Interleukin 17-Producing γδ T Cell Induced Demyelination of the Brain in Angiostrongylus Cantonensis Infection

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Research

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Abstract

**Background** Angiostrongylus cantonensis infection is a typical cause of eosinophilic encephalitis (EM), which has been reported to induce serious damage in the central nervous system. Both parasite and host factors contribute to the onset of EM, but the related immune-inflammation pathogenesis remains poorly characterised.

**Methods** An *A. cantonensis* infection model was generated through infection of mice by gavage. Transmission electron microscopy and immunohistochemistry were used to assess the pathologic changes in the brain. The mRNA expression of inflammatory factors was tested using qRT-PCR. A combination of flow cytometry and western blotting was used to evaluate the alteration of leukocytes and related cytokines. A critical role of IL-17 was found by injecting IL-17A monoclonal antibody into naïve and *A. cantonensis*-infected mice.

**Results** *A. cantonensis* larvae altered the immune homeostasis in the brain, leading to the destruction of myelin sheaths and activation of microglia. During this process, IL-17A accumulation was observed, and IL-17RA was expressed in oligodendrocytes and microglia during the infection. Notably, γδ T cells were the origin of IL-17A production induced by the parasite. After an IL-17A-neutralising antibody was applied, alterations in myelination and the state of the microglia were discovered; the neurobehavioral scores of the mice also improved.

**Conclusion** Our study reveals a previously unrecognised impact of the IL-17⁺ γδ T cells in parasitic encephalopathy and emphasizes that blocking IL-17A signalling can attenuate microglial activation, thus reducing CNS demyelination and ameliorating the behavioural deficit in *A. cantonensis*-infected mice.

**Background**

Angiostrongyliasis is a food-borne parasitic disease caused by *Angiostrongylus cantonensis* and mainly occurs in Asia and the Pacific islands [1]. It is thought that humans are infected by *A. cantonensis* by consuming intermediate hosts or vegetables that contain the third stage larvae [2]. The infective larvae invade through the small intestine, spread through the blood circulation, and then damage the heart and lungs, which results in enteritis, cough, and fever. As both mice and humans are non-permissive hosts, *A. cantonensis* ultimately migrates to the host brain, leading to eosinophilic encephalitis or meningoencephalitis [3]. Previous research has demonstrated that *A. cantonensis* infection induces serious central nervous system (CNS) damage, and anti-helminthic treatment alone had no noticeable effect on the recovery of neurological symptoms [4]. Infiltration of lymphocytes, macrophages, eosinophils, and neutrophils is a hallmark of angiostrongyliasis and has also been reported in human cases, where intense inflammatory responses occur in the brain [5]. However, the actual impact of the immune response to *A. cantonensis* remains poorly characterised, which underlines the need to examine the pathogenesis in more detail.
Among numerous cytokines, we focused on interleukin 17A (IL-17A) because IL-17A is important in multiple inflammatory encephalopathies, such as experimental autoimmune encephalomyelitis (EAE), ischemic brain injury, and cerebral trauma [6, 7]. Injection of exogenous IL-17A into the brain can induce neuroinflammatory responses and activate glial cells, which indicate that brain cells express IL-17 receptor A/C (RA/C) complexes [8]. Furthermore, IL-17A participates in neutrophil recruitment, autoimmune disease, and extracellular pathogen immunity [9]. In the neuropathic pain model of partial ligation of the sciatic nerve, inflammatory infiltration of a damaged spinal cord is alleviated following IL-17 gene knockout [10]. Sources of IL-17A are abundant, including TH-17, γδ T, NK, and LTi cells [11]. A very small number of leukocytes is present in the naïve mouse brain, which are activated and accumulate upon pathogen intrusion, autoimmune encephalopathy, or stroke [12].

γδ T cells make up a small fraction of T cells [13]; they are enriched in the skin, small intestine, lungs, and reproductive organs, and they are particularly enriched in the epidermal lymphocytes of the skin and the epithelial lymphocytes of the mucosal tissues [14]. γδ T cells are innate immune cells, which can directly induce immune responses to causative agents. Moreover, γδ T cells can promote the progression of EAE by reducing the immunosuppression of regulatory T (T-reg) cells and enhancing the functions of αβ effector T cells [15]. IL-17 is a major factor secreted by γδ T cells. IL-17+ γδ T cells cooperate with TH-17 cells and form an amplification loop, which continues to release IL-17 throughout the early stage of cerebral inflammation [16]. Important data have highlighted a critical role for microglia in induced myelin damage in the model of multiple sclerosis (MS) and neuronal apoptosis in the cerebral ischemia reperfusion model Moreover, microglia can receive stimulation of IL-17 from other cells and secrete IL-17 in response to IL-1β and IL-23 [17] [18].

