Interleukin 17-producing γδ T cell induced demyelination of brain during *Angiostrongylus cantonensis* infection

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Research

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Abstract

Background: *Angiostrongylus cantonensis* infection is one typical reason 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 remain poorly characterized.

Methods: *A. cantonensis* infection model was generated through infecting mice by gavage. TEM and IF were used to assess the pathologic changes of brain. The mRNA expression of inflammatory factors was tested by qRT-PCR. Combining flow cytometry with western blotting to evaluate the alteration of inflammatory cells and related cytokines. Critical role of IL-17 was found, to evaluate the function of IL-17A, we injected IL-17A mAb to naive and *A. cantonensis* infected mice.

Results: *A. cantonensis* larvae altered immune homeostasis in the brain, led to the destruction of myelin shealth and activation of microglia. IL-17A accumulation was observed, IL-17RA was expressed on oligodendrocyte and microglia during the infection. More important, γδ T cells are the source of IL-17A induced by parasite. After IL-17A neutralizing antibody was applied, alterations of microglia state and myelination were discorved, mice neurobehavioral test also improved.

Conclusion: Our study reveals a previously unrecognized impact of the IL-17^{+} γδ T cells in parasitic encephalopathy and emphasizes that blocking IL17A signaling can attenuate microglia activation, thus reducing CNS demyelination and ameliorate behavioral deficit in *A. cantonensis* infected mice.

Background

Angiostrongyliasis is a food-borne parasitic disease caused by Angiostrongylus cantonensis and prevailingly occurs in Asia and the Pacific islands. Human infect A. cantonensis presumably because of eating the intermediate hosts or vegetables that contain the larvae of the third stage[1]. The infective larvae invade through the small intestine causing enteritis,then travel within the body blood circulation and damage heart and lung, which result in cough and fever. In the suitable host, these parasitic nematode worms migrate to brain, finally leading to eosinophilic encephalitis or meningoencephalitis. We and other researchers demonstrated that A. cantonensis infection induced obvious central nerves system (CNS) damage in mice and only anti-helminthic treatment had no obvious effect. These findings underscore the need to identify more detailed pathogenesis. Infiltration of lymphocytes, macrophages, eosinophils and neutrophils is the dominant pathogenic manifestation of infected brain, not only that, demyelination is a typical injury which brings out limitation of nerve impulse conduction in which animal motor function is bound to be impacted [2, 3, 4].

Interleukin 17A (IL-17A) is an important interleukin in both innate and adaptive immunity, belonged to a member of IL-17family, mainly participating in neutrophil recruitment, autoimmune disease, and extracellular pathogens immunity [5]. As previously reported, multiple inflammatory encephalopathy, i.e., experimental autoimmune encephalomyelitis (EAE), ischemic brain injury, are to varying degrees affected

by IL-17A [6]. Despite this, IL-17A involved pathways have not been fully elucidated and require further study. One research showed that IL-17A expression alone was able to activate glial cells and enhance neuroinflammatory responses, indicating that brain cells express functional IL-17 receptor A/C (RA/C) complexes [7]. Sources of IL-17A are abundant, including TH-17 cells, γδ T cells, NK cells and LTi cells [8].

CNS normally contains a very small amount of T cells,these cells will activate and accumulate when autoimmune encephalopathy or stroke happen. T cell can be divided into two groups according to T cell receptor (TCR), one group is called γδ T cell which has TCR1. TCR1 is composed of γ and δ chain□the later can be further classified by chain types [9]. γδ T cells make up a minor fraction of T cells in lymphoid tissue, but are enriched in the skin, small intestine, lungs, reproductive organs and other mucous membranes and subcutaneous tissues. γδ T cells are part of the epidermal lymphocytes of the skin and the epithelial lymphocytes of the mucosal tissues, which can directly induce immune responses to invading pathogens [10]. γδ T cells belong to innate immune cells, effector function including antiviral infection, anti-tumor immunity, participation of autoimmune diseases, and immune regulation of inflammatory response. However, γδ T cells cause the tissue injury when they are performing function as well [11].

IL-17 and microglia are related to inflammation in the CNS, so inhibiting IL-17 cytokine family function and inactivating microglia could have beneficial effects on pathogenic CNS conditions [12]. Microglia can secrete IL-17 response to IL-1β and IL-23, and receive stimulation of IL-17 from other cells [13]. Microglia have two distinct functional phenotypes: pro-inflammatory (M1) and anti-inflammatory (M2). M1 is classically activated phenotype associated with antigen-presentation properties, secretion of pro-inflammatory cytokines and reactive oxygen and nitrogen species, while M2 secrete anti-inflammatory cytokines and growth factors which have neuroprotective properties [14]. Microglia induce myelin damage in model of MS and neuronal apoptosis in cerebral ischemia reperfusion model [15, 16]. Whether M1 and M2 microglia polarization participates in demyelination induced by A. cantonensis has never been studied.

Here we show that $IL17^+$ γδ T cells and microglia are activated during the A. cantonensis infection, and this is followed by obvious CNS demyelination. Application of IL-17A neutralizing antibody distinctly attenuated demyelination, microglia activation was simultaneously decreased. These results indicate that IL-17A may play a key role in demyelination caused by A. cantonensis, and microglia polarization could be the possible mechanism for the effect of IL-17 on demyelination in Angiostrongyliasis. Specific blocking of IL-17A could be a therapeutic target for treating demyelination associated with neuroinflammatory conditions.

