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Peripheral *Taenia* infection increases immunoglobulins in the central nervous system

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ABSTRACT

Human cysticercosis is a disease caused by larvae of the cestode *Taenia solium*. It is an important common cause of adult-onset seizures world-wide where it exacts a debilitating toll on the health and well-being of affected communities. It is commonly assumed that the major symptoms associated with cysticercosis are a result of the direct presence of larvae in the brain. As a result, the possible effects of peripherally located larvae on the central nervous system are not well understood. To address this question, we utilised the *Taenia crassiceps* intra-peritoneal murine model of cysticercosis, where larvae are restricted to the peritoneal cavity. In this model, previous research has observed behavioural changes in rodents but not the development of seizures. Here we used ELISAs, immunoblotting and the Evans Blue test for blood–brain barrier permeability to explore the central effects of peripheral infection of mice with *T. crassiceps*. We identified high levels of parasite-targeting immunoglobulins in the sera of *T. crassiceps*-infected mice. We show that the *T. crassciceps* larvae themselves also contain and release host immunoglobulins over time. Additionally, we describe, for the first known time, significantly increased levels of IgG within the hippocampi of infected mice, which are accompanied by changes in blood–brain barrier permeability. However, these *T. crassiceps*-induced changes were not accompanied by alterations to the levels of proinflammatory, pro-seizure cytokines in the hippocampus. These findings contribute to the understanding of systemic and neuroimmune responses in the *T. crassiceps* model of cysticercosis, with implications for the pathogenesis of human cysticercosis.

1. Introduction

Human cysticercosis is a common debilitating disease caused by larvae of the cestode *Taenia solium*. An outdated conservative estimate suggests that up to 50 million people worldwide have cysticercosis (Centres for Disease Control, USA, 1993). It is particularly common in rural or peri-urban areas of central and South America, Sub-Saharan Africa, India and Asia (White, 2018). Humans are the definitive host of the adult lifestage of the cestode *T. solium*. Cysticercosis occurs when humans act as accidental intermediate hosts (instead of pigs) following ingestion of the eggs in faeces from an infected host. The eggs change into L1s after exposure to gastric acid, translocate across the intestinal epithelium and travel to in various tissues, with a strong predilection for the CNS. This results in what is termed neurocysticercosis. The major symptom associated with cysticercosis in humans is recurrent seizures. Cysticercosis is an important cause of adult-onset seizures in endemic countries (Ndimubanzi et al., 2010). In addition to seizures, patients with neurocysticercosis may experience deficits in learning and memory (Nau et al., 2018). Whether this is a result of a direct function of the parasite, an immune response, recurrent seizures, or a combination of these, is uncertain.

It is generally assumed that the symptoms associated with neurocysticercosis are a result of the direct presence of larvae in the brain and the resulting pericystic inflammatory host response (Cangalaya et al., 2016). There are, however, interesting precedents for generalised parasitic infections having effects on the brain. For example, nodding syndrome caused by the parasite *Onchocerca volvulus*, which infects the skin and connective tissue without entering the brain, is characterised by head dropping and other seizure-like activity, together with learning deficits (Lagoro and Arony, 2017). Interestingly, the parasite does not invade the nervous system, and symptoms are due to host-generated antibodies against the parasite having a central, autoimmune, neurotoxic

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effect (Johnson et al., 2017). Furthermore, there is evidence that infection by other helminths such as *Ascaris lumbricoides*, *Trichuris trichiura* and *Schistosoma* subspecies can affect learning and cognition in infected individuals without infection of the CNS (Ezeamama et al., 2018; Pabalan et al., 2018). In the study of cysticercosis, little consideration has been given to the possibility that a systemic immune response to *Taenia* larvae, whether centrally or peripherally located, could be responsible for, or contribute to, neurological symptoms.

A valuable model system in which to explore this possibility is one in which mice are infected intra-peritoneally with larvae of the cestode *Taenia Crassiceps*. *Taenia Crassiceps* is a related and antigenically similar cestode to *T. solium*. *Taenia Crassiceps* typically infect canids, mustelids and felids as definitive hosts and various rodents as intermediate hosts (Willms and Zurabian, 2010). However, human infection as an accidental intermediate host has also been observed (Ntoukas et al., 2013). In the intraperitoneal murine *T. crassiceps* model, larvae remain within the peritoneal cavity and do not invade the nervous system. As a result, it is a useful model system for studying potential effects of systemic responses to *Taenia* larvae on the brain (de Lange et al., 2019).

Previous research using this model has shown that, following infection, *Taenia* larvae are able to shift an initial, protective, systemic T helper type 1 immune response toward a T-helper type 2 response, which is more permissive for chronic infection. This Th2-type response is associated with high serum levels of cytokines IL-4, IL-5, IL-10 and antibodies IgG1, IgG4 and IgE (Dissanayake et al., 2004; Terrazas et al., 2010). To modulate the host immune response, *Taenia* larvae produce and excrete/secrete molecules which impair dendritic cell maturation and promote Th2-driving ability (Terrazas et al., 2010). Furthermore, the larvae can sequester and dispose of host immune proteins including IgG (Flores-Bautista et al., 2018). The effects of these systemic parasite-induced host immune responses on the brain are still relatively uncertain. However, one study utilised the murine intraperitoneal *T. crassiceps* infection model and reported behavioural changes, including impaired learning and memory, without larval invasion of the nervous system (Morales-Montor et al., 2014). This was found to be associated with variable changes in cytokine mRNA, for example relatively elevated mRNA levels of TNF-α and IL-6, but no change in IL-1β mRNA expression in the hippocampi of infected mice. One mechanism through which peripheral larvae may affect the brain could be via host-generated immunoglobulins against the larvae entering the nervous system via disruptions in the blood–brain barrier.