Here, inspired by their crucial pathological roles in brain inflammation, we hypothesized that IL-17+ γδ T cells might also contribute to cerebral lesion in A. cantonensis infection. We show that IL-17+ γδ T cells and microglia are activated after infection, and this is followed by obvious marked CNS demyelination. Application of IL-17A neutralising antibody distinctly attenuated demyelination, and microglial activation was simultaneously decreased. These results indicate that disrupting IL-17A function could be a therapeutic target to treat the demyelination associated with neuroinflammatory conditions.

Methods

A. cantonensis infection and anti-IL-17A antibody treatment Male BALB/c mice (aged 5–8 weeks) were purchased from the Animal Center Laboratory of Sun Yat-Sen University (Guangzhou, China). The Institutional Animal Care and Use Committee approved all animal procedures. Larvae III (L3) of A. cantonensis were collected from Biomphalaria glabrata, which were digested and homogenised by placing in a pepsin-HCl solution and incubating at 37 °C for 2 h. L3 were washed from the snail sediment with phosphate-buffered saline (PBS) and counted under an anatomical microscope. Experimental group mice were infected with 30 A. cantonensis L3 by gavage. All mice were housed in the same room and were randomly divided into experimental (n = 5) and control (n = 5) groups. The experimental group and control group were then treated with IL-17A-neutralising antibody (0.05 mg/kg/day, eBioscience) or
immunoglobulin G1 (IgG1) isotype control (clone MOPC-21) for 3 weeks. Intraperitoneal injection antibody was discontinued 3 days before parasitic infection to avoid off-target effects of antibiotics.

**Neurobehavioral test** Neurological assessments for motor and sensory function were performed by staff blinded to group assignments. Motor function tests comprised freedom of movement (open field test), limb symmetry (tail suspension test), climbing (climbing ability on a reversed mesh plate), and balance (stability on a 2 cm diameter rod). Sensory function tests included proprioception; tactile reaction (bilateral stimulation of the neck); and visual (tip to the eye with a swab), olfactory (lemon juice stimulation), and pain responses (needle stab to the palm). Each function corresponded to one test. Every test was graded on a scale from 0 to 3, and the total score for all items was out of 24 (for scoring details please see [19]). Dead mice were assigned a score of 0. The neurological function score of mice was quantified in a double-blinded manner.

**Histology and immunofluorescence** Mice were anaesthetised with isoflurane and perfused transcardially with ice-cold PBS followed by 4% paraformaldehyde (PFA). After fixing with PFA, 10 µm brain sections were cut at -20 °C and mounted on glass slides. Next, sections were permeabilised with 0.3% Triton X-100 and blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h before incubation with primary antibody in 1% BSA at 4 °C overnight. Sections were washed three times in PBS, incubated with fluorescence isothiocyanate-labelled secondary antibody, diluted 1:500 in 1% BSA at 37 °C for 1 h, and washed again in PBS. DAPI (1:1000 dilution, Beyotime Biotechnology, 4083S) was then applied for 5 min to stain nuclei. Slides were observed under a fluorescence microscope. The antibodies used to detect the cells were as follows: rabbit anti-iNOS (1:100, Abcam, ab95866), rabbit anti-Arg-1 (1:200, eBioscience, 17369782), mouse anti-Iba-1 (1:200, Novus, NB100-1028 ), mouse anti-CC1 (1:300, Millipore,OP80), and rabbit anti-IL-17RA/CD217 (1:100, Absin, abs124310). Specimens stained without the primary antibody were used as negative controls.