Methods

A. cantonensis larvae infection and anti-IL-17A treatment Male BALB/c mice (aged 5–8 weeks) were purchased from the animal center laboratory at 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 via homogenization and digestion of minced snail tissue that was placed in a pepsin-HCl solution and incubated at 37 °C for 2 h. L3 in the sediment were washed with phosphate-buffered saline (PBS) and counted under an anatomical microscope. All mice were housed in the same room and randomly allocated into two groups: wild type group and A. cantonensis infection group. Additional mice were treated with IL-17 neutralizing antibody (0.05 mg/kg/day, eBioscience) or with immunoglobulin G1 (IgG1) isotype control (clone MOPC-21) for 3 weeks. Administration of 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 encompassed freedom of movement, limb symmetry, climbing, and balance. Sensory function included proprioception; tentacles reaction; and visual, olfactory, and pain responses. Every test was graded on a scale from 0 to 3, and the total for all items was 24 (for scoring details please refer to literature [33]). The neurological function score of each mouse was the sum of the scores on all of the above tests.

Histology and immunofluorescence Mice were anesthetized 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. Then, sections were permeabilized 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 fluorescein isothiocyanate-labeled and tetramethylrhodamine-labeled (for the others) secondary antibody (Abcam), diluted 1:500 in 1% BSA at 37 °C for 1 h, and washed again in PBS. Then DAPI (1:1000 dilution, Beyotime Biotechnology) was applied for 5 min to stain nuclei. Antibodies used to detect cells are as follows: rabbit anti-iNOS (Abcam, ab95866), rabbit anti-Arg-1 (eBioscience, 17369782), mouse anti-Iba-1 (Novus,NB100-1028), mouse anti-CC1(Millipore□OP80), rabbit anti-IL17RA/CD217 (Absin, abs124310). Specimens stained without the primary antibody were used as negative controls. Slides were finally observed under a fluorescence microscope.

RNA isolation and real-time quantitative polymerase chain reaction (PCR) Total RNA was extracted from the cerebrum with TRIzol reagent according to the manufacturer's instructions (Invitrogen). For cDNA synthesis, RNA was reverse transcribed with a PrimeScript RT reagent kit (TaKaRa). The expression levels of genes encoding IL-17, IL17R,CD86, CD206, Ym-1, CD11b, Arg-1, and iNOS were measured by real-time PCR with SYBR Premix Ex Taq kit (TaKaRa). Amplification of cDNA was performed on an ABI Prism 7900 HT cycler. mRNA levels were measured using the specific primers, primer sequences were as follows: iNOS, 5'ACCTTGTTCAGCTACGCCTT-3' and 3'-CATTCCCAAATGTGCTTGTC-5', CD86, 5'-GTCTAAGCAAGGTCACCCGAAAC-3', 3'-TCCAGAACACACACAACGGTCATA-5', CD206, 5'-GCATCAGTGAAGGTGGATAGAG-3' and 3'-TGAGAACAACAGACAGGAGGAC-5', CD11b, 5'-CACGACGAGCACAAGTCCA-AC-3' and 3'-AGTCCTCAACTCCACGGGTCA-5', Arg-1, 5'-TGTCTGCTTTGCTGTGATGCC-3' and 3'-GGAACTCAACGGGAGGGTAAC-5', IL-17, 5'-TCATGTGGTGGTCCAGCTTTC-3' and 3'-CTCAGACTACCTCAACCGTTCC-5', IL-17RA, 5'-

GTGGGTCTTCAAACACTTCTTCA-3' and 3'-CAAGGAGCTGTGCTTAG-GTT-5'and expression of the gene of interest was normalized to that of the housekeeping gene GAPDH (reduced glyceraldehyde-phosphate dehydrogenase).

Western blotting analysis Brain tissue was washed twice with cold PBS and lysed in extraction buffer (20 mM HEPES [pH 7.4], 2 mM EDTA, 50 mM XXX-glycerophosphate, 1 mM dithiothreitol, 1 mM Na3VO4, 1% Triton X-100, and 10% glycerol) on ice. The lysates were centrifuged at 12,000 rpm for 15 min. Supernatants were collected, and protein concentrations were determined by bicinchoninic acid protein assay. Proteins were heated with sample buffer, separated in 12% sodium dodecyl sulfate-polyacrylamide gels by electrophoresis, and electroblotted onto nitrocellulose membrane. Transferred blots were incubated sequentially with blocking agent (5% non-fat milk in Tris-buffered saline). Anti-iNOS antibody (1:125 dilution, Abcam), anti-Arg-1(1:500 dilution, Abcam) and secondary antibody blots were developed by the enhanced chemifluorescence detection kit on Hyperfilm (Fuji, Japan) according to the manufacturer's directions. The same blots were subsequently stripped and reblotted with an antibody to β-tubulin to verify equal protein loading. Graphs of blots were obtained in the linear range of detection and to quantify protein levels using ImageJ software.

Transmission electron microscopy observation After anesthesia, the animals were euthanatized by transcardially perfusion with 4% PFA. Brain tissue were crosscut in 15 μm at -20 °C and mounted on glass slides. The corpus callosum was quickly dissected and post-fixed overnight in 2.5%. glutaraldehyde. Next, corpus callosum fragments were post-fixed in a solution containing 1% osmium tetroxide, then dehydrated in acetone series and embedded in SPIN-PON resin. Resin polymerization was performed at 60 °C for 3 days. Semi-thin sections (0.5 μm thickness) were placed on glass slides and stained with toluidine blue. Finally, demyelination was detected with a 300KV transmission electronic microscope and myelin sheath thickness was measured with ImageJ software. G ratio is calculated through dividing axon diameter by nerve tract diameter.