In this study we set out to investigate possible mechanisms by which a peripheral infection with *T. crassiceps*, and the resulting systemic immune response, could result in neurological changes such as those described by Morales-Montor et al. (2014). We employed the murine intra-peritoneal *T. crassiceps* infection model to elicit a systemic immune response, as demonstrated by high levels of parasite-targeting immunoglobulins in the serum of *T. crassiceps*-infected mice. We describe, for the first known time, significantly higher levels of IgG within the hippocampi of infected mice which are accompanied by changes in blood–brain barrier permeability. Unexpectedly, these *T. crassiceps*-induced alterations were not accompanied by changes in the levels of proinflammatory cytokines in the hippocampus. We further show that the *T. crassciceps* larvae contain host immunoglobulins and cytokines, and describe how these are released in vitro by the larvae over 3–10 days. These findings demonstrate that systemic host immune responses to infection with *Taenia* larvae can result in alterations in the brain, and thereby contribute to a wider understanding of the neuropathology in cysticercosis.

2. Materials and methods

2.1. Animals

2.1.1. Ethics

Mice were closely monitored for signs of distress (at least once a day), and all animal handling, care and procedures were carried out in accordance with South African national guidelines (South African National Standard: The care and use of animals for scientific purposes, 2008) and with authorisation from the University of Cape Town Animal Ethics Committee (AEC), South Africa (Protocol No: AEC 015/015, AEC 019/025).

2.1.2. Mice

In accordance with experiments conducted by Mahanty et al. (2013), female C57BL/6 mice were housed in the animal care unit at the Faculty of Health Sciences, University of Cape Town, under controlled temperature conditions and a 12:12 h dark:light cycle. Control mice were housed in separate cages in the same facility.

2.1.3. Infection

In accordance with the model described by Morales-Montor et al. (2014), 5–8 week old female C57BL/6 mice each received a single i.p. injection of 20 non-budding *T. crassiceps* larvae (ORF strain). Mice were killed 12–14 weeks later and the larvae harvested from the peritoneal cavity. Each mouse harboured between 200 and 500 larvae. Age-matched control mice were injected with PBS (Fig. 1).

2.1.4. Euthanasia

Mice were euthanised by halothane overdose followed by cervical dislocation.

2.2. Sample preparation

2.2.1. Preparation of T. crassiceps whole cyst homogenate

Larvae were frozen at −80 °C after harvesting and washing. Upon thawing, larvae were suspended in a volume of PBS (1X, pH 7.4) threefold that of the larval volume. A protease inhibitor cocktail was added to this suspension (1% vol/vol, Sigma-Aldrich, USA). The larvae were then homogenised on ice using a glass tissue homogeniser. The resulting mixture was centrifuged at 1500*g* for 20 min at 4 °C. The supernatant, excluding the low density white floating layer, was collected, sterile filtered through a 0.22 μm size filter (Millex-GV syringe filter, Merck, Germany), aliquoted and stored at −80 °C until use.

2.2.2. Preparation of T. crassiceps excretory/secretory (E/S) products

After harvesting, approximately 15 ml of PBS-washed larvae (+/− 15 ml) were transferred into a culture flask with 50 ml of culture medium (Earle's Balanced Salt Solution with glucose (5.3 g/litre), Glutamax (1X) (ThermoFisher Scientific, USA), penicillin–streptomycin solution (50 U/ml: 50 μg/ml) (Sigma-Aldrich, stock solution 5000 U/ml: 5 mg/ml), gentamicin sulphate

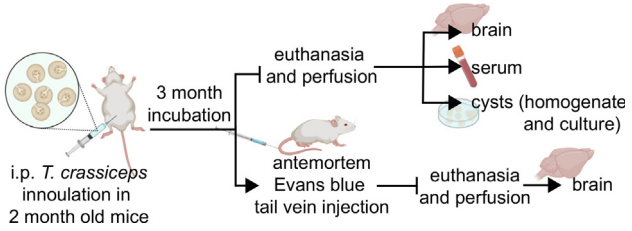

Fig. 1. Outline of sample collection protocol.

(100 μg/ml) (Lonza BioWhittaker, USA) and nystatin (11.4 U/ml) (Sigma-Aldrich). Larvae were maintained at 37 °C in 5% CO_2.

2.2.2.1. Pooled E/S product collection. After 24 h the medium was discarded and replaced with 50 ml of fresh medium. Medium was collected every 3–5 days (and temporarily stored at −20 °C) and replaced with fresh medium for up to 20 days. After 20 days all the collected media was pooled and concentrated/buffer exchanged to PBS (1X, pH 7.4) using an Amicon stirred cell (Merck) with a 3 kDa molecular weight cut-off membrane.

2.2.2.2. E/S product collection separated by day. Medium was collected and replaced with 50 ml of fresh medium at the end of day 1 in vitro, and again at the end of days 3, 5, 8 and 10 in vitro. Each medium collection was concentrated/buffer exchanged to PBS (1X, pH 7.4) using an Amicon stirred cell (Merck) with a 3 kDa molecular weight cut-off membrane. This protocol was performed on two separate harvests of *T. crassiceps* larvae.

