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Effects of *Toxocara* larvae on brain cell survival by *in vitro* model assessment

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SUMMARY

Neuroinvasive larvae of the common dog and cat roundworms, *Toxocara canis* and *Toxocara cati*, may cause severe neurological and neuropsychological disturbances in humans. Despite their pathogenic potential and high prevalence worldwide, little is known about their cell-specific influences and cerebral host–pathogen interactions in neurotoxocarosis. To address this discrepancy, a co-culture system of viable larvae with murine neuronal (CAD), oligodendrocytal (BO-1) and microglial (BV-2) cell lines has been established. Additionally, murine adult brain slices have been co-cultured with *Toxocara* larvae to consider complex organotypic cell–cell interplay. Cytotoxicity of larval presence was measured enzymatically and microscopically. Microscopic evaluation using trypan blue exclusion assay revealed to be less reliable and sensitive than the lactate dehydrogenase activity assay. Ultimately, even low numbers of both *T. canis* and *T. cati* larvae have impaired survival of differentiated CAD cells, which morphologically resemble primary neurons. In contrast, viability of oligodendrocytal and microglial cells as well as brain slices was not impaired by larval presence. Therefore, immune-mediated mechanisms or trauma by migrating larvae presumably induce the *in vivo* pathology rather than acute cytotoxic effects. Conclusively, the helminthic larvae co-culture system presented here is a valuable *in vitro* tool to study cell-specific effects of parasitic larvae and their products.

Key words: CAD cells, BO-1 cells, brain slices, BV-2 cells, neurotoxocarosis, parasitic zoonosis, *Toxocara canis*, *Toxocara cati*.

INTRODUCTION

Human neurotoxocarosis is a zoonotic infection caused by neuroinvasive larvae of *Toxocara canis* (Werner, 1782) or *Toxocara cati* (Schrank, 1788), the common roundworms of dogs and cats, respectively (Finsterer and Auer, 2007). After infection, larvae enter the host's tissue by penetrating the intestinal wall. Ultimately, passive blood transport and active migration may take larvae to the central nervous system (CNS). Such neuroinfections might manifest clinically in motor impairments such as ataxia, paresis or paralysis (Russegger and Schmutzhard, 1989; Villano *et al*. 1992; Sommer *et al*. 1994; Moreira-Silva *et al*. 2004). Furthermore, strong associations with epilepsy (Quattrocchi *et al*. 2012) as well as cases of neuropsychological disturbances, such as dementia or depression, have been described (Fortenberry *et al*. 1991; Rüttinger and Hadidi, 1991; Richartz and Buchkremer, 2002).

Migrating *Toxocara* larvae may remain viable for years in host tissues and produce *Toxocara* excretory/secretory (TES) antigen (reviewed by Maizels, 2013). Despite remarkable worldwide prevalence of anti-TES antibodies (reviewed by Maizels, 2013), the well-characterized clinical syndromes (reviewed by Strube *et al*. 2013) and suggested implications of this parasite infection in allergic conditions (Pinelli and Aranzamendi, 2012) as well as neurodegenerative diseases (Söndergaard and Theorell, 2004; Liao *et al*. 2008), the true pathogenic potential of *Toxocara* larvae is still poorly understood. Particularly, the underlying cause for parenchymal damage and demyelination in chronic CNS infections, as demonstrated in mice (Epe *et al*. 1994; Heuer *et al*. 2015), remains unclear. Mechanical effects of larvae perforating the brain tissue as well as exposure to larval TES and immune-mediated processes might play a role.

To address this question *in vitro*, co-cultures of viable *T. canis* and *T. cati* larvae with different CNS cell populations as well as a CNS tissue culture, so-called brain slices, were established. Neuronal, oligodendrocytal and microglial murine cell lines were employed to monitor consequences of cellular exposure to larvae. Additionally, suitability of brain slice co-culture as a replacement for *in vivo* studies on neurotoxocarosis was assessed by histological analysis.

MATERIALS AND METHODS

Animals

Experimental infection of dogs and cats for maintenance of *T. canis* and *T. cati* was approved by the ethics commission of the State Office for Consumer

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Protection and Food Safety of the German federal state of Lower Saxony under reference number 33.9-42502-05-01A038. For brain slice generation, adult C57Bl/6J mice were housed in Makrolon cages in a 12 h light/dark cycle. Standard rodent diet and water were supplied *ad libitum*. For organ removal, mice were euthanized by cervical dislocation. According to the German Animal Welfare Act, euthanasia of mice for brain removal was reported to the University's animal welfare officer.