Flow cytometry Single cell suspensions of the brain were processed by digestion for 40 min at 200 rpm, 37 °C with type IV collagenase (1 mg/ml, Sigma-Aldrich) and DNase I (100 ug/ml, Roche) in 5% fetal bovine serum RPMI1640 solution, followed by filtration of tissue suspensions through 70um strainer. Then 7 ml of cell suspensions and 3 ml of 100% Percoll (final concentration 30% Percoll) were mixed and overlaid over 2 ml of 70% Percoll. Samples were centrifuged at 500 g with gradient rate, for 30 min at 18 °C. Cells of white mist removed from the interface, washed twice with PBS containing serum. Cells were incubated with Fc block (CD16/CD32), and were stained with fluorescence-conjugated anti-CD11b (M1/70), anti-CD45 (30-F11), anti-TCRγδ (GL3, all from eBioscience). For IL-17A intracellular staining, cells need to be 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 stained with fluorescence-conjugated anti-IL-17A (eBio17B7; eBioscience). Fixable viability dye EF780 (65086514; eBioscience) was used for dead cells exclusion. Samples were analyzed by flow cytometry with a CytoFLEX (BECHMAN COULTER) and date were dealt with FlowJo10. Software.

Quantification and statistical analysis Mice randomization were based on the random number generator function in Microsoft Excel software. Lesion pixel counts and area quantification for western blotting result were performed using Image J. GraphPad Prism 8.0 was used for statistical analysis to compare data on myelin sheath thickness, real-time PCR, and western blot graphs among different groups. Data were expressed as mean ± s.e.m. and were analyzed by two-tailed t-test or one-way ANOVA and Tukey's test, as appropriate. $p < 0.05$ was considered statistically significant.

Results

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

After A. cantonensis infect mice, they could invade body through blood circulation. Larvae III of A. cantonensis move into intestinal mucosa of small intestine, migrate into liver and then lung, ultimately arrive into brain, cause severe central nervous system damage, the most severe damage is myelin sheath. Transmission electron microscope (TEM) images of corpus callosum showed obvious demyelination at 14 days post infection (14 dpi) and this damage was more pronounced at 21 dpi. Myelin sheath structure became incompact and thickness continuously decreased. The myelin G ratio, in infected animals, was higher than their controls, which is contrary to myelin thickness. And axon arrangement also became disordered (Fig. 1a). A. cantonensis infection induced not only demyelination injury but also inflammatory cytokine storm. Earlier research showed brain damage due to mechanical damage caused by parasite movement, but recent evidences indicate inflammatory injury may be more important [17].

Flow cytometric analysis of brain immune cell revealed increased infiltration of blood-borne cells ($CD45^+$) after 7 dpi in infected mice. Although leukocytes were increased, the number of microglia did not show prominently change (Fig. 1b). Even so, microglia occupy the main position of inflammatory cells in the brain, we detected the expression pattern of cell type that might be involved in eosinophils recruitment. Real-time PCR result showed increased levels of markers for M1 (CD86, iNOS, CD11b) and M2 (CD206, Arg-1, YM1) microglia, with the highest levels at 21 dpi (Fig. 1c). We also detected the protein levels of iNOS and Arg-1 of infected brain tissue. iNOS expression persistently increased and peaked at 21 dpi, whereas Arg-1 was highly expressed beginning at 14 dpi and continued to increase until 21 dpi (Fig. 1d). Double immunofluorescence for Iba-1/iNOS and Iba-1/Arg-1 showed obvious increases in iNOS and Arg-1 expression in brain tissue, especially around corpus callosum (Fig. 1e). Collectively, these results indicate that A. cantonensis infection could activate both M1 and M2 microglia.

IL-17A and IL-17RA expression level obviously rose in brain after A. cantonensis infection

To evaluate the impact of inflammatory factor on the brain, we compared the level of interleukin in A. cantonensis-infected versus WT mice injected with normal saline. Among several types of interleukin, we focused on IL-17A as it had been implicated in EAE and multiple sclerosis (MS) [18]. We previously described the level of some interleukins increased during A. cantonensis infection [19], but we did not

explore their effect to the brain damages and inflammatory responses. The transcriptional level of IL-17A and IL-17RA in brain were greater in infected mice than in control mice treated with saline (Fig. 2a). IL-17A protein production was assessed after infection, but was detectable at low amounts in the first seven days. Intracellular cytokine staining (ICS) confirmed that A. cantonensis induced IL-17A production, the content of IL-17A increased with lastingness of infection (Fig. 2b).

Furthermore, to determine the location of IL-17RA within the brain, we examine intact brain sections of mice using immunofluorescence. Some IL-17RA were observed on the oligodendrocyte, especially on the axon, this change was particularly pronounced at 14 dpi (Fig. 2c upper). IL-17A influences oligodendrocyte lineage cell proliferation and differentiation through multiple pathways mediated by IL-17RA in inflammatory disease[20]. It is known microglia as a type of macrophage can secret IL-17, and in the meanwhile, it can be active by IL-17, but it was unclear whether IL-17 plays a role during A. cantonensis-infection. To clarify the relationship between IL-17 and demyelination, microglial IL-17RA expression was also detected with immunofluorescence double labeling. IL-17RA was expressed in Iba-1-positive microglia (Fig. 2c inferior), suggesting a possible link between IL-17A and microglia. Whereas another important glia cell—astrocyte,which is indicated by GFAP□was devoid of IL17RA (data not shown). It has previously reported microglia produce IL-1β, IL-6 and TNFα when cocultured with IL-17 producing Th1/Th17 cells, and then promote tissue damage[21]. Demyelination injury as typical injury of A. cantonensis infection, partly because the attack of direct damage of IL-17A on oligodendrocyte, and may also due to the microglia effects.