2.2.3. Serum

Blood was withdrawn prior to transcardial perfusion and allowed to clot undisturbed at room temperature for 20 min. Samples were centrifuged at 1500*g* for 10 min at 4 °C. The supernatant was removed and stored at −80 °C until use. Sera from infected (n = 5) and control mice (n = 5) were aggregated to provide adequate volumes for ELISAs and immunoblots.

2.2.4. Hippocampal tissue

Mice were perfused with ice-cold PBS and both hippocampi were collected from control (n = 6) and infected (n = 8) mice. These samples were then frozen in liquid nitrogen and stored at −80 °C. Each hippocampus was thawed in 400 μl of RIPA buffer with 0.2 % HALT™ Protease Inhibitor Cocktail (ThermoFisher Scientific) and sonicated for 15 s on ice. Samples were then centrifuged at 17,200*g* for 30 min at 4 °C and supernatants were saved and stored at −80 °C.

2.2.5. Evans Blue (EB) injections

A 0.5 % solution of Evans Blue (EB) (Sigma-Aldrich) dye in PBS was prepared and filtered through Sartorius™ grade 3-HW smooth filter paper discs (ThermoFisher Scientific). Thirty minutes before euthanasia, each mouse received 200 μl of EB solution, injected into the lateral tail vein of infected (n = 5) and control (n = 3) mice. Animals were overdosed with halothane and transcardially perfused with ice-cold PBS. The hippocampi, frontal and remaining cortices and cerebellum were collected, dried and weighed. A solution of 50% trichloroacetic acid (TCA) (Sigma-Aldrich) at 1:3 w/v with 0.9 % NaCl solution (Merck) was added to each sample, and they were sonicated for 15 s on ice. Samples were centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant was collected and diluted 1:3 in ethanol (Sigma-Aldrich), before being stored at −80 °C (Kaya and Ahishali, 2011; Radu and Chernoff, 2013).

2.3. Experimental procedures

2.3.1. ELISAs

Levels of the inflammatory cytokines IL-1β, IL-6 and TNF-α were measured in supernatants from brain homogenates, serum and *T. crassiceps* E/S products using commercially available ELISA reagents according to the manufacturer's instructions (R&D Systems, Germany). Plates (96 well) were coated with 50 μl of one of three antibody solutions, either goat anti-mouse IL-1β (3 μg/ml) (R&D Systems, BAF401), goat anti-mouse IL-6 (3 μg/ml) (R&D systems, BAF406) or goat anti-mouse TNF-α (1 μg/ml) (R&D Systems, BAF410), all blocked with 4% BSA (Sigma-Aldrich). They were then incubated with donkey anti-goat biotinylated detection antibodies (50 μl of 0.3 μl/ml antibody solution), before adding streptavidin alkaline phosphatase (BD, USA) solution. The phosphatase substrate was added 45 min before absorbance measurement. Absorbances were measure at 405 nm with a reference wavelength of 492 nm using a Glomax Explorer Microplate Reader (Promega, USA), and unknown sample concentrations were interpolated from a standard curve from samples consisting of serial dilutions of recombinant cytokines. A detailed version of the protocol followed is available online (dx.doi.org/10.17504/protocols.io.bh2fj8bn). Where samples fell below the detection limit of ELISAs, they were allocated the value of the lower detection limit for the purposes of statistical comparison.

2.3.2. Western blotting

2.3.2.1. General protocol. Hippocampal protein concentrations were determined according to the Pierce™ BCA Protein Assay (Thermo Fisher Scientific). Aliquots (20 μg) of the protein samples were denatured in SDS at 100 °C, separated by electrophoresis on 12% SDS-PAGE, and then blotted to nitrocellulose membranes. Protein transfer was confirmed with Ponceau S stain (0.1% w/v Sigma-Aldrich). Membranes were blocked for 5 min using 10 ml undiluted EveryBlot Blocking Buffer (Bio-Rad, USA) and then incubated with primary antibodies (specified in sections 2.3.2.2. and 2.3.2.3.) overnight at 4 °C. Membranes were then treated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies , and imaged using a SynGene G:Box.

2.3.2.2. Serum T. crassiceps-specific IgG detection. Two 12% gels were each loaded with 15 μg of pooled E/S products and 15 μg of cyst homogenate, and were transferred to individual nitrocellulose membranes. Each membrane was then incubated overnight at 4 °C in 500 μl of serum from either *T. crassiceps*-infected mice or control mice, such that any *T. crassiceps*-specific antibodies in the serum would bind to the antigens separated by electrophoresis in the E/S product or homogenate samples. The membranes were then incubated in goat-anti-mouse IgG-HRP (0.33 μl/ml) (Bio-Rad, 170–6516) in 5 ml EveryBlot Blocking Buffer (Bio-Rad), developed and digitally imaged for analysis.

2.3.2.3. E/S product IgG detection. E/S samples collected in two batches at days 3, 5, 8 and 10 in culture were transferred onto a nitrocellulose membrane, incubated in a rabbit anti-mouse-IgG IgG (0.5 μl/ml) (Abcam, UK, ab133470) and rabbit anti-beta-actin IgG (0.2 μl/ml) (Abcam, ab16039) mixture, and then in donkey-anti-rabbit IgG-HRP (0.5 μl/ml) (Abcam, ab16039) in 5 ml EveryBlot Blocking Buffer (Bio-Rad), before the membrane was developed and digitally imaged for analysis.