In vitro *hatching of* Toxocara *larvae*

T. canis and *T. cati* eggs were isolated from feces of experimentally infected dogs and cats by standard sedimentation–flotation technique using saturated sodium chloride solution. Afterwards, eggs were allowed to embryonate for 4–6 weeks in tap water at 25 °C in an incubator (BT5C42E, Heraeus, Hanau, Germany). Larval hatching *in vitro* was performed according to Rajapakse *et al.* (1992) with slight modifications. Briefly, eggs were concentrated in 1 mL tap water followed by adding 10 mL sodium hypochlorite (Carl Roth, Karlsruhe, Germany). The mixture was incubated at room temperature for 19 min. Afterwards, 40 mL of deionized water were added and the solution was centrifuged at 100 ***g*** for 5 min. Furthermore, eggs were washed eight times with 0·85% sterile NaCl solution with subsequent centrifugation at 1500 ***g*** for 5 min each. After the last centrifugation step, saline solution was removed to 1 mL of total volume and 10 mL phosphate-buffered saline (PBS) supplemented with 1% penicillin/streptomycin (GE Healthcare, Freiburg, Germany) were added. Eggs were gassed for 5 min with 100% CO_2 from a gas cylinder and then centrifuged at 3000 ***g*** for 5 min. Subsequently, 10 mL of supernatant were removed carefully and 5 mL of larval culture medium [RPMI 1640 with 1% (w/v) glucose, 0·85% (w/v) sodium hydrogen carbonate, 10 U mL^{-1} penicillin/streptomycin (GE Healthcare) and 0·25 μg per mL amphotericin B (Genaxxon BioScience, Ulm, Germany)] were added. Larvae were then allowed to migrate through a gauze-covered Baermann-funnel overnight to eliminate any remaining egg shells, dead larvae and faeces particles from the medium (de Savigny, 1975). Funnels were placed in a standard cell culture incubator (NU-4950E, NuAire, Plymouth, Minnesota, USA) at 37 °C with 5% CO_2 supply. Larvae collected at the bottom of the funnel were transferred into six-well plates and fresh larval culture medium was added. Larval culture medium was replaced by the respective cell line medium (see section below) after 24 h of incubation. Both larval species (*T. canis* and *T. cati*) were maintained in media of the respective cell lines with daily media changes for at least 2 days before use in cell culture experiments.

Maintenance of central nervous cell cultures

CAD cells (Qi *et al.* 1997), a murine neuronal cell line, were obtained from Sigma-Aldrich (Taufkirchen, Germany) and maintained as described by Qi *et al.* (1997). Briefly, undifferentiated CAD cells were grown in 25 and 75 cm^2 tissue culture flasks (Sarstedt, Nürnbrecht, Germany) in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 1% GlutaMAX™ (Life Technologies, Darmstadt, Germany), 8% fetal bovine serum (FBS; GE Healthcare) and 2% penicillin/streptomycin (GE Healthcare). CAD cells differentiate into a cell division arrested primary neuron-like phenotype under conditions of serum deprivation. Differentiation was induced by switching to DMEM/F12 medium (Life Technologies) with 50 ng mL^{-1} sodium selenite (Sigma-Aldrich) and 2% penicillin/streptomycin after plating the cells in serum-containing medium 24 h before. Cells were incubated at 37 °C and 5% CO_2 supply. Undifferentiated CAD cells were passaged at confluence every 3–4 days by gently rinsing them off the cell culture flask bottom with 5–10 mL of fresh medium and re-plating them at a 1:5–1:10 dilution.

BV-2 cells (Blasi *et al.* 1990), which are considered a valid model for the brain's resident microglia (Henn *et al.* 2009), were purchased from Banca Biologica e Cell Factory (ICLC ATL03001, Interlab Cell Line Collection, IRCCS Azienda Ospedaliera Universitaria San Martino – IST Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). Cells were maintained in 25 cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 2 mM L-glutamine (Life Technologies), 10% FBS and 2% penicillin/streptomycin at 37 °C and 5% CO_2 supply. Every 3–4 days, cells were passaged at a 1:5–1:10 dilution after trypsination and centrifugation at 300 ***g*** for 5 min.

As oligodendrocytal cells, BO-1 cells (Pringproa *et al.* 2008) were cultured in 25 cm^2 tissue culture flasks pre-coated with poly-L-lysine (Sigma-Aldrich). Cells were maintained in B104-conditioned medium containing the N1 supplements as described by Louis *et al.* (1992) and passaged at confluence, every 2–5 days at a 1:2–1:5 dilution after trypsination and centrifugation at 300 ***g*** for 5 min.