γδ T cell is the major source of IL-17A in A. cantonensis infected mice brain

To determine the source of IL-17A in infected mice brain, we measured several types of cell that produce IL-17, finally found γδ T cells are the major source of IL-17A. High diversity of γδ TCR, MHC-independent and antigen-independent process and presentation suggest that γδ T cell can be the first line of defense against infection. γδ T cells often appear in mucosal immunity but rarely in CNS neuroimmunity, similar to IL-17A, this cell is most extensively studied in stroke and EAE [22]. There were only a few γδ T cells detected in the normal mice brain. The frequency of brain γδ T cells was obviously increased after infection, as compared to that in sham operated mice (Fig. 3a). Approximately 60% of γδ T cells from infected mice brain expressed IL-17A. After 14dpi, $IL17^+$ γδ T cells accounted the majority of $IL17^+$ leukocyte (Fig. 3b). It has been reported A. cantonensis infection led to the immunosuppression of mice, the level of several important cytokines decreased, the total number of B and T cells declined at 21dpi [23], nevertheless, infection status of the brain continued worse, it may indicate a class of cells including γδ T cells still did the damage. The relation of γδ T cell with eosinophil had been studied, these two cells have synergistic effect in allergic response[24], eosinophilic meningeal encephalitis caused by A. cantonensis may also have a γδ T cell impact.

To determine whether the functional γδ T cells are directly from peripheral lymphoid organs or finish the differentiation in the leision, we detected the level of $IL17^+$ γδ T cells in the thymus and spleen. Thymus is the birthplace of T cells, γδ T cells are different with αβ T cells, they may acquire the functions with the

development of thymus[11]. We tested the level of γδ T cell and $IL17^+$ cell in the thymus during infection, the result was different with brain, γδ T cells number maintained a high level even in wild type mice, and had a peak around 7 dpi, whereas $IL17^+$ γδ T cells did not change appreciably even in the later phase of infection (data not show). Moreover, thymus morphology showed evident atrophy at 21dpi. Spleen as another lymphoid organ represented immunosuppressive status after infection, both γδ T cell (data not show) and $IL17^+$ γδ cell numbers sharply fell at 21dpi. $IL17^+$ γδ T cell merely rose at 7 dpi, which may be account of small intestinal infection (Fig. 3c). In order to acquire better survival environment, A. cantonensis suppress the body immunity function along with prolonged time. Moreover, we speculated γδ T cell owned corresponding function after migrated to the brain lesions caused by A. cantonensis.

Microglia Activation Weakened And Demyelination Relieved After IL-17 Neutralization

IL-17A is key factor involved in promoting the survival, recruitment and activation of other inflammatory cells via the regulation of cytokines and chemokines expression in several neuroimmune responses. Our above-mentioned data demonstrated that IL-17A expression is also outstanding in the A. cantonensis-infection, to test the real impact of IL-17A, we neutralized IL-17A by injecting specific blocking monoclonal antibodies (mAbs) through the intraperitoneal route. Both LFB (Luxol Fast Blue) staining and TEM were applied to examine whether demyelination condition improved, and myelin sheath thickness was estimated with measurement software. Previous results showed after 21 dpi of A. cantonensis infection, LFB staining of the mouse cerebral medulla decreased, indicating demyelination. While IL-17A neutralizing antibody was applied, the color of cerebral medulla was obviously darker, suggesting less demyelination. TEM results also support this conclusion. IL-17 neutralizing antibody treatment had no effect on myelin sheath thickness in normal mice (Fig. 4a). Because myelin damage can cause unpaired motor function, the neurobehavioral scores of mice were evaluated in each group. We found A. cantonensis-infected group got lower scores than WT group, whereas IL-17 neutralizing antibody attenuated this decrease (Fig. 4b). Given the relationship between demyelination and neurobehavioral scores, we proposed that IL-17A damage effect on myelin during infection cannot be ignored.

Previous research presented that inhibiting activation of microglia by minocycline can efficaciously relieve the injury from A. cantonensis [25]. Microglia maintain the steady statue of immune microenvironment of brain, the mRNA expression levels of CD86, iNOS, CD11b, Arg-1and YM1 were accordingly affected by IL-17 neutralization (Fig. 4c). On protein level, iNOS and Arg-1 had no alteration in the control mice after IL-17 inhibition but decreased in the A. cantonensis infected mice treated with the inhibitor (Fig. 4d). Moreover, the active state of microglia generally performs amebiform, this phenomenon also vanished by contrast (Fig. 4e). These findings implicate microglia inhibition in the ameliorating effect of IL-17 neutralizing antibody on demyelination caused by A. cantonensis infection.

Discussion

The immune system is traditionally regarded as a network which interacts cells and cytokines. Meanwhile, immunoreaction is a double-edged sword, while vanishing pathogens and tumors, it also

causes damage to the body itself. We need to look at the impact of the immune system dialectically. Brain is the most severely damaged organ caused by the infection with A. cantonensis, whose related theory was various, but no one can fully explain the causes of death for this animal disease model [3, 26]. Eosinophils infiltration, as the most outstanding characteristics, has long been studied, [27] where better prognosis was got through anti-inflammatory drug combination than using antiparasitic drugs alone. The present study provides evidence that IL-17A secreted by γδ T cells play an important role in demyelination and cytokine storm caused by A. cantonensis infection through activation of microglia. Inhibiting IL-17A may be a therapeutic intervention for dampening microglial activation in inflammatory demyelination.