2.3.2.4. Hippocampal and serum IgG detection. Hippocampal protein aliquots (20 μg) from infected and control animals were loaded onto SDS-polyacrylamide gels. A single sample from an infected mouse was repeated on every blot for the purpose of data normalisation. For comparison of systemic IgG levels, another gel was loaded with serial dilutions of serum in RIPA buffer (0.5 μg, 0.05 μg and 0.005 μg of serum) from infected mice and control mice, alongside 20 μg of the same hippocampal sample used for blot normalisation. All membranes were then incubated with antibodies as described in Section 2.3.2.3, developed and digitally imaged for analysis.

2.3.3. EB assessment of blood–brain barrier integrity

Samples were diluted in 95% EtOH and a standard curve contained 0.05–100 μg/ml of EB dye (Sigma-Aldrich) in 50% TCA and 95% EtOH. Fluorescence was measured at 620 nm excitation and 680 nm emission using a Glomax Explorer Microplate Reader (Promega).

2.4. Data analysis

For ELISAs, cytokine concentrations were calculated from absorbance readings using the Glomax Explorer Microplate Reader (Promega).

For IgG quantification, the optical densities for actin (43 kDa) and IgG (50 kDa) were determined for each exposure (30–180 s, in 30 s intervals) using ImageJ. The IgG value was divided by the actin value, calculated as a ratio to the reference sample that remained the same across blots. The IgG amount in that reference sample was interpolated as an average across exposures from a concentration curve calculated in GraphPad Prism 6 from a blot of known concentrations (IgG from Abcam ab133470, lot concentration at 0.6 mg/ml). The IgG amount was then calculated for each value at each exposure. Colourisation and background removal for image creation was performed post-analysis.

Due to skewed data distributions, non-parametric Mann–Whitney *U* tests were performed to compare the infected versus the control mice in all experiments. A value of $P < 0.05$ was considered significant, and the Hodges–Lehmann difference between means was used in lieu of an effect size calculation. All graphs show the median with the interquartile range. GraphPad Prism v6.0 was used for data analysis and graph creation.

3. Results

3.1. Chronic peritoneal T. crassiceps infection induces a robust humoral immune response

All *T. crassiceps* mice in our study had typical larval burdens (200–500 larvae), confirming that our mice were susceptible to peritoneal infection by the parasite. The infection was confined to the peritoneal cavity as no larvae were present in any of the brain samples harvested. We first wished to confirm that there was a systemic immune response to the intra-peritoneal infection of mice with *T. crassiceps*. To do so, we used western blotting to compare the amount of IgG in the sera of infected and uninfected mice. IgG was markedly increased in the sera of infected mice (pooled from five animals) compared with control animals (pooled from five animals) (Fig. 2A).

Next, we sought to determine whether infected mice might have raised specific antibodies circulating in their serum against antigens in the *T. crassiceps* antigens from the larval homogenate or pooled E/S products. To do so, two polyacrylamide gels were each loaded with *T. crassiceps* homogenate and E/S products and transferred onto nitrocellulose membranes. One membrane was then incubated with infected serum, while the other was incubated in serum from a control mouse. Both were then probed with anti-mouse-IgG primary antibodies. These blots clearly show specific binding of serum IgG to multiple different antigens of both larval homogenate and E/S products in *T. crassiceps*-infected mice (Fig. 2B).

We next sought to determine the serum levels of the proinflammatory cytokines TNF-α and IL-1β which have known roles in epileptogenesis (Vezzani et al., 2016), using ELISAs. We did not detect significant differences in serum cytokine levels between infected and control mice (Fig. 2C) with the values from the majority of the samples below the detection threshold of 7.8 pg/ml for TNF-α (6/8 infected mice and 5/7 control mice were below 7.8 pg/ml for TNF-α) and 31.3 pg/ml for IL-1β (8/8 infected samples and 4/7 control mice were below 31.3 pg/ml for IL-1β).