Co-culture of central nervous cell lines and Toxocara *larvae*

Co-culture experiments were run in six-well plates and repeated at least once. Seeding densities were 1×10^4 cells per well for undifferentiated CAD- and BV-2 cells, 2×10^4 cells per well for BO-1 cells and 6×10^4 cells per well for differentiated CAD cells. After seeding into six-well plates, cells were

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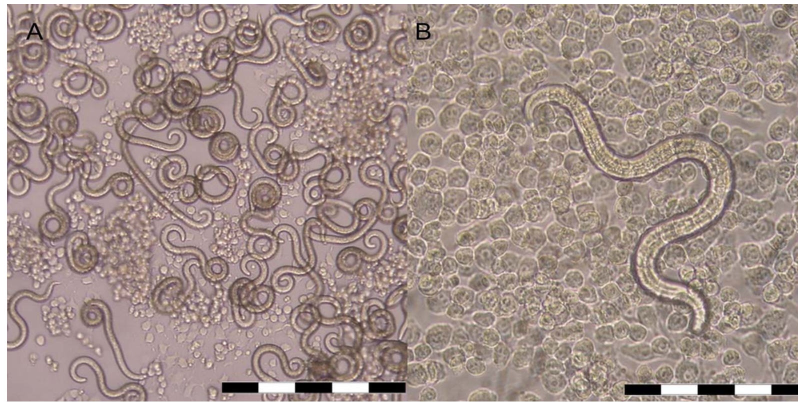

Fig. 1. *Toxocara canis* larvae and undifferentiated CAD neuronal cells (A) or BV-2 microglial cells (B), respectively, after 5 days in co-culture. Microscopically, both larvae and cells appear viable. Scale bars represent 200 μM (A) and 50 μM (B).

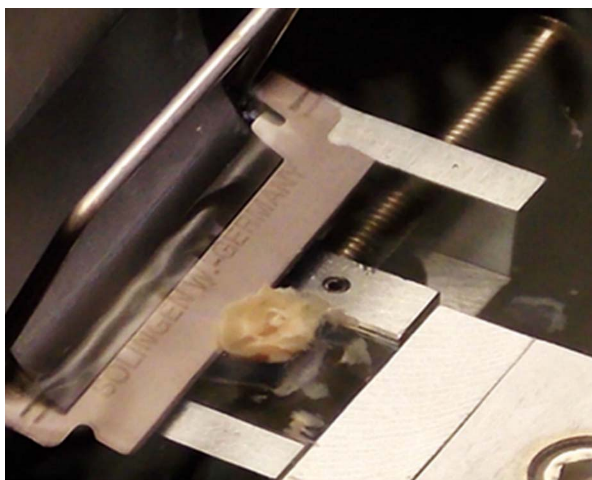

Fig. 2. Generation of adult mouse brain slices using the Lancer® vibratome series 1000 sectioning system (The Vibratome Company).

allowed to attach for at least 24 h before addition of larvae. Per experiment and group, six wells of cells each were exposed to 10, 50, 100 or 500 *T. canis* or *T. cati* larvae resulting in $n = 12$ replicates per larval quantity of each parasite species (exposure group). Undifferentiated CAD cells and BV-2 cells in co-culture with larvae are depicted in Fig. 1. Wells with unexposed cells served as controls. Viability of cells in culture after exposure to larvae was assessed using two different methods. On day 5 after addition of *T. canis* and *T. cati* larvae, cells were harvested to assess cell viability via trypan blue exclusion assay. This dye accumulates in damaged or dead cells. Assay performance and microscopic counting were carried out following a standard protocol (Strober, 2001). Additionally, cytotoxicity was evaluated enzymatically. As the cytoplasmic enzyme lactate dehydrogenase (LDH) is released into the supernatant if the cell membrane's integrity is impaired, it is a reliable marker for natural cytotoxicity (Korzeniewski and Callewaert, 1983). Thus, cell culture supernatants were assayed using a commercial LDH assay [CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Mannheim, Germany)] twice and in triplicates ($n = 72$ per exposure group) according to the manufacturer's instructions. The respective media as well as supernatants of *T. canis* and *T. cati* larval cultures without central nervous cells served as negative controls.