At the beginning of immunological research, central nervous system was considered to be an immune privileged area, and brain was different from other organs because of the protection of the blood-brain barrier, which prevented the immune cells and cytokines entering [28].But as more and more studies have demonstrated that the nervous system also has the inherent immunity and adaptive immunity, especially after rich lymphatic vessels are found in the sinus durae matris. The meninges is identified as the third lymphoid tissue, also becomes an important channel of white blood cells into the brain. The immune system plays an indispensable role in maintaining the homeostasis of the nervous system [29]. As mentioned before, IL-17A has a typical proinflammatory effect, in which it causes inflammatory cell migration and tissue destruction by inducing some cells to secrete cytokines, whose role in EAE model and its relationship with demyelinating diseases were widely reported in recent years. NF-kB activator 1 (Act1) is the key element of the IL-17 downstream signaling pathways, Act1 level in mature oligodendrocytes and neurons did not influence the progress of disease, but in the $NG2^{+}$ glial cells, knockout Act1 gene led to a serious myelin damage even increased the levels of IL-17 [30].However, the degree of inflammatory damage in IL-$17A^{-/-}$ knockout mice only changed slightly after receiving antigen stimulation, which suggested that the immune activity of IL-17A in EAE model was redundant [31].Similar experiments were carried out in homozygous mutations of IL-21 and IL-22 [32], but the experiments above only showed that T-helper17 cells were not decisive, did not show the role of γδ T cells. In our study, there was a great change of IL-17A expression level, based on which, we treated A. cantonensis infected mice with an IL-17A neutralizing antibody to determine whether it could alleviate brain injury, while multiple results all indicated this suppression was effective. What's more, we observed IL-17A receptor expression on oligodendrocyte and microglia, which indicated some association among them. The attack of IL17A to oligodendrocyte can be direct without other cells mediate, not only resulting in cell damage but also inducing cell apoptosis[33]. Microglia are activated by IL-17A, and secrete IL-17A at the same time, which form the positive feedback. It was reported that the neutralization of IL-17 decreased the M1/M2 microglia ratio and concomitantly suppressed a bisphosphonate-related osteonecrosis of the jaw-like condition in mice [34], which is partially in accordance with our results. In this study, M1 and M2 microglia activation both occurred. Therefore, we speculate that the main pathological changes after infection were partially attributable to M1 microglia activation, the state of immunosuppression is on account of M2 microglia. Whereas there are some aspects that are worth to discuss and improve that: If only looking at the brain, we can just in situ inject neutralizing antibodies into brain; In addition to

microglia, whether there are other inflammatory cells that are worth exploring; Whether antibody injection in human infection sample will have the same effect with animal in this helminth infection.

γδ T cells increasing Th17 levels by producing IL-17A has been reported in EAE model, and the synthesis of IL-23 by Th17 promoted the activation of $IL17^+$ γδ T cells [35]. $IL17^+$ γδ T cells can also reduce the number of T-reg cells (regulatory T cell) by inhibiting the conversion of conventional T cells to Foxp3 + T-reg cells, and can inhibit the immunosuppressive effect of T-reg cells. In $Tcrd^{-/-}$ knockout mice, the increase in the number of T-reg cells and the enhancement of the associated inflammatory inhibition effect effectively control the antigenic activation of αβ T cells [36]. In addition, the effect of changes in intestinal flora on ischemic brain injury is also mediated by γδ T cells. The researchers found dysbacteriosis in the intestinal tract would change the number of T-reg cells and IL-17 $^+$ γδ T cells in the meninges. Affected by the result of antigen presentation, dendritic cells in the intestinal tract of animals with dysbacteria-like flora were more inclined to promote the differentiation of T-reg cells, γδ T cells were inhibited by IL-10 from T-reg cells, thus inhibiting the production of IL-17 $^+$ γδ T cells, which is also the reason for the reduction of ischemic brain injury [37]. However, the study of the role of γδ T cell in cerebral lesions caused by helminth infection was rarely published. In the experimental cerebral malaria animal model, TCR $\delta^{-/-}$ mice showed reduced brain swelling and cerebral hemorrhage. In addition, there is almost no damage of blood-brain barrier, and animal survival indicators were good [38]. TCR $\delta^{-/-}$ mice had lowed number of worms and slighter brain damage after the tapeworm infection, while the infiltration of inflammatory cells and related cytokines of type I immune response also decreased in the brain[39]. Our result also proved γδ T cells hold a place in A. cantonensis infection and the majority of IL-17A was secreted by γδ T cells which centralized in the brain and mainly increased at the late stage of infection. However, our date showed the change of γδ T cells in the thymus was not as intense as in the brain, and the level of IL-17 $^+$ γδ T cells was not high during infection. We speculated that a certain amount of γδ T cells are reserved in the brain or meninges under normal condition, once sense the parasite antigens from the in vivo environment through the TCR and co-stimulatory receptors and then process appropriate functions. Effector functions do not only depend on the outside,but the type and constitution of γδ TCR segments are equally important [40]. It is necessary to explore which γδ T cell subset plays a leading role and whether γδ T cells have pathogenic effects to the body. Except IL-17A, γδ T cells can secrete other cytokines, such as IL-5 and eotaxin, that directly attract eosinophils to migrate to the site of inflammation, but also secrete IFN-γ and IL-4 to regulate the activity of αβ T cells and macrophages, thus promoting the chemotaxis of eosinophils[24]. Both IL-5 and eotaxin were tested obviously increasing in A. cantonensis infected animal[41], thus γδ T cells probably have other functions that is worth studying in this model.

Conclusion

The study about intracerebral parasite disease related to γδ T cell is rare, and $IL17^+$ γδ T cell more so. We present evidences support a key role of IL17A in A. cantonensis infection. IL-17A secreted by γδ T cells act on demyelination and microglia polarization Specifically blocking IL-17A could be considered as a potential therapeutic target for treating demyelination associated with neuroinflammatory conditions

involving microgliosis. The results above show clearly A. cantonensis not only cause mechanical injury, but also destroy the cerebral immunologic balance and bring cytokine storm. We should pay more attention to the indirect damage of nematode to the 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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Availability of data and mateials