**RNA isolation and real-time quantitative polymerase chain reaction (PCR)** Total RNA was extracted from the brain using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). For cDNA synthesis, mRNA was reverse-transcribed using the Prime Script RT reagent kit (TaKaRa). cDNA was amplified on an ABI Prism 7900 HT cycler. The cDNA was then used for performing real-time quantitative PCR using the SYBR Premix Ex Taq kit (TaKaRa). The relative expression level of mRNA was determined using the 2^{ΔΔCt} method. mRNA levels were measured using the following specific primer sequences: iNOS, 5'-ACCTTGTTCAGCTACGGCTT-3' and 3'-CATTCCCATAATGTGGTTC5'; CD86, 5'-GCTATAGCAAGGTCAAGGCGAAAC-3' and 3'-CTAGAAGACACACAAGGCTCAA-5'; CD206, 5'-GCATGAGGGGTGGATAGAG-3' and 3'-GAGAAGACAGACAGAGGAGACG-5'; CD11b, 5'-CAGGAGAGCAAGCTCAACTCCACGGTCA-3' and 3'-AGTGGTCAACTCCACGGTCA-5'; Arg-1, 5'-TGCTGCTTGTGTGATGC-3' and 3'-GGAACCTCAACGGGGAGCTAAC-5'; IL-17, 5'-TCATGTGGTGGGTCCAGCCTT-3' and 3'-CTGAGACTACCTCAACCGTCC-5'; and IL-17RA, 5'-GTGGTCTTTTCAACTCCTTC-3' and 3'-CCAAGGCTGTGCTGGTTAGTT-5'. Expression of the gene of interest was normalised to that of the housekeeping gene GAPDH (reduced glyceraldehyde-phosphate dehydrogenase).
**Western blotting** Brain tissue was washed twice with cold PBS and lysed in extraction buffer (20 mM HEPES [pH 7.4], 2 mM EDTA, 50 mM glycerophosphate, 1 mM dithiothreitol, 1 mM Na3VO4, 1% Triton X-100, and 10% glycerol) on ice. The lysates were centrifuged at 500 x g for 15 min. Supernatants were collected, following which protein concentrations were determined using bicinchoninic acid protein assay. Proteins were heated with sample buffer, separated in 12% sodium dodecyl sulfate-polyacrylamide gels by electrophoresis, and electroblotted onto a nitrocellulose membrane. Transferred blots were incubated sequentially with a blocking agent (5% non-fat milk in Tris-buffered saline). Anti-iNOS (1:125 dilution, Abcam), anti-Arg-1 (1:500 dilution, Abcam), and secondary antibody blots were developed using the enhanced chemiluminescence detection kit on Hyperfilm (Fuji) according to the manufacturer's directions. The same blots were subsequently stripped and reblotted with an antibody of β-tubulin to verify equal protein loading. Graphs of blots were obtained in the linear range of detection and protein levels were quantified using ImageJ software (NIH).

**Transmission electron microscopy** Following anaesthesia, the animals were euthanised by transcardial perfusion with 4% PFA. Brain tissues were crosscut into 15 µm sections at -20 °C and mounted on glass slides. The corpus callosum was rapidly dissected and post-fixed overnight in 2.5% glutaraldehyde. Next, corpus callosum fragments were post-fixed in a solution containing 1% osmium tetroxide and then dehydrated using an acetone gradient. Dehydrated samples were embedded in SPIN-PON resin, which was polymerised at 60 °C for 3 days. Semi-thin sections (0.5 µm thickness) were placed on glass slides and stained with toluidine blue. Finally, the corpus callosum was observed under a 300KV transmission electronic microscope. Three grids per sample and 10 photographs per grid were acquired. Myelin sheath thickness and axon diameter were measured in a double-blinded manner by ImageJ software, and the g ratio was calculated by dividing the axon diameter by that of the nerve tract.

**Luxol fast blue staining** Brain samples were evaluated by LFB staining at several time points post-infection. The brain tissue was dehydrated in a gradient (100% and 95%) ethanol series, cleared in xylene, and embedded in paraffin. Serial brain transverse cryosections (20-µm thick) were cut on an RM 2035 microtome (Leica). The sections were then stained with LFB staining solution (Sigma, 0.1% LFB in 95% ethanol with 0.5% acetic acid) at 60 °C, rinsed in 95% ethanol, and finally differentiated in 0.05% lithium chloride solution. The staining images in the corresponding CNS areas between control and infections were obtained under an inverted microscope equipped with a camera (DM 2500B, Leica).