Brain slices

Generation of brain slices was based on methods published previously (Stoppini *et al.* 1991; Daza *et al.* 2007; Kim *et al.* 2013). Briefly, adult C57Bl/6J mice (>6 months of age) were euthanized by cervical dislocation, decapitated and brains were removed under sterile conditions. Cerebella were disconnected and cerebra were halved vertically and immediately embedded in liquid 4% low melting agarose (Sigma-Aldrich) using a 24-well tissue culture plate kept on ice. After approximately 5 min, agarose had hardened and was removed from the tissue culture plate in one piece. The embedded brain tissue was then attached to the vibratome block (Lancer® vibratome series 1000 sectioning system, The Vibratome Company, St. Louis, Missouri, USA) using cyanoacrylate adhesive and vibratome block and attached tissue were entirely submerged in ice-cold PBS with 1% antibiotics (GE Healthcare) in the vibratome basin. Slices of approximately 200 μM were cut (Fig. 2), placed in ice-cold dissection medium [Hibernate A with 2% Gibco® B27® supplement (Life Technologies, Darmstadt, Germany), 1% GlutaMAX™ supplement, 1% penicillin/streptomycin and 0·25 μg mL^{-1} amphotericin B (Genaxxon BioScience, Ulm, Germany)] according to Kim *et al.* (2013) for at least 5 min and then transferred onto cell culture inserts (PICM0RG50, Millipore, Molsheim, France) insix-well plates with 1 mL of serum-free brain slice culture medium [Neurobasal A with 2% Gibco® B27® supplement, 1% GlutaMAX™

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supplement, 1% penicillin/streptomycin and 0·25 $\mu g\,mL^{-1}$ amphotericin B (Genaxxon BioScience, Ulm, Germany)] according to Kim *et al*. (2013). Initially, slices were incubated at 37 °C with 5% CO_2 supply for 24 h. After media change, duplicate slices of six different animals per group were incubated for 6 days with 10, 50 or 100 *T. canis* or *T. cati* larvae. Daily media changes were performed by carefully removing 0·5 mL of medium from each well and adding 0·5 mL of fresh brain slice medium. Control slices of all animals, at least one slice per animal, were not exposed to larvae but otherwise treated identically. Supernatants of day 6 after addition of *T. canis* and *T. cati* larvae were analysed for LDH by CytoTox 96® Non-Radioactive Cytotoxicity Assay twice and in triplicates ($n \geq 36$ per exposure group) as described above.

For histological analysis, brain slices were submerged in 10% phosphate-buffered formalin (Roti®-Histofix, Carl Roth) and embedded in paraffin wax. Slices were stained with haematoxylin and eosin (H&E). In H&E stains, damaged and viable neurons were counted in three fields of vision per slice at 40× magnification. Thus, between 430 and 1430 cells were evaluated per group. Subsequently, numbers of damaged and viable neurons were expressed as percentage of total cells per group.

Statistical analyses

Control and exposure groups were compared by Mann–Whitney *U* tests and subsequent Bonferroni–Holm corrections using the GraphPad Prism 6 software (version 6.03; GraphPad Software, La Jolla, CA, USA). Differences were considered significant, if $P \leq 0·05$.

RESULTS

Trypan blue exclusion assay of central nervous cell–Toxocara co-cultures

Microscopic identification and enumeration of viable and dead cells showed that cell death was increased significantly in undifferentiated CAD cells compared with unexposed controls when co-cultivated with 100 and 500 *T. canis* larvae (Fig. 3A). *Toxocara cati* larvae did not increase cell death in undifferentiated CAD cells. Successful differentiation of CAD cells was confirmed by growth of long, axon-like processes. In these cells, only addition of 100 *T. cati* larvae induced a significant effect on cell viability (Fig. 3C). Proportions of dead cells in differentiated CAD cells were markedly higher than in undifferentiated CAD cells. In BO-1 cells, death rates were close to 0% and only 500 *T. canis* larvae caused a significantly higher degree of cell death (Fig. 3E). In BV-2 cells, less dead cells were detected in setups exposed to 10 and 50 *T. canis* larvae as well as 10, 100 and 500 *T. cati* larvae compared with controls (Fig. 3G).

LDH activity assay of central nervous cell–Toxocara co-cultures

In undifferentiated CAD cells, LDH activity was enhanced compared with unexposed controls by co-cultivation with 500 viable *T. canis* larvae (Fig. 3B). Less than 500 *T. canis* or *T. cati* larvae did not have a significant effect on LDH release of undifferentiated CAD cells. In contrast, a significantly higher LDH activity compared with controls was detected in supernatants of differentiated CAD cell co-cultures when incubated with 50, 100, 500 *T. canis* larvae or 10, 50, 100 or 500 *T. cati* larvae (Fig. 3D). In supernatants of BO-1 cell–*Toxocara* co-cultures, LDH activity of control and exposure groups was very low (Fig. 3F). Thus, statistical analysis was not feasible. In supernatants of BV-2 cell co-culture setups, LDH activity was not significantly affected by presence of either larval species (Fig. 3H). Larval viability monitored by microscopic inspection did not appear to be influenced by co-culture conditions (Fig. 1). Additionally, LDH activity of *T. canis*- and *T. cati* larval culture supernatants was measured and did not differ from the negative control.