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

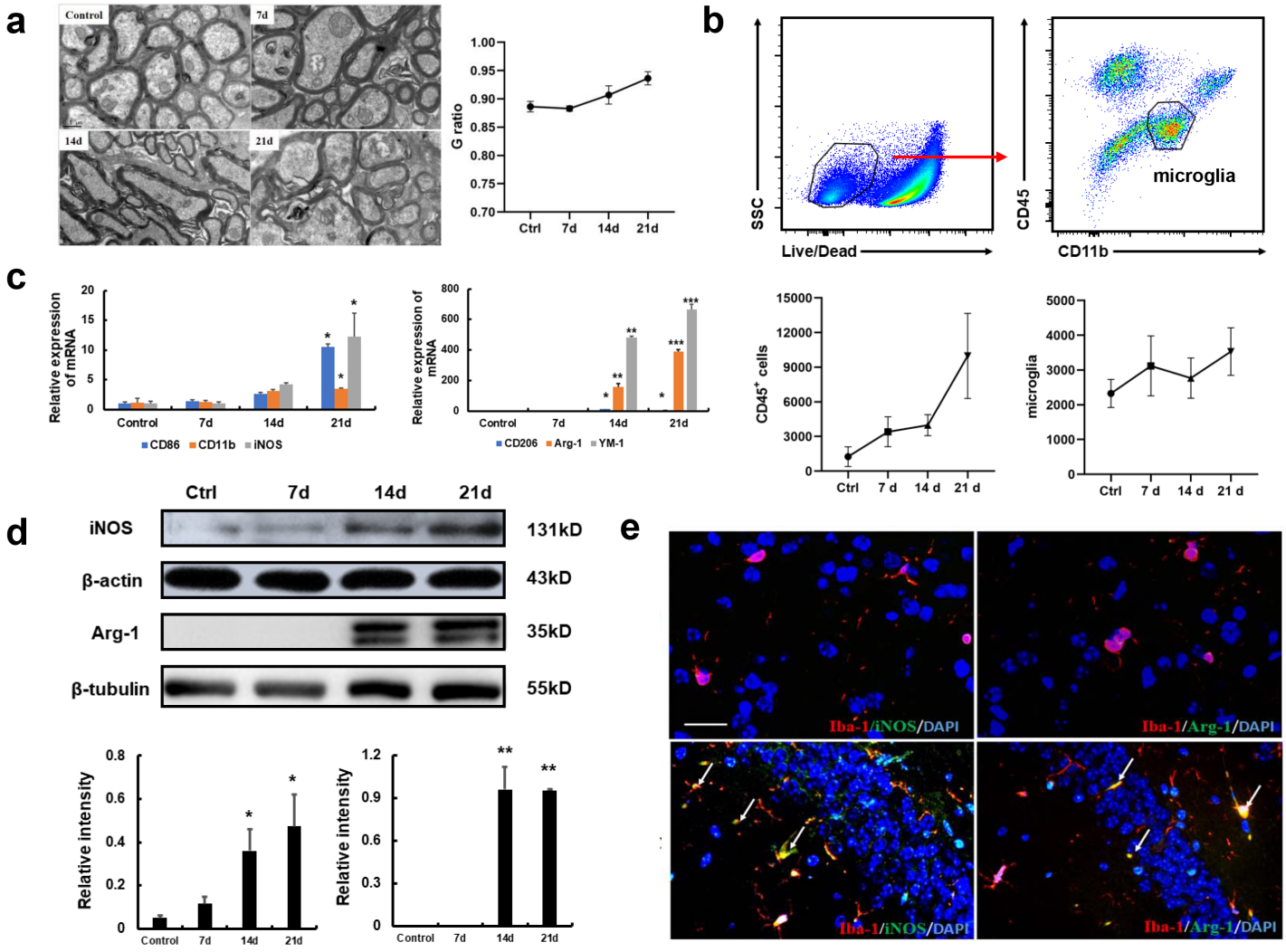

Figure 1

Demyelination and microglia activation were observed in brain tissue of mice during A. cantonensis infection. (a) Transmission electronic image of corpus callosum at 7, 14, and 21 days after A. cantonensis infection. Scale bar = 0.5 μm. Right offside is the graph of g ratio in each group. (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-1and YM1 after A. cantonensis infection. (d)Western blots showing iNOS and Arg-1 protein levels of individual group, relative densitometric analysis of Western blots was represented in the below, as normalized 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 21dpi 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 is presented as means ± SEM.