**Flow cytometry** Single cell suspensions of brain tissue were processed by digestion for 40 min at 200 rpm and 37 °C with type IV collagenase (1 mg/ml, Sigma-Aldrich) and DNase I (100 µg/ml, Roche) in 5% fetal bovine serum RPMI1640 solution, followed by filtration of tissue suspensions through a 70 µm strainer. 7 ml of cell suspensions and 3 ml of 100% Percoll (final concentration 30% Percoll) were mixed. The mixture was then overlaid on 2 ml of 70% Percoll and centrifuged at 500 x g for 30 min at 18 °C, with minimal deceleration. Cells of grey mist were removed from the 30–70% interface with a Pasteur pipette and washed twice with PBS containing 5% serum. Cells were incubated with Fc block (CD16/CD32) to avoid nonspecific binding and then stained with fluorescence-conjugated anti-CD11b (M1/70), anti-CD45 (30-F11), anti-T cell receptor (TCR) γδ antibodies (GL3, all from eBioscience). For IL-17A intracellular
staining, cells were stimulated for 5 h at 37 °C with phorbol myristate acetate (PMA; 1 µg/ml) and ionomycin (1 µg/ml) and for 3 h at 37 °C with brefaldin A (BFA, 10 µg/ml). Cultured cells were collected and washed with PBS and sequentially stained with fluorescence-conjugated anti-IL-17A antibody (eBio17B7; eBioscience). Fixable viability dye EF780 (65086514; eBioscience) was used for dead cell exclusion. Samples were analysed using flow cytometry with a CytoFLEX (BECHMAN COULTER) and data were handled using FlowJo10 software (Tree Star).

Quantification and statistical analysis Randomisation of mice was based on the random number generator function in Microsoft Excel. Lesion pixel counts and area quantification for western blotting results were performed using ImageJ. GraphPad Prism 8.0 was used to statistically compare the data on myelin sheath thickness, real-time quantitative PCR, and western blot graphs between different groups. Data are expressed as the mean ± SEM and were analysed using two-tailed t-test or one-way ANOVA and Tukey's test, as appropriate. p < 0.05 was considered statistically significant.

Results

Demyelination and microglial activation were observed in the brain tissue of mice during A. cantonensis infection

Eosinophilic encephalitis is by far the most extensively reported pathological change of A. cantonensis infection [20]. However, we observed that a number of mice appeared to experience partial paralysis and death due to serious brain parenchyma injury during the late stage of infection. To gain further insights into the potential mechanisms responsible for motor dysfunction, we focused on the state of axons in the brain. Transmission electron microscope (TEM) images of the corpus callosum showed noticeable axon demyelination at 14 days post-infection (dpi), which was more pronounced at 21 dpi. Myelin sheath structure became incompact and thickness continuously decreased. The myelin g ratio, which was calculated by dividing the axon diameter by that of the nerve tract, was higher in infected animals than that in the controls. The change in g ratio was contrary to the myelin thickness, and the axon arrangement became disordered (Fig. 1a). A. cantonensis infection induced not only demyelination injury but also an inflammatory cytokine storm. Previous studies have shown that cerebral injuries occur due to mechanical damage caused by parasite movement; however, a recent study has evidenced that inflammatory responses may be more important [1].

Flow cytometric analysis of brain immune cells revealed increased infiltration of blood-borne cells (CD45+) at 7 dpi. Although the number of leukocytes increased, the number of microglia did not show notable change (Fig. 1b). However, we detected the expression pattern of cell types that might be involved in eosinophil recruitment. Real-time qPCR results showed increased mRNA levels of markers for M1 (CD86, iNOS, and CD11b) and M2 (CD206, Arg-1, and YM1) microglia in the infected brain tissue [21], with the highest levels at 21 dpi (Fig. 1c). We also detected the protein levels of iNOS and Arg-1. iNOS expression continually increased and peaked at 21 dpi, whereas high levels of Arg-1 expression started from 14 dpi and continued to increase until 21 dpi (Fig. 1d). There were more Iba-1+iNOS+ M1 and Iba-
$1^\text{Arg-1}^+$ M2 cells in the brains of infected mice than those of the control mice, particularly around the corpus callosum (Fig. 1e). Collectively, these results indicate that *A. cantonensis* infection can lead to both M1 and M2 microglial activation but may not be sufficient to promote microglial proliferation.

**IL-17A and IL-17RA expression levels increased considerably in the brain following *A. cantonensis* infection**

To evaluate the impact of inflammatory factors on the brain, we compared the levels of interleukins in *A. cantonensis*-infected versus control mice injected with normal saline. We previously described how the expression of certain interleukins changed during *A. cantonensis* infection [22], but we did not explore their effect in damage to the brain and inflammatory responses. Among several interleukin types, we focused on IL-17A, as it has been reported to be implicated in EAE and MS [23]. The transcriptional levels of IL-17A and IL-17RA in the brain were greater in *A. cantonensis*-infected mice than those in control mice (Fig. 2a). IL-17A protein production was detected at low levels in the first few days of infection. Intracellular cytokine staining confirmed that *A. cantonensis* induced IL-17A production, and the content of IL-17A increased with the duration of infection (Fig. 2b). We noticed that there were some differences in IL-17A levels at 21 dpi, which may be ascribed to resistance to *A. cantonensis*. Some mice showed serious cerebral haemorrhaging and even died, but the others only showed abnormal motor functions.