*Brain slice–*Toxocara *co-cultures*

LDH activity of brain slice–*Toxocara* co-culture media on day 6 of co-incubation did not differ significantly from controls. Histological analysis of H&E stained slices showed medium to high numbers of damaged or dying neurons (Fig. 4), macro-and microglia cells, gliosis, proliferation of endothelial cells and single gitter cells in *Toxocara*-exposed and control slices. Larvae were not detected in the tissue. Microscopic evaluation of neuron viability and LDH measurements did not reveal any significant differences between control and exposure groups. LDH activity in brain slices and proportions of damaged neurons in these slices are depicted in Fig. 5.

DISCUSSION

Severe histopathological changes have been described in brains of mice infected with *T. canis*, while animals present with central nervous impairments (Epe *et al*. 1994; Heuer *et al*. 2015). Although lesions have been reported to be predominantly located in the white matter (Summers *et al*. 1983; Dolinsky *et al*. 1985) and demyelination as well as indication of axonal damage have been documented in *T. canis*-infected animals (Epe *et al*. 1994; Janecek *et al*. 2014; Heuer *et al*. 2015), limited

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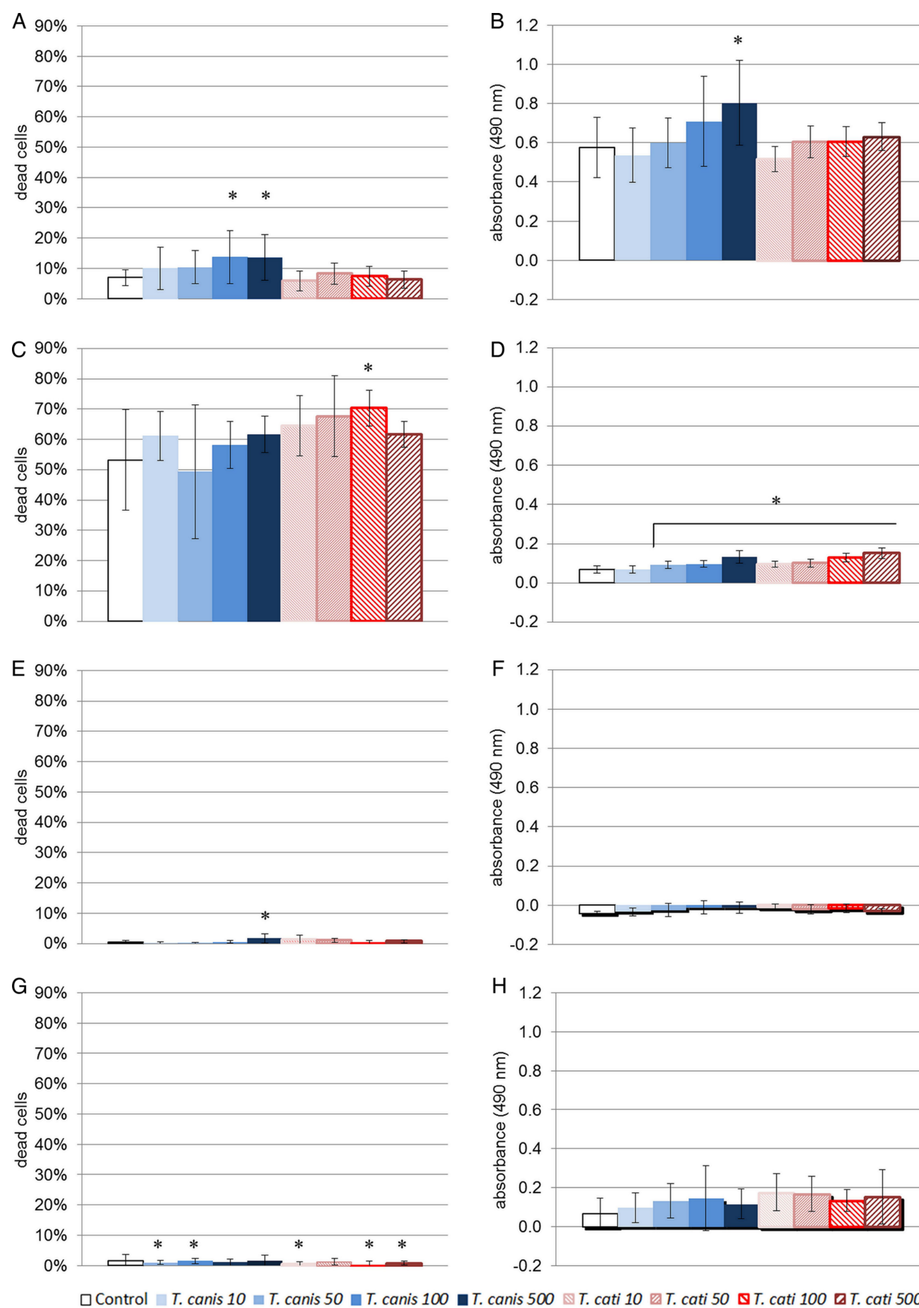