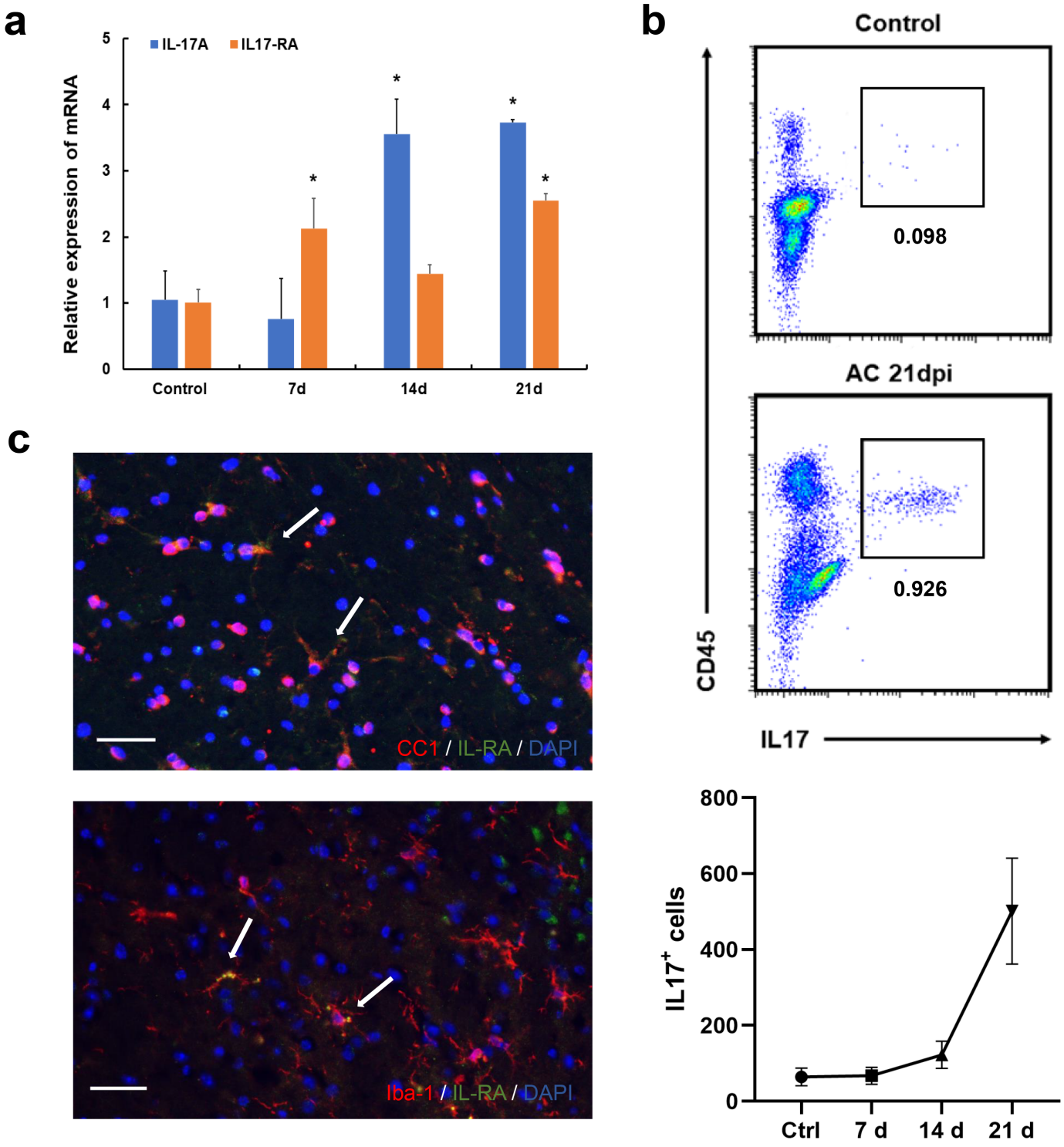

Figure 3

Figure 2. IL-17A and IL-17RA expression level obviously rose in brain after A. cantonensis infection (a) RNA expression of IL-17A and IL17RA at 7, 14, and 21 dpi. (b) Flow cytometry result of brain infiltrating IL17+ leukocytes (numbers represent events within the gate as a percentage of leukocyte). Right is the quantification of the absolute number of IL17+ cells for each time point. (c) Immunofluorescence of CC1/IL-17RA and Iba-1/IL-17RA double staining of brain tissue in the 21dpi group. Co-expression (white

arrows) were 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 is presented as means ± SEM.

