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Original Article

IgE antibody responses in cerebrospinal fluids relate to the brain pathologic injury of hosts with *Angiostrongylus cantonensis* infection

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IgE;
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Mouse strain

Abstract *Background:* The immunoglobulin E (IgE) response to *Angiostrongylus cantonensis* infection increases in the host. This study analyzed the IgG and IgE responses detected in different body fluids of *A. cantonensis*-infected mice.
Methods: BALB/c (high susceptibility), CBA (medium), and C57BL/6 and C57BL/10 (resistance) strain mice were used in this study. The levels of IgM, IgG, and IgE in the serum and cerebrospinal fluid (CSF) from infected mice were compared. *A. cantonensis*-reactive antigens from BALB/c and C57BL/6 mice CSF were also analyzed.
Results: Antibodies against fifth-stage larvae (L5) antigens increased in mice CSF, particularly IgE, relate to worm rejection and the susceptibility of different mouse strains. The increased IgE level in BALB/c mice CSF is lower than that from others, suggesting IgE response in brain is more important than that in serum. Anti-L5 and anti-excretory/secretory (ES) antigen IgE and IgG responses in CSF were analyzed. In addition, the antibody-dependent eosinophil-mediated

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cytotoxicity induced by anti-excretory/secretory (ES) antigen antibodies may be the reason of severe brain inflammation in infected BALB/c mice. IgE and IgG antibodies against a 105 kDa protein of L5 antigen was detected at week 3 post-infection in C57BL/6 mice and week 5 post-infection in BALB/c mice. We suggest that 105 kDa protein is related with the antibody response of *A. cantonensis*-infected mice.

Conclusion: We found that IgE antibodies in mice CSF against L5 antigens related to worm rejection in mice brains. This study may help to identify specific angiostrongyliasis markers that can be applied for clinical diagnosis and treatment in future.

Introduction

Angiostrongylus cantonensis is