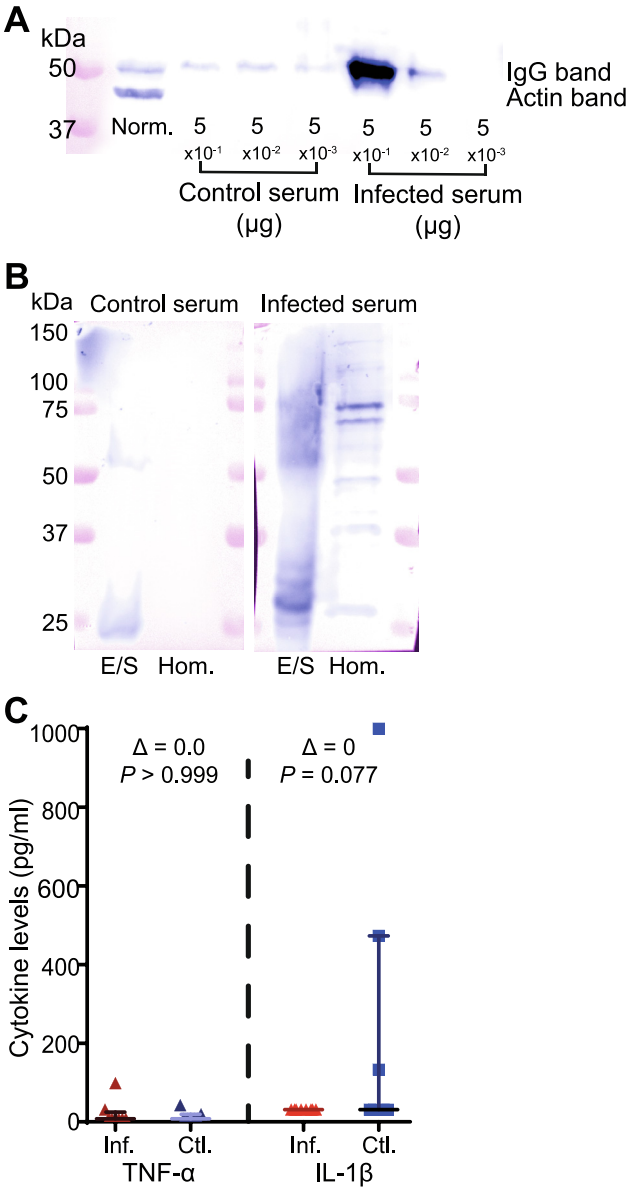

Fig. 2. Chronic peritoneal *Taenia crassiceps* infection induces a robust humoral immune response. (A) IgG levels in serial dilutions of control versus infected serum (amount loaded in µg shown below the bands) show high levels of IgG in the sera of infected mice. (B) Two membranes each containing a lane run with *T. crassiceps* pooled excretory/secretory (E/S) products and one run with larval homogenate (Hom.). These were incubated in sera from control and infected mice, and probed with anti-mouse IgG. This demonstrates the presence of *T. crassiceps*-specific antibodies in the sera of infected animals. (C) There was no significant difference in the serum cytokine levels of TNF-α and IL-1β between infected (Inf.) and control (Ctl.) animals. In addition, most serum samples had below detectable levels of these cytokines (7.8 pg/ml for TNF-α and 31.3 pg/ml for IL-1β). Norm., normalisation sample (20 µg of hippocampal homogenate from a *T. crassiceps*-infected mouse); Δ, Hodges–Lehmann difference between the medians.

3.2. Taenia crassiceps larvae release host IgG and inflammatory cytokines over time

Given previous reports that *T. crassiceps* larvae can sequester and release host IgG (Flores-Bautista et al., 2018), we next sought to corroborate this observation and to further determine whether the larvae release host cytokines. To do so we evaluated the larval E/S products for IgG and cytokines TNF-α, IL-1β and IL6 following 3, 5, 8 and 10 days in culture (Fig. 3). Western blots probing the *T. crassiceps* larval homogenate and E/S products for mouse IgG

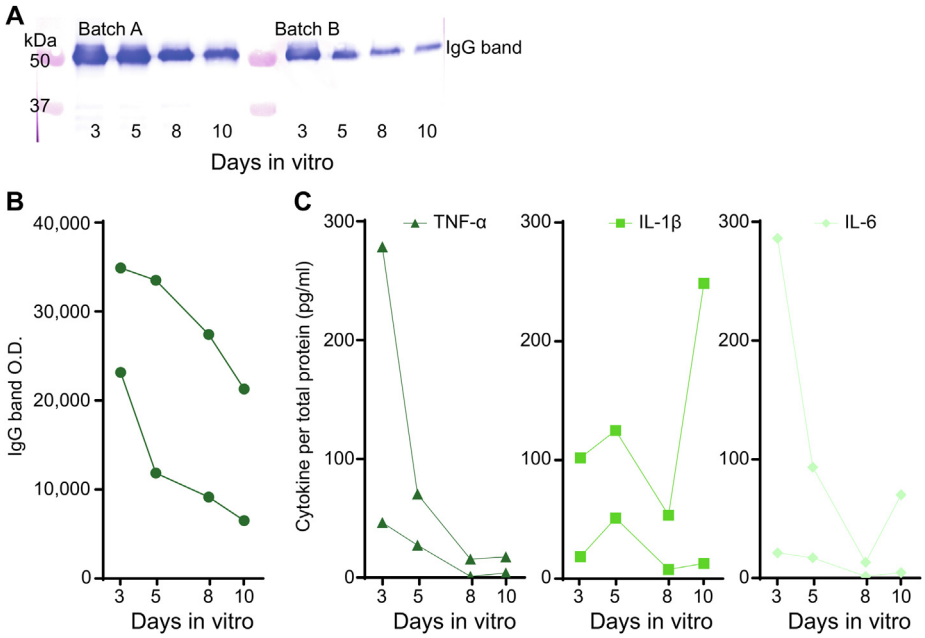

Fig. 3. *Taenia crassiceps* larvae release host proteins over time. Culture medium from two separate batches of *T. crassiceps* larvae were collected on days 3, 5, 8 and 10 in culture. (A) Host IgG was detected in the larval excretory/secretory (E/S) product, with levels decreasing with extended time in culture. (B) Measured optical density from blots shown in A, no proteins usable as a loading control were present for quantification. (C) The cytokines TNF-α, IL-1β and IL-6 were detected in the E/S product at all time points tested.

showed clear evidence of host IgG, which steadily decreased over time (Fig. 3A,B). After 10 days in culture, mouse IgG could nevertheless still be detected in the larval E/S product. Moreover, although there was variation in the total cytokine levels between the two batches of *T. crassiceps* larval cultures, we were able to reliably detect the presence of the cytokines TNF-α, IL-1β and IL-6 in the E/S products at all time points tested using ELISAs (Fig. 3C).