The location of IL-17RA within the brain was determined using immunofluorescence staining. Some amount of IL-17RA was observed on the oligodendrocytes, especially on the axons. This change was particularly pronounced around the corpus callosum (Fig. 2c). IL-17A can influence oligodendrocyte lineage cell proliferation and differentiation through multiple pathways mediated by IL-17RA in inflammatory diseases [24]. In addition, the microglia, which are a type of macrophage, have been known to secrete IL-17 and can be activated by IL-17. It is still unclear whether IL-17 plays a role in microglial activation during *A. cantonensis* infection. Similarly, IL-17RA was detected in Iba-1$^+$ microglia (Fig. 2c), suggesting a possible link between IL-17A and microglia. However, another important group of glial cells, the astrocytes, were devoid of IL-17RA (data not shown). It has previously been reported that microglia secrete functional cytokines, such as IL-1$\beta$, IL-6, and TNF$\alpha$, when co-cultured with IL-17-producing Th1/Th17 cells [25]. Overall, our results demonstrate the relevance of IL-17A to the brain injury caused by *A. cantonensis* infection.

**γδ T cells were the major source of IL-17A in the *A. cantonensis*-infected mouse brain**

To determine the source of IL-17A following infection with *A. cantonensis*, we stimulated cerebral leukocytes *ex vivo* with PMA, BFA, and ionomycin, and stained the intracellular IL-17A. There was a small proportion of CD4$^+$ CD8$^+$ T cells or macrophages that was IL-17A$^+$ (data not shown), whereas IL-17$^+$ γδ T cells accounted for the majority of IL-17$^+$ leukocytes after 14 dpi (Fig. 3a). The high diversity of γδ TCR, major histocompatibility complex-independent and antigen-independent processes and presentation suggest that γδ T cells may be the first line of defence against infection. However, only a few γδ T cells were detected in the naive mouse brains. γδ T cells often appear in mucosal immunity but rarely in CNS
neuroimmunity, similar to IL-17A, and this cell has been most extensively studied in stroke and EAE [26]. The frequency of brain γδ T cells increased considerably during the late stage of infection, compared to that in the sham-operated mice. Moreover, approximately 60% of γδ T cells expressed IL-17A (Fig. 3b). The level of several important cytokines decreased and the total number of B and T cells declined at 21 dpi. It has been reported that *A. cantonensis* infection leads to the immunosuppression of mice [27]. Nevertheless, the infection status of the brain continued to worsen, possibly indicating that a class of cells including γδ T cells continued to contribute to brain injury.

To determine whether the functional γδ T cells originate directly from peripheral lymphoid organs, we detected the level of IL-17+ γδ T cells in the thymus and spleen during infection. The thymus is the source of γδ T cells, part of γδ T cells acquire their specialized functions before leaving the thymus [29]. We tested the levels of γδ T and IL-17+ cells in the thymus during infection and the results was different with the brain. The peak number of γδ T cells was at around 7 dpi. In contrast, the amount of IL-17+ γδ T cells in the thymus did not change appreciably even in the later phase of infection. Moreover, thymus morphology showed evident atrophy at 21 dpi (data not shown). The spleen as another lymphoid organ presented an immunosuppressive state after infection, with both T cell and γδ cell numbers falling sharply at 21 dpi. The numbers of IL-17+ γδ T cells only rose at 7 dpi, which may be due to the small intestinal infection (Fig. 3c). To create a better environment for survival, *A. cantonensis* suppresses the body's immunity function over a prolonged period of time. We speculate γδ T cells owned the corresponding function after migrating to the brain lesions caused by *A. cantonensis*.