Fig. 3. Trypan blue exclusion assay (A, C, E, G) and LDH release assay (B, D, F, H) of undifferentiated CAD cells (A, B), differentiated CAD cells (C, D), BO-1 cells (E, F) and BV-2 cells (G, H) 5 days after exposure to 10, 50, 100 and 500 viable *T. canis* and *T. cati* larvae, respectively. Differentiated CAD cells appear to be most affected by larval presence of both *T. canis* and *T. cati*. Error bars indicate standard deviation (S.D.), asterisks statistically significant differences ($P \leq 0.05$).

information about the susceptibility of individual CNS cell types to *Toxocara*-mediated damage is available to date. Cell culture-based approaches to unravel neuropathology caused by this parasite are scarce. The only CNS cell type investigated in the context of neurotoxocarosis is the astrocyte, where *T. canis*-TES has been found to trigger apoptosis (Hsiao *et al*. 2013). The impact of *T. cati* as well as the reaction of other CNS cell types to TES or *Toxocara* larvae themselves is completely unknown.

Focusing on the histological damage described above oligodendrocytal (BO-1), neuronal (CAD) and microglial (BV-2) cell lines as well as brain slice cultures were selected for co-cultivation with viable *Toxocara* larvae. With the successful establishment of a co-culture of *T. canis* or *T. cati* larvae with murine CNS cell lines or brain slices, respectively, a tool for research on cell-specific effects of larval presence *in vitro* is provided. Brain slice cultures in particular offer the possibility to investigate parasite-host interplay in three-dimensional, organotypic tissue organization (Stoppini *et al*. 1991, 2000). Typically, perinatal brain tissue is used for cultivation as it shows enhanced viability (Kim

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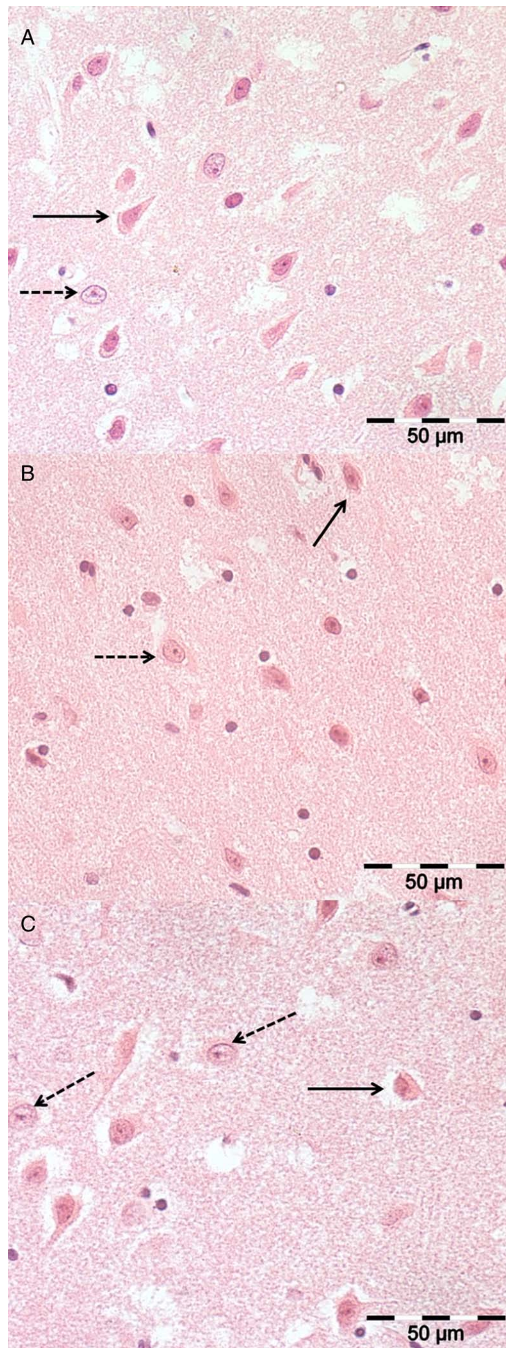

Fig. 4. Viable and damaged neurons in control (A) and brain slices infected with 100 *T. cati* larvae (B) and 100 *T. canis* larvae (C) (H&E stain). Dashed arrows indicate viable neurons with round cores and nuclei. Continuous arrows indicate damaged neurons with swollen or shrunken eosinophilic cytoplasm. Scale bars represent 50 μM (A–C).

et al. 2013). Nevertheless, this study aimed to further simulate natural conditions by use of adult mouse brain slices, as neurotoxocarosis in paratenic hosts is a chronic disease process resulting generally from postnatal infections. Thus, the use of perinatal brain slices for researches of the adult brain is controversial (Kim *et al*. 2013).

