (D, E, F) Dot plots and bar graph from spinal cord cells

Fig. 4. Inflammatory monocytes and neutrophil granulocytes invade the central nervous system of chronically infected mice. Recruited myeloid cells (CD45^{hi}CD11b⁺) were further stained for Ly6C and Ly6G to distinguish between inflammatory monocytes (Ly6C^{hi}Ly6G⁻) and neutrophil granulocytes (Ly6C^{hi}Ly6G⁺). Both populations were recruited to the brain and the spinal cord during chronic *T. gondii* infection. Data are shown as dot plots of control (A) and *T. gondii*-infected (B) brain cells, (C) displays the percentages of respective gates as bars (White bars – Control, Black bars – *T. Gondii*). (D, E, F) Dot plots and bar graph from spinal cord cells

Enhanced MHC expression on the cell surface indicated activation

To further elucidate the activation status of the particular cell populations, we analyzed the expression of MHC classes I and II molecules by flow cytometer comparing the median fluorescence intensity. In brains as well as in spinal cords of infected mice, we found that MHC classes I and II molecules were upregulated on the surface of resident microglia cells as well as recruited Ly6C^{hi} inflammatory monocytes. Examination of the activation status of neutrophil granulocytes in the spinal cord revealed that only MHC class I but not class II molecules were upregulated (Fig. 5).

Furthermore, microglia in the infected spinal cord showed a higher expression of MHC classes I and II molecules than microglia in the infected brain. Though, Ly6C^{hi} inflammatory monocytes revealed the highest amount of MHC class II both in the spinal cord and in the brain compared to microglia and neutrophil granulocytes (Fig. 5).

Chronic infection with *T. gondii*-induced enhanced expression of pro-inflammatory cytokines in brain and spinal cord

Inflammatory processes are characterized by altered expression of numerous genes, among them most importantly genes for cytokines facilitating intercellular communication. To address this aspect, we performed semi-quantitative RT-PCR for several pro-inflammatory cytokines.

First, we compared brains and spinal cords of non-infected mice and found increased mRNA levels of *IFNG* (fold change: 4.9 ± 1.9), *TNF* (fold change: 3.0 ± 0.4), *IL1B* (fold change: 2.5 ± 0.6) and *IL6* (fold change: 1.7 ± 0.3) in spinal cords vs. brains. Contrary to that, there was less expression of *IL12A* in the spinal cord (fold change: 0.1 ± 0) (Fig. 6A).

After investigating the base line, we measured mRNA levels 8 weeks p.i. and found that in brains, as well as in spinal cords, pro-inflammatory cytokines were upregulated. We detected the highest increase for *IFNG* (brain fold change: 7213 ± 1071 , spinal cord fold change: 1113 ± 215), followed by *TNF* (brain fold change: 386 ± 77 , spinal cord fold change: 207 ± 12), *IL1B* (brain fold change: 298 ± 78 , spinal cord fold change: 112 ± 32) and *IL6* (brain fold change: 31 ± 5 , spinal cord fold change: 12 ± 4). Interestingly, although the expression of *IL12A* was similar in control as well as in infected brains (fold change: 1.3 ± 0.2), we discovered a significant upregulation of this cytokine in the spinal cord upon chronic *T. gondii* infection (fold change: 6.3 ± 1.7) (Fig. 6B).

Comparing expression levels post infection in the brain with those in the spinal cord, we found only subtle differences. *TNF* mRNA levels were higher in the spinal cord (fold change: 1.6 ± 0.1). Yet, expression of *IFNG* (fold change: 1.5 ± 0.3), *IL1B* (fold change: 0.9 ± 0.3) and *IL6* (fold change: 0.7 ± 0.2) was similar in both organs. Consistent with the results from controls, we found lower expression of *IL12A* in infected spinal cords (fold change: 0.4 ± 0.1) (Fig. 6C).