**Microglial activation weakened and demyelination was relieved after IL-17 neutralisation**

We next tested whether suppression of IL-17A in *A. cantonensis*-infected mice was sufficient to provide a neuroprotective effect. IL-17A is involved in promoting the survival, activation and recruitment of other inflammatory cells by regulating cytokine and chemokine expression in several neuroimmune responses [30]. To evaluate the impact of IL-17A, we neutralised IL-17A by injecting specific blocking monoclonal antibodies (mAbs) through the intraperitoneal route (Fig. 4a). Because myelin damage causes impaired motor function, the neurobehavioral scores of mice were evaluated in each group. We found that the *A. cantonensis*-infected group got lower scores than the control group, but IL-17 neutralising antibody attenuated this effect (Fig. 4b). Both Luxol Fast Blue (LFB) staining and TEM were applied to examine the myelin sheath condition. At 21 dpi, demyelination of infected mice with IL-17A suppression was clearly relieved (Fig. 4c). Given the relationship between demyelination and neurobehavioral scores, we proposed that the effect of IL-17A damage on myelin during infection cannot be ignored.

Previous research has demonstrated that inhibiting the activation of the microglia using minocycline can effectively relieve the injury from *A. cantonensis* [31]. We therefore assessed the condition of the microglia after IL-17 inhibition. The mRNA expression levels of CD86, iNOS, CD11b, Arg-1, and YM1 were distinctly decreased in *A. cantonensis*-infected mice treated with the inhibitor (Fig. 5a). We obtained similar results when iNOS and Arg-1 protein levels were detected (Fig. 5b). Moreover, the active state of microglia generally performs ameboid; this shape was also not detected (Fig. 5c). These findings in *A.
cantonensis-infected mice indicate that microglial activation is involved in the effect of IL-17A on demyelination.

**Discussion**

The present study provides evidence that IL-17A expression was dominant in the brain during the late stage of *A. cantonensis* infection. IL-17A was mainly secreted by γδ T cells, and played an important role in the process of demyelination and microglial activation. Inhibiting IL-17A may therefore serve as a route for therapeutic intervention by dampening microglial activation in inflammatory demyelination. This research is also a continuation of our previous study where we demonstrated that IL-17A is involved in eosinophil accumulation and the NF-κB /Traf6 signalling pathway [32]. The brain is the most severely damaged organ during the migration of *A. cantonensis* through the body [33]. Eosinophil infiltration in the meninges, as the most outstanding characteristic, has been studied extensively [34]. A better prognosis was achieved by applying an anti-inflammatory drug combination than by using an antiparasitic drug alone [35].

The central nervous system was previously considered to be an immune-privileged area because the blood-brain barrier blocks immune cells and cytokines from entering the brain [36]. However, recent studies have demonstrated that the nervous system also has inherent and adaptive immunity, particularly since rich lymphatic vessels have been found in the sinus durae matris [37]. IL-17 is regarded as a proinflammatory factor, whose role in brain inflammation-linked diseases has been considerably discussed [9, 29]. There are different perspectives on the relationship of IL-17 to demyelination. It has been reported that the severity of EAE in IL-17−/− mouse models is reduced due to a decrease in MOG-specific T cell sensitisation [38]. Act1 (NF-kB activator1), as a key element of the IL-17 signalling pathway, influences the progress of EAE. Knockout of the Act1 gene in NG2+ glial cells leads to severe myelin damage [39]. However, the degree of inflammatory damage in IL-17A−/− mice changes only slightly after receiving antigen stimulation, which suggests that the immune activity of IL-17A in the EAE model is redundant [40]. Similar experiments have been conducted in homozygous mutations of IL-21 and IL-22 [41].

In our study, there was a considerable change in IL-17A expression level in *A. cantonensis*-infected mice; accordingly, we tested whether an IL-17A-neutralising antibody could alleviate brain injury, and multiple results indicated that this suppression was effective. Moreover, we observed IL-17A receptor expression in oligodendrocytes and microglia, which indicated some association with them. The attack of IL-17A on oligodendrocytes can be direct without the mediation of other cells, not only resulting in cell damage but also inducing cell apoptosis [7].

Microglia can be activated by IL-17A and also secrete other cytokines to promote IL-17A production, which forms a positive feedback loop [42]. It has been reported that the suppression of IL-17 decreases the M1/M2 microglia ratio and concomitantly suppresses a bisphosphonate-related osteonecrosis of the jaw-like condition in mice [43]. M1 and M2 microglial activation both occurred in the late stage of *A.
cantonensis infection. We speculate that the main pathological changes after infection were partially attributable to M1 microglial activation, and the state of immunosuppression was attributable to the M2 microglia. Recently, IL-17 has been discovered to control synaptic plasticity and short-term memory, we expect the study of this interleukin to other aspects of brain function, thus defining new neuroimmunology concepts [44].