3.3. Chronic peritoneal *T. crassiceps* infection increases intra-hippocampal IgG levels

Noting the clear systemic antibody response in Fig. 2, we next wanted to determine if mice with intra-peritoneal *T. crassiceps* infection had higher levels of IgG within the brain parenchyma itself. All mice were PBS-perfused prior to dissection to prevent interference from intravascular IgG within the hippocampi, and hippocampal samples from infected and control mice were immunoblotted for IgG. There was a striking difference between infected (n = 16) and control (n = 12) samples, with no IgG detectable in any of the control samples, and a clear presence of IgG present in most of the samples from infected mice (Fig. 4A). Quantification of hippocampal IgG was performed using a normalisation sample and compared with a separately measured calibration curve with known amounts of IgG. There was a highly significant difference in hippocampal IgG levels between infected and control mice (infected mice had a median of 0.0198 (Interquartile range (IQR): 0.0049–0.0266) ng per 20 μg while control mice had a median of 0.0002 (IQR: 0.0–0.0004) ng per 20 μg, P < 0.0001, Mann–Whitney test).

3.4. Chronic peripheral *T. crassiceps* infection disrupts the blood–brain barrier but does not increase hippocampal proinflammatory cytokines

We next sought to determine a potential cause for the elevated intra-hippocampal IgG levels observed in infected mice (Fig. 4), by injecting EB dye into the tail vein and tracked its presence within the brain parenchyma with fluorometry. This would inform us if peritoneal infection with *T. crassiceps* was also associated with blood–brain barrier dysfunction. We saw a clear trend in the amount of dye present in the brain, suggesting enhanced blood–brain barrier permeability in infected animals compared with controls. This was particularly evident within the cortex, with a median of 0.849 μg of EB (IQR 0.797–1.230 μg) in the frontal cortex of infected mice compared with uninfected mice, with a median of 0.0.521 μg of EB (IQR 0.055–0.630 μg), a Hodges–Lehmann difference of 0.732 μg of EB (P = 0.008, Mann Whitney U test), and in the remaining cortex of infected mice there was a median of 1.213 μg of EB (IQR 0.678–2.267 μg), versus a median of 0.623 μg of EB (IQR 0.143–0.784 μg) in the uninfected mice, a Hodges–Lehmann difference of 0.591 μg of EB (P = 0.032, Mann Whitney U test) (Fig. 5B).

Finally, following our observation of an increase in intra-hippocampal IgG and a disruption in blood–brain barrier function in mice with peritoneal *T. crassiceps* infection, it is reasonable to assume that this could lead to enhanced parenchymal inflammation. This in turn could modulate network excitability. We therefore performed ELISAs of proinflammatory cytokines (TNF-α, IL-1β and IL-6), with previously described roles in neuroinflammation and epilepsy (Vezzani et al., 2016). Interestingly, the hippocampal cytokine levels were uniformly low and we did not detect significant differences in any of the three cytokines between infected and control mice (Fig. 5A). The median hippocampal TNF-α levels (detection threshold of 7.8 pg/ml) in infected compared with control mice were 56.9 pg/ml (IQR 34.1–84.2 pg/ml) versus 50.9 pg/ml (IQR 40.4–89.7 pg/ml), respectively, a Hodges–Lehmann difference of 0.6 (P = 0.925, Mann Whitney U test). The median hippocampal IL-1β levels (detection threshold of 31.3 pg/ml) in infected versus control hippocampi were 574 pg/ml (IQR 506–735 pg/ml) versus 623 pg/ml (IQR 519–0.751 pg/ml), respectively, a Hodges–Lehmann difference of 265 (P = 0.598, Mann Whitney U test). Finally, the median IL-6 levels (detection threshold of 3.9 pg/ml) were not significantly different in infected and control mice, with 132 pg/ml

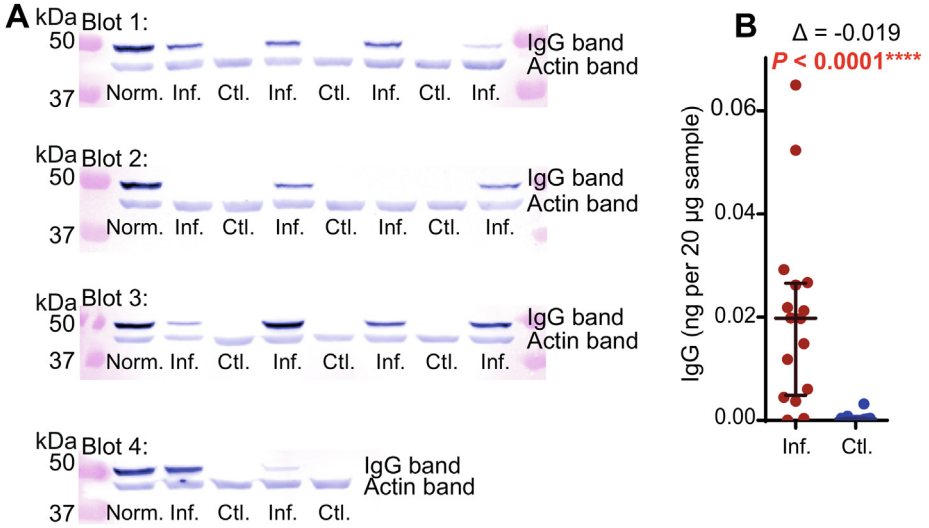

Fig. 4. Chronic peritoneal *Taenia crassiceps* infection increases intra-hippocampal IgG levels. (A) Blots probing for IgG in homogenised hippocampi of infected (n = 16) and control (n = 12) mice (exposed for 3 min) (multiple blots needed to include all the samples). (B) Population data of quantified hippocampal IgG levels from the blots in A. Inf., infected; Ctl., control; Norm., infected hippocampal normalisation sample repeated between bots; Δ, Hodges–Lehmann difference between the medians.