Prevention of excess damage is crucial especially dur-

Fig. 5. Surface presentation of MHC molecules is enhanced upon chronic infection with *T. gondii*. Presentation of major histocompatibility complex (MHC) proteins class I and II on the surface of cells isolated from brain or spinal cord was quantified using flow cytometry by measuring the median fluorescence intensity levels of FITC (MHC class I; A,C) and APC-eFluor780 (MHC class II; B,D) before (control, white bars) and eight weeks after intraperitoneal infection (*T. gondii*, black bars) of C57BL/6 mice. Base levels were determined using antibodies with matched isotypes and were subtracted from the measured intensities. Data are displayed as mean $\pm$ SEM. Student's *t* test was performed and *p* values of $p \leq 0.05$ were considered significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

Fig. 6. Expression of pro- and anti-inflammatory cytokines is increased in *T. gondii*-infected brains and spinal cords. Brains (C: $n = 6$; I: $n = 5$) and spinal cords (C: $n = 6$; I: $n = 5$) were harvested during chronic infection (8 weeks post infection). Total RNA was isolated from tissues and subjected to semiquantitative RT-PCR amplification for different cytokines as well as the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). Relative mRNA expression was calculated by normalization on HPRT expression and as n -fold change of HPRT expression in non-infected brains (A) or non-infected brains/spinal cords (B) or infected brains (C). Data are displayed as mean $\pm$ SEM. Student's t test was performed and p values ≤ 0.05 were considered significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

ing long-lasting inflammation. Regulatory or anti-inflammatory cytokines, among them IL-10, play a major role in mediating this task.

We measured elevated *IL10* mRNA levels in control spinal cords compared to control brains (fold change: 2.5 ± 0.3 ; Fig. 5A). Interestingly, we made an inverse observation 8 weeks p.i. where expression was lower in the spinal cord (fold change: 0.5 ± 0.1) than in the brain (Fig. 6C). Nevertheless, in both organs, expression was enhanced comparing *T. gondii*-infected animals with non-infected controls (Fig. 6B).

Discussion

The parasite *T. gondii* has received increasing attention in the last years especially due to behavioral and neurological changes observed in rodents and humans [16–22, 26–30]. Prompted on revealing the mechanisms used by the parasite to contribute to neurological and psychiatric disorders, research focused mainly on pathological changes in the brain affected by the infection. The spinal cord is, along with the brain, a fundamental component of the CNS with the primary function such as the transmission of neural signals between the brain and the rest of the body. Few papers have reported *Toxoplasma* induced myelopathy especially in immunocompromised patients [6, 9–12]. Yet, no study focused on the spinal cord pathology in immunocompetent individuals, underestimating the possible contribution of the spinal cord lesions to the behavioral changes triggered by latent *T. gondii* infection. Therefore, the present study was designed to investigate the pathological effects of chronic *T. gondii* in-

fection in the murine spinal cords and to compare these changes with the ones occurring in the brain.

Histopathological evaluation of brains and spinal cords of *T. gondii*-infected mice revealed comparable pathological processes. Inflammatory foci and *T. gondii* cysts were widely recognized in the brain and the spinal cord without any preference for a specific area. Moreover, despite the presence of recruited inflammatory cells and generalized activation of resident cells no obvious changes in the myelin staining intensity or axonal density could be observed in the spinal cords. The cortical and spinal cord neurons showed slight changes due to the initiation of neurodegeneration in the vicinity of inflammatory foci. These results suggest rather altered function of the spinal cord neurons and less or no structural alterations.

The exchange between the blood and the CNS is limited by a specialized endothelium, the blood brain barrier (BBB) and the blood spinal cord barrier (BSCB), respectively. Whereas both serve a similar purpose, several differences have been described, such as increased BSCB permeability to cytokines like IFN- γ and TNF, but also decreased tight/adhere junction protein expression [59]. These differences might be a potential explanation of the detected increase in the number of neutrophils and higher expression of pro-inflammatory cytokines in the control spinal cord compared to the control brain.