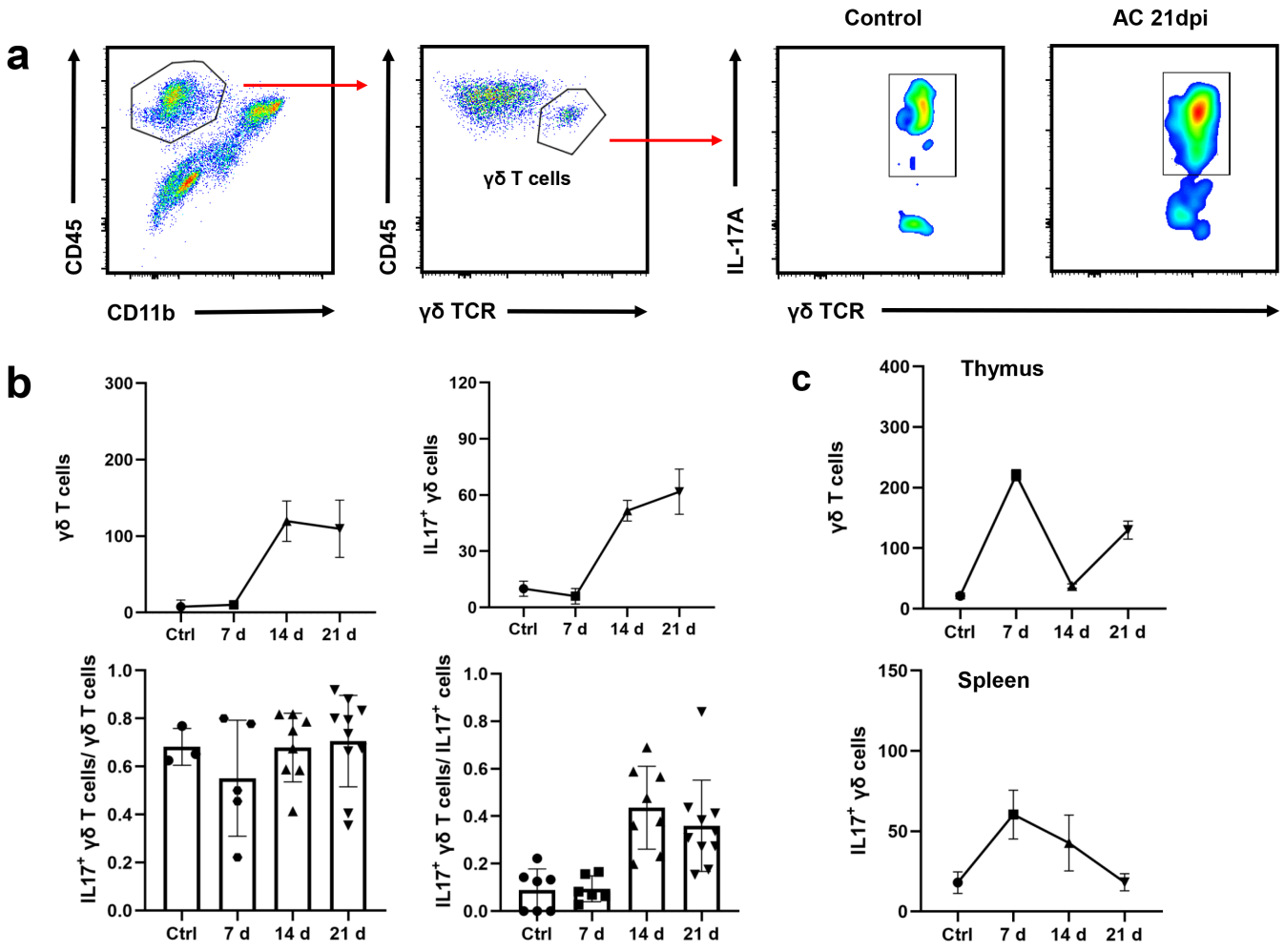

Figure 5

γδ T cell is the major source of IL-17A in A. cantonensis infected mice brain. (a) Representative flow cytometry analysis and (b) quantification of total γδ T cells and IL-17+ brain γδ T cells (CD45highCD11b−γδTCR+IL17A+) at at 7, 14, and 21 dpi. Bottom, graphs depict percentages from indicated populations. (c) The above is the absolute number of IL17+ γδ T cells in thymus, the below is γδ T cells amount in spleen. Data in each statistical graph is presented as means ± SEM

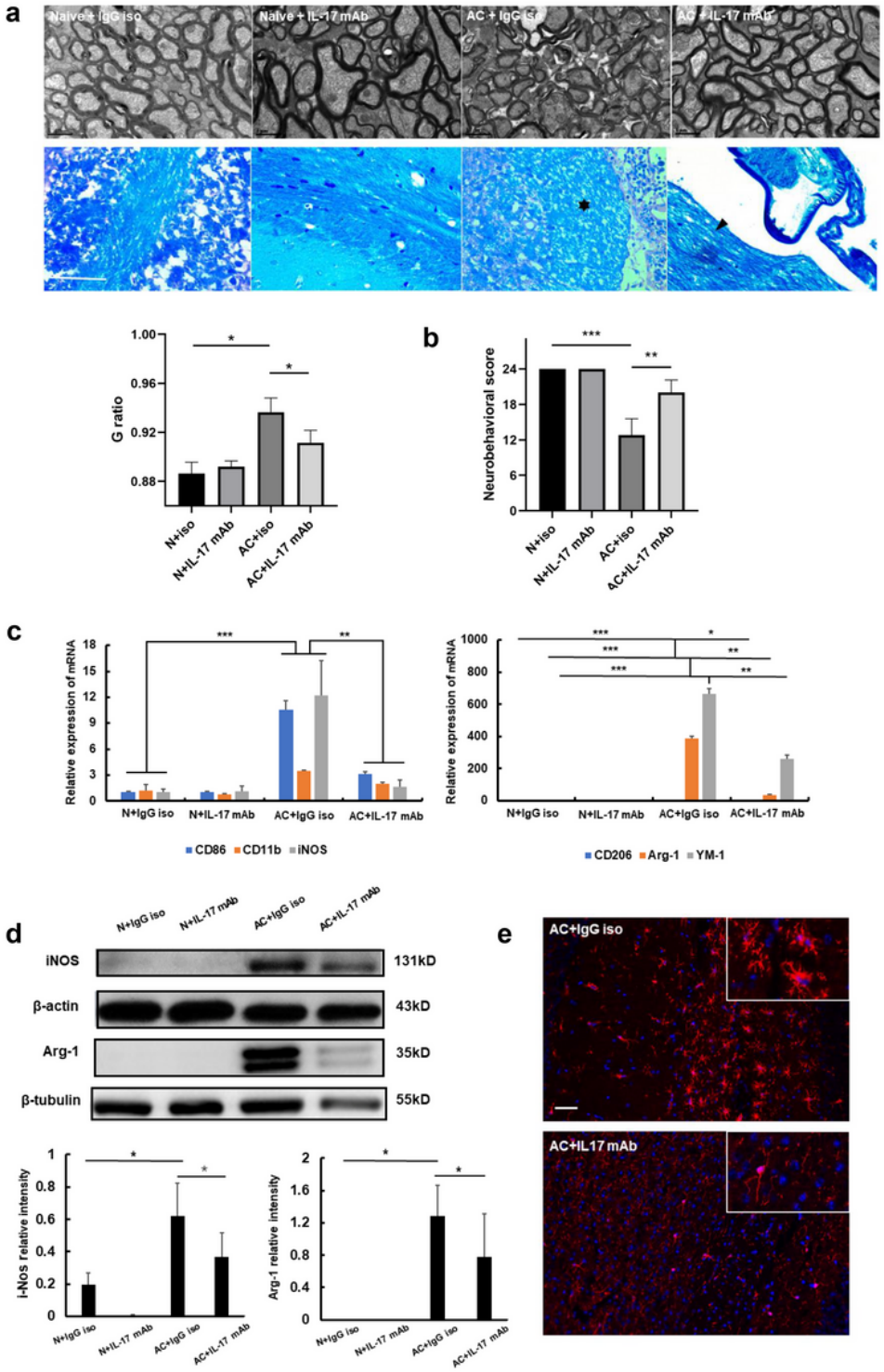

Figure 7

Microglia activation weakened and demyelination relieved after IL-17 neutralization. (a) TEM and LFB images of corpus callosum in the control group and experimental group (inject IL17A mAb). Black symbol indicates the cerebral medulla change region. TEM scale bar = 0.5 μm, LFB scale bar=100um. Below is the graph of g ratio in each group. (b) Neurological function scores in each group. (c) RNA expression of CD86, iNOS, CD11b, CD206, Arg-1and YM1 after A. cantonensis infection and injection with IL17A mAb.

(d)Western blots showing iNOS and Arg-1 protein levels of individual group, relative densitometric analysis of Western blots was represented in the below, as normalized to β-actin and β-tubulin. (e) Immunofluorescence of Iba-1 staining of brain tissue in the 21dpi infection groups with IgG1 isotype Ab and IL17A 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 is presented as means ± SEM.