(IQR 113–139 pg/ml) versus 132 pg/ml (119–140 pg/ml), respectively, a Hodges–Lehmann difference of 2 (P = 0.635, Mann Whitney U test).

4. Discussion

Here we used ELISAs, western blots and the EB test in a murine model of peripheral *T. crassiceps* infection to investigate possible mechanisms by which a peripheral cysticercosis infection could result in neurological changes. We found high levels of parasite-targeting immunoglobulins in the serum of *T. crassiceps*-infected mice. In addition, we found that the *T. crassiceps* larvae themselves also contain and release host immunoglobulins over time, indicating that a systemic immune response has been mounted. Furthermore, we observed significantly increased levels of IgG within the hippocampi of infected mice, with data suggesting an increase in blood–brain permeability. These changes could contribute to behaviour disturbances and/or seizures. However, these *T. crassiceps*-induced alterations were not accompanied by alterations to the levels of proinflammatory cytokines in the hippocampus, as described by previous authors (Morales-Montor et al., 2014).

We found that intra-peritoneal infection of C57BL/6 mice produced robust and reliable chronic infections, with approximately 500 cysts harvested from each mouse. This is in contrast to work suggesting that C57BL/6 mice are resistant to the ORF strain of *T. crassiceps* (Reyes et al., 2009). This likely points to significant variability in susceptibility to infection between strains or even perhaps sub-strains of mice. In our experiments, C57BL/6 mice were easily infected and mounted a significant humoral response to the parasite, as demonstrated by the presence of *T. crassiceps*-targeting antibodies in the serum. In addition, we were able to corroborate recent research, which has shown that the larvae themselves can take up and release host protein, including IgG (Flores-Bautista et al., 2018). We found that even after 10 days in culture *T. crassiceps* larvae were still releasing host IgG and cytokines into the culture media. This is interesting considering that cytokine levels in the serum were undetectable. We postulate that the cytokines taken up by the larvae may be from an early initial systemic inflammation that had since resolved and the cysts had not yet released the internalised cytokines, or, more likely, that the local inflammatory response in the peritoneal cavity (from where the cysts were harvested) resulted in elevated cytokines there, but not in the systemic circulation, nor in the brain. Furthermore, this is important to note for those using *T. crassiceps* larvae-derived homogenate or E/S products to investigate the modulation of host immune responses and to exercise caution as these products likely contain both host IgG and cytokines, which could influence their results.

Other research using the intra-peritoneal *T. crassiceps* infection model in BALB/c mice has shown that cysticercosis results in behavioural changes without larval invasion of the nervous system, including impairment in behavioural tasks such as the object recognition test and the forced swim test (Morales-Montor et al., 2014). It is well-known that peripheral infections in mammals are characterised by local, systemic and CNS effects. CNS effects may give rise to 'sickness behaviour' (Ghai et al., 2015) and IL-1β and TNF-α, produced particularly as part of the acute phase response, are thought to be important mediators of this neuro-immune signalling (Cartmell et al., 1999). In our experiments, following 12 weeks of infection, we found low to undetectable levels of IL-1β and TNF-α in serum, and no difference between control and infected animals. This is consistent with previous reports that although *T. crassiceps* infection results in a T helper type 1 proinflammatory immune response in the first 2 weeks following infection, this is followed by a sustained Th2 response with low levels of IL-1β and TNF-α and high levels of IgG (Peon et al., 2013). This suggests that although IL-1β and TNF-α can cross the blood–brain barrier (Banks et al., 1995), centrally acting serum IL-1β and TNF-α are unlikely to be responsible for any sickness associated behaviours in the animals due to low serum levels following chronic *T. crassiceps* infection.

Interestingly, we found raised IgG in the hippocampi of infected mice compared with control mice. It is possible that this IgG could have some functional effect through local immune activation (Kadota et al., 2000), although we did not demonstrate this here. We hypothesised that the increase in hippocampal IgG was secondary to increased translocation of the serum IgG from a breakdown in blood–brain barrier integrity.

The blood–brain barrier, consisting of negatively charged endothelial cells cross-linked through tight junctions, smooth muscles cells and pericytes, ensures that only small molecules that are primarily lipophilic and cationic are able to cross from blood vessels into brain parenchyma without specialised transport channels (Ribeiro et al., 2012; Finke and Banks, 2017). This would nor-