It has been previously shown that during chronic infection with *T. gondii*, cells are recruited to the brain [34, 35, 37]. We confirmed the recruitment of CD45^{hi}CD11b⁺ and CD45^{hi}CD11b⁻ cells to the brain and revealed a similar pattern of recruited cells in the spinal cord by flow cytometric analysis. Together with our histopathological findings, this strengthens the concept that inflammation is not limited to the brain but similar inflammatory processes take place in the spinal cord.

Supporting the idea of generalized and extensive changes, we detected increased expression of MHC classes I and II molecules on recruited cells as well as on resident microglia. MHC molecules are important for the recognition of an infection. While MHC class I molecules mainly bind peptides originating from digestion of cytosolic and nuclear proteins and are present on every nucleated cell, MHC class II molecules hold peptides derived from extracellular proteins and are therefore essential for phagocytic cells. Upregulation of both types of molecules indicates an activation of cells during an ongoing inflammatory process [60]. Comparing surface expression levels of MHC molecules, we measured higher levels of MHC classes I and II on microglia from the chronically infected spinal cord than from the infected brain. Furthermore, Ly6C^{hi} inflammatory monocytes had the highest MHC class II levels from the populations we investigated in the infected brain as well as in the spinal cord. Interestingly, the expression of MHC class II on neutrophils from the brain and the spinal cord was present, but not significantly increased compared to controls. Even though neutrophils can present peptides on MHC class II when they are activated under certain conditions

like rheumatoid arthritis [61], this does not seem to be the case in our study.

An ongoing and active inflammation is also characterized by production of various cytokines. All cytokine expression levels we measured (*IFNG*, *TNF*, *IL1B*, *IL6*, *IL12A*, *IL10*) were significantly increased in brains and spinal cords compared to controls, except for *IL12A* in the brain. Therefore, multiple ways are introduced as to how the spinal cord could be affected by the inflammation, e.g., by *IL-1 β* -driven excitotoxic motor neuron injury [62]. Despite the overall similar inflammation in the spinal cord compared to the brain, we found several significant differences. With respect to pro-inflammatory cytokines, we found higher *TNF* expression but lower *IL12A* expression in the infected spinal cord 8 weeks p.i. compared to the brain at the same time point. It remains unclear whether those differences matter in the face of the generally overwhelming inflammatory processes. *TNF* being a coordinator of inflammatory responses, it has been used in trials for treatment of spinal cord injury [63]. Considering the higher *TNF* levels in the spinal cord than in the brain, future research should be done to address this issue.

Downstream of *TNF*, there is *IL-12*, which is produced by dendritic cells and macrophages to control differentiation of T cells toward the Th1 type [42, 64]. Previous studies described divergent roles for *IL-12* in the CNS, one being the induction of neurogenesis and remyelination followed by improved locomotor functions after experimental spinal cord injury [65]. Conflicting results have been reported in the context of autoimmunity, where lack of *IL-12* can either inhibit autoimmunity or lead to exacerbated pathology [66, 67]. Overall, the effects of *IL-12* are not yet fully understood, and further studies are necessary to describe its exact role and effects in the spinal cord during chronic toxoplasmosis.

For *IL10* expression representing the immunoregulation we observed an inversion upon infection: while the increased *IL10* expression in the control spinal cord compared to the brain may be a result of the overall higher expression of pro-inflammatory cytokines, *IL-10* was shown to inhibit production of *TNF* [48]; thus, in the chronically infected spinal cord, the lower *IL10* and higher *TNF* expression may be connected.

Our findings demonstrate, for the first time, the presence and the character of distinct spinal cord pathology upon *T. gondii* infection in a murine model. Further studies should aim to elucidate the definite effects of the ongoing inflammation on specific neuronal populations of the spinal cord, especially on motor neurons, well known for their role in movement production.

Acknowledgments

This work was partially supported by the DFG (to I.R.D.).

Conflict of interest statement. The authors declare that no conflict of interests exists.

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