A new and unexpected finding of our study was the source of IL-17A. It has been reported that Vδ1+ and Vδ2+ γδ T cells accumulate around acute MS plaques and chronically demyelinated areas [45]. γδ T cells are known to enhance Th17 functions by producing IL-17A in an EAE model, and the synthesis of IL-23 from Th17 promotes the activation of IL-17+ γδ T cells [16]. IL-17+ γδ T cells can also reduce the conversion of conventional T cells to Foxp3+ T-reg cells and inhibit the immunosuppressive effect of T-reg cells to enhance autoimmunity [15]. In addition to MS and EAE, the outcome of ischemic brain injury is also affected by γδ T cells. IL-17A, expressed by γδ T cells, recruit neutrophils through promoting CXCL-1 expression in infarcted lesions. Neutralisation of IL-17A relieved the ischemia-reperfusion injury [46]. T-reg cells, which are affected by dysbacteria-like flora, migrate from the intestine to the meninges and secrete IL-10 to suppress IL-17+ γδ T cell differentiation. The reduction of IL-17+ γδ T cells decreases following post-ischemic leukocyte and chemokine infiltration [47]. However, the role of γδ T cells in cerebral lesions caused by helminth infection has rarely been reported. In an experimental cerebral malaria animal model, knocking out the TCRδ gene ameliorated brain swelling and haemorrhage, and protected the blood-brain barrier [48]. TCRδ−/− mice also had a lower number of worms and less severe brain damage following tapeworm infection. The infiltration of inflammatory cells and related cytokines of the type I immune response also decreased in the brain [49].

Our experiments demonstrated that γδ T cells play a role in A. cantonensis infection and are major IL-17A-producing cells. However, our data showed that the change of γδ T cells in the thymus was not as intense as in the brain, and the amount of IL-17+ γδ T cells did not increase during infection. We speculated that a certain amount of γδ T cells are reserved in the brain under normal conditions, and once these γδ T cells sense the parasitic antigens in the in vivo environment they develop appropriate functions [50]. The effector functions of γδ T cells also depend on the type and constitution of TCR segments [51]. It is necessary to explore which γδ T cell subset plays a key role in A. cantonensis infection. Except IL-17A, γδ T cells can secrete other cytokines, such as IL-5 and eotaxin, that directly induce eosinophils to migrate to the site of inflammation, and they also secrete IFN-γ and IL-4 to regulate the activity of αβ T cells and macrophages, thus promoting the chemotaxis of eosinophils [28]. Both IL-5 and eotaxin levels have been observed to clearly increase in A. cantonensis-infected animals [52]. γδ T cells probably have other functions that are worth studying in this model. Overall, our study demonstrated that IL-17A was involved in limiting A. cantonensis, but at the cost of increased brain damage. This could have implications for human immune defence against parasites, and thus, innate-like T cells should be studied further.

**Conclusion**
Studies into the role of γδ T cells in intracerebral parasitic diseases are rare, and those on IL-17+ γδ T cells are even rarer. We present evidence to support a key role of IL-17A in *A. cantonensis* infection. IL-17A secreted by γδ T cells acts on demyelination and microglial polarisation. Specifically blocking IL-17A could be considered a potential therapeutic target for treating demyelination associated with neuroinflammatory conditions involving microgliosis. Our results show that *A. cantonensis* not only cause mechanical injury but also disrupt the cerebral immunological balance and induces a cytokine storm. Future studies should focus on the indirect damage elicited by nematodes to the brain tissue.

**Abbreviations**

CNS
central nervous system; IL-17:interleukin 17; iNOS:inducible nitric oxide synthase; Arg-1:arginase 1;
MS:multiple sclerosis; EAE:experimental Autoimmune Encephalomyelitis; TCR:T cell receptor;
mAb:monoclonal antibody; TEM:transmission electron microscopy

**Declarations**

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Not applicable

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**Availability of data and materials**

The research article data used to support the findings of this study are included within the article.

**Authors’ contributions**

Ying Feng, Zongpu Zhou, Cunjing Zheng, and Feng Feng carried out the experiments and performed the statistical analyses. Ying Feng drafted the manuscript. Zhongdao Wu and Fukang Xie conceived and coordinated the study. All authors read and approved the final manuscript.

**Ethics approval**

All procedures applied to the canines were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and approved by the Laboratory Animal Regulations of Guangdong Province.
Animal welfare was in compliance with Laboratory animal Guideline for Ethical Review of Animal Welfare, General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China/Standardization Administration of China (GB/T35892-2018).