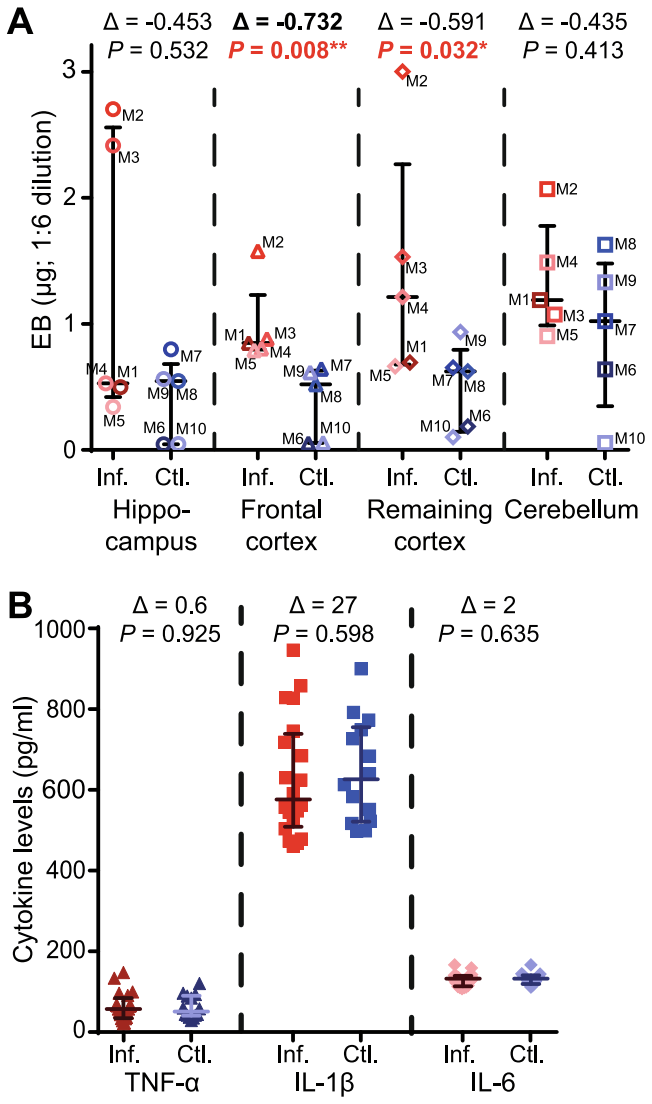

Fig. 5. Chronic peripheral *Taenia crassiceps* infection disrupts the blood–brain barrier but does not increase hippocampal proinflammatory cytokines. (A) Levels of Evans blue in homogenised samples from various areas in the brains of infected and uninfected mice. There was a trend towards an increased level of Evans Blue in the brains of infected mice, particularly in the cortex. (B) Cytokine levels in the hippocampi were above the detection threshold, however, no difference between infected and control samples was observed. Inf., infected; Ctl., control; EB, Evans Blue; Δ, Hodges–Lehmann difference between the medians.

mally exclude immunoglobulins from entering the brain tissue, however, blood–brain barrier breakdown has been shown to increase parenchymal IgG, either via passive diffusion or via transmigration of immune cells such as plasma cells (Sweeney et al., 2019). Our EB experiment was not definitive (Fig. 5A), although results were suggestive of impaired blood–brain barrier integrity. An alternative explanation for the elevated hippocampal IgG could be from local cytokine production through resident plasma cells, potentially activated by cyst antigenic products or immune factors from a local or remote source. Furthermore, local inflammation may be a cause, rather than a result, of blood–brain barrier breakdown. Further research including in-situ histological examination of the hippocampal milieu is recommended.

It is possible that the raised parenchymal IgG observed here could precipitate a local inflammatory response, including the local production of cytokines, with consequent effects on neuronal networks (Prieto and Cotman, 2017). However, we found low or undetectable levels of the cytokines IL-1β, TNF-α and IL-6, with no differences between infected and control animals. These findings contrast those of Lopez-Griego et al. (2015) who, using quantitative PCR for cytokine mRNA, found a modest but significant increase in TNF-α and IL-6 but not IL-1β mRNA in the hippocampi of infected female mice. This technique may be more sensitive than ELISAs, although to what degree mRNA expression relates to differences in translated cytokine levels is uncertain.

Given that IL-1β and TNF-α acting directly within the brain parenchyma have been strongly linked to changes in network excitability and seizures (Vezzani et al., 2011), our finding that there were no differences in these cytokines in the brains of *T. crassiceps*-infected versus control animals is important in the context of seizures secondary to cysticercosis. Along these lines, previous work has demonstrated that *T. crassiceps* induces an anti-inflammatory cytokine microenvironment, including decreased TNF-α and IFN-γ production, and high IL-4 and IL-10 expression (Reyes et al., 2011). These cytokines are important for antibody class switching. Future work should determine the possible local production of IL-4 and IL-10, and determine which classes of antibody IgG1, IgG2a, IgG2b or IgE are produced and present within the brain parenchyma. As noted in our introduction, there is a significant body of literature regarding the pro- versus permissive anti-inflammatory responses and immune modulation that occurs over time in peripheral taeniid infections, however, the neurological immunological milieu has not been studied in this longitudinal context.

A more coherent understanding of the local peritoneal, systemic, and local neurological immunological milieux over time would be exceptionally valuable in explaining why and when seizures occur. We observed no clear signs of inflammation besides increased levels of hippocampal IgG, and findings which may suggest blood–brain barrier dysfunction. This lack of significant inflammation may explain why seizures are not observed in the *T. crassiceps* intra-peritoneal murine model of cysticercosis. In humans, it would be immensely helpful to corroborate symptoms with local and systemic immunological states, although it is critical to note that findings from animal models are not necessarily applicable to human beings. Evidence is lacking for the possibility of learning and memory deficits in systemic cysticercosis infection, although it seems plausible based on observations of other parasites. Furthermore, determining whether cyst infiltration of the CNS is a prerequisite for the higher incidence of seizures seen in neurocysticercosis, and which immune states are predominant systemically and locally would be critical information. This paper shows that a more holistic approach to cysticercosis and neuroinflammation in peripheral and systemic disease is needed to understand the complex processes involved in this host–parasite interaction.

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