To investigate potential cytotoxic effects of larval presence, release of LDH into the culture medium was employed in brain slice and CNS cell co-cultures. Additionally, trypan blue staining were performed on CNS cell co-cultures as usage of more than one assay method has been recommended in cytotoxicity testing to avoid experimental artefacts (Ciapetti *et al*. 1998). Trypan blue exclusion stain was chosen in addition to the LDH assay as sometimes simple, inexpensive manual techniques are less prone to interferences than enzymatic kits (Weyermann *et al*. 2005). Indeed, results differed between the two methods used in all the examined cell types. Although the trypan blue exclusion test is commonly used for routine viability checks in cell culture, its downfalls are time-consuming manual counting, investigator's bias and sampling errors (Uliasz and Hewett, 2000). For brain slices, LDH activity coincided with microscopic evaluation of neurons in H&E stains. Furthermore, LDH activity measurement has previously been described as a reliable method to quantify cell death in neurons (Koh and Choi, 1987), oligodendrocytes, microglia (Lyons and Kettenmann, 1998) and brain slices (Mozes *et al*. 2012). Therefore, LDH assay results were considered sustainable.

Depending on cell lines and media used, results from trypan blue staining differed significantly from those obtained by LDH activity measurement. In differentiated CAD cells, for example, the groups *T. canis* 50, 100, 500 and *T. cati* 10, 50 and 500 did not show statistically significant differences to controls in the dye exclusion test, whereas they did in LDH measurements. Such false negative results of trypan blue stains have been reported previously (Krause *et al*. 1984). Cell type-dependent differences and staining of serum protein in the media have been described (Black and Berenbaum, 1964; Strober, 2001) and may explain why results of manual cell counts for BV-2 cells, a model for the brain's resident microglia (Henn *et al*. 2009), and undifferentiated neuronal CAD cells, grown in serum-containing media, cannot or just partially be reproduced in the LDH assay. Furthermore, LDH tests have been shown to detect early events of cell injury, which cannot be detected by trypan blue stain (Mitchell *et al*. 1980). This may serve to explain why in differentiated CAD cells almost all parasite exposed groups showed a significantly higher LDH level than controls, whereas in the dye exclusion test only one group (exposed to 100 *T. cati* larvae) revealed significantly more stained cells. Overall, the LDH assay appears to be a feasible test for parasite-induced cytotoxicity.

Looking at the effect of viable *T. canis* or *T. cati* larvae on the different cell types in detail, viability of microglial and oligodendrocytal cells did not appear to be significantly affected. As *in vivo* oligodendrocytal damage occurs weeks after larvae have entered the brain, the lack of an effect may be due to the short incubation period of 5 days. On the

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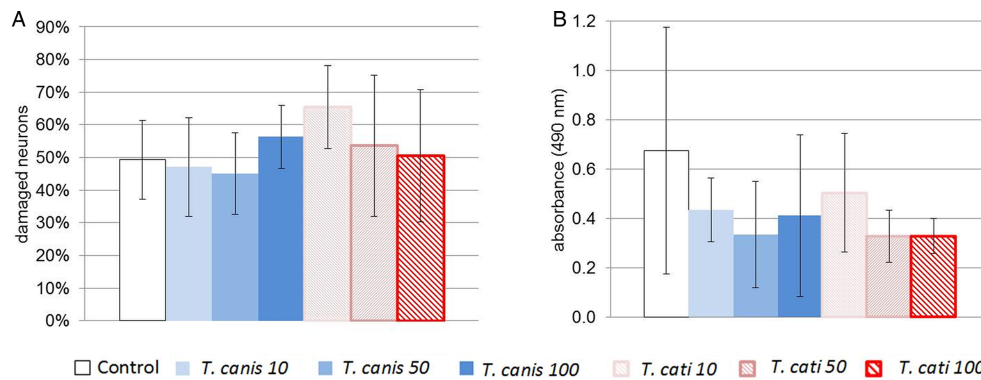

Fig. 5. Analysis of cell death and damage in brain slice–*Toxocara* co-cultures after 6 days of incubation with 10, 50 and 100 *T. canis* or *T. cati* larvae, respectively. Microscopic evaluation of damaged neurons in H&E stained slides (A) and LDH activity in co-culture media (B) did not reveal any significant differences of *T. canis*- or *T. cati*-exposed brain slices compared with controls. Error bars indicate S.D..