**Consent for publication**

Written informed consent for publication was obtained from all participants.

**Competing interests**

The authors declare that they have no competing interests.

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Figures
Figure 1
Demyelination and microglial activation were observed in the brain tissue of mice during *A. cantonensis* infection (a) Transmission electron microscopy image of corpus callosum from control and *A. cantonensis*-infected mice. Scale bar = 1 μm. The percentage of myelinated axons (middle) and myelin g ratio (right) of control and *A. cantonensis*-infected mice. (b) Flow cytometric gating strategy of brain-infiltrating leukocytes (CD45+) and microglia (CD45intCD11bint). Bottom: quantification of the absolute number of cells for leukocyte and microglia in the infected brains. (c) RNA expression of CD86, iNOS, CD11b, CD206, Arg-1, and YM1 following *A. cantonensis* infection. (d) Western blots showing iNOS and Arg-1 protein levels in control and *A. cantonensis*-infected mice 7, 14, and 21 days post-infection (dpi); relative densitometric analysis of Western blots was represented in the below, as normalised to β-actin and β-tubulin. (e) Immunofluorescence of Iba-1/iNOS and Iba-1/Arg-1 double staining of brain tissue in the control and 21 dpi infection groups. Prominent expression (white arrows) was observed. Scale bar = 100 μm. n = 5 animals/group, *P<0.05, **P<0.01, ***P<0.001, Student’s t-test. Data in each statistical graph are presented as means ± SEM.
IL-17A and IL-17RA expression level noticeably increase in the brain following A. cantonensis infection (a) RNA expression of IL-17A and IL-17RA at 7, 14, and 21 dpi. (b) Flow cytometry result of brain-infiltrating IL-17+ leukocytes (numbers represent events within the gate as a percentage of leukocytes). Below is the quantification of the absolute number of IL-17+ cells for each time point. (c) Immunofluorescence images of CC1/IL-17RA and Iba-1/IL-17RA double staining of brain tissue in the control and 21dpi groups. Co-expression (white arrows) was observed. Scale bar = 100 μm. n = 6 animals/group, *P<0.05, **P<0.01, ***P<0.001, Student’s t-test. Data in each statistical graph are presented as means ± SEM.
Figure 3

γδ T cell is the major source of IL-17A in A. cantonensis-infected mouse brains (a) Representative flow cytometry analysis and (b) quantification of total γδ T cells and IL-17+ brain γδ T cells (CD45highCD11b−γδTCR+IL-17A+) at 7, 14, and 21 dpi. Bottom, graphs depict percentages from indicated populations. (c) Above: absolute number of γδ T cells and IL-17+ γδ T cells in thymus; below: total amount of γδ T cells and IL-17+ γδ T cells in the spleen. Data in each statistical graph are presented as means ± SEM.
IL-17 neutralisation relieved brain demyelination (a) IL-17A neutralising antibody and IgG1 isotype antibody were injected into BALB/c mice intraperitoneally 3 days before infection, followed by five intraperitoneal challenges for 21 days. At 21 dpi, neurobehavioral tests were processed and the brain tissues were collected. (b) Neurological function scores in each group. (c) TEM and LFB images of the
corpus callosum in the control group and experimental group (injected with IL-17A mAb). Black symbol indicates the cerebral medulla change region. TEM scale bar = 0.5 μm, LFB scale bar = 100 μm. Below is the percentage of remyelinated axons in the corpus callosum from mice treated with IL-17A-neutralising antibody and IgG1 isotype antibody, and a scatterplot of g ratio versus axon diameter. n = 5 animals/group, *P<0.05, **P<0.01, ***P<0.001, by Student’s t-test and one-way ANOVA. Data in each statistical graph are presented as means ± SEM.
Figure 5

Microglial activation weakens after IL-17A suppression (a) RNA expression of CD86, iNOS, CD11b, CD206, Arg-1, and YM1 following A. cantonensis infection and injection with IL-17A mAb. (b) Western blots showing iNOS and Arg-1 protein levels of individual groups; relative densitometric analysis of western blots is represented below, as normalised to β-actin and β-tubulin. (c) Immunofluorescence of Iba-1 staining of brain tissue in the 21 dpi infection groups with IgG1 isotype Ab and IL-17A mAb. Scale bar = 200 μm. n = 5 animals/group, *P<0.05, **P<0.01, ***P<0.001, by Student’s t-test and one-way ANOVA. Data in each statistical graph are presented as means ± SEM.