other hand, the relation of larvae to cells and TES concentration were certainly higher in this *in vitro* assay than in an *in vivo* infection. Eight weeks post infection (pi) with 1000 *T. canis* larvae, Epe *et al.* (1994) reported a maximum of 110 larvae in the entire brain of C57Bl6 mice. Janecek *et al.* (2014) detected a maximum of 36·3 *T. canis* larvae (day 42 pi) and 9·4 *T. cati* larvae (day 98 pi) in the CNS of C57Bl6 mice in an infection setting with 2000 embryonated *T. canis* or *T. cati* eggs. Therefore, the lack of compromised viability in BO-1 cells could also indicate, that oligodendrocytal damage *in vivo* is not the primary result of TES exposure. However, the methods used only detect cellular damage associated with impairments of membrane integrity. Further research is needed to evaluate potential intracellular effects, which might lead to apoptosis in the long term. In contrast, the lack of an effect on microglial (BV-2) cells and on brain slices appears to demonstrate that the histopathological picture presented *in vivo* with gitter cell accumulations and demyelination (Janecek *et al.* 2014; Heuer *et al.* 2015) is neither a direct effect of TES nor a very local primary immune response to such. In the light of these findings, *in vivo* pathology appears to be the result of other processes, e.g. chronic trauma responses to migrating larvae as well as more extended or even global immune-mediated brain damage. Another explanation might be that TES-mediated neuronal damage and axonal loss could lead to secondary demyelination and subsequent activation of microglia and this process might just take more than 6 days.

Although histological examination of brain slices co-cultured with *Toxocara* larvae did not reveal a clear predilection of any cell type to TES-mediated cytotoxicity and evaluation of neuronal survival did not reveal differences to controls, further research is needed to understand the mechanisms of a possible *Toxocara*-associated neurotoxicity. As the histopathological alterations observed *in vivo* (Epe *et al.* 1994; Janecek *et al.* 2014; Heuer *et al.* 2015) could not be reproduced in the brain slice model, the system cannot serve as a full replacement for animal trials in neurotoxocarosis research. Additionally, it cannot be determined whether the neuronal damage observed is due to brain slice generation or parasitic effects. In contrast, other studies using neonatal hippocampal rat brain slices and protozoan parasites, such as *Toxoplasma gondii* (Scheidegger *et al.* 2005), *Neospora caninum* (Vonlaufen *et al.* 2002) and *Trypanosoma brucei brucei* (Stoppini *et al.* 2000), highlight the usefulness of the model for the study of parasite–host interactions. Stoppini *et al.* (2000) propose incubation of organotypic brain slices in the presence of proinflammatory agents for trypanosomiasis research, as the CNS pathology is predominantly an inflammatory process. Molecular and histopathological examinations on neurotoxocarosis draw a similar picture with immune-mediated factors and traumatic injuries playing an important part in pathogenesis (Epe *et al.* 1994; Janecek *et al.* 2014, 2015). Therefore, further optimization of the *Toxocara* brain slice co-culture with injection of larvae into the slice and administration of proinflammatory agents might aid in simulating a broader spectrum of the disease's aspects and natural route of exposure to TES.

However, the presence of 10, 50, 100 and 500 *T. cati* as well as 50, 100 and 500 *T. canis* larvae has a cytotoxic effect on differentiated CAD cells. Undifferentiated CAD cells are only affected by 500 *T. canis* larvae, suggesting an enhanced susceptibility of TES-mediated cytotoxicity of differentiated over undifferentiated CAD cells and overall, of the neuronal cell line over microglial and oligodendrocytal ones. Interestingly, *T. cati* and *T. canis* larvae reveal a similar cytotoxic potential in differentiated, primary neuron-like, CAD cells, although *T. canis* larvae cause more extensive parenchymal brain damage *in vivo* (Janecek *et al.* 2014;

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Heuer *et al*. 2015). Therefore, long-term effects of *T. cati* larvae in the host's brain should not be underestimated as they appear to mediate neurotoxic effects.

Concluding remarks

The successful establishment of a co-culture system of *Toxocara* larvae with different murine cell lines and brain tissue culture enables further molecular and cell type-specific research on this zoonotic pathogen. Although the brain slice co-culture does not appear to be a satisfying substitute for *in vivo* experiments to date, optimization of this system might lead to its employment for specific questions regarding neurotoxocarosis. Observed cytotoxic effects of both *T. canis* and *T. cati* on murine neuronal cells highlight their destructive potential and raise more questions about consequences of their presence in the human brain. Therefore, *in vitro* approaches leading to a deeper understanding of the molecular mechanisms of this disease are highly desirable as long-term effects of human infections with *Toxocara* spp. as well as their impact on public health are still poorly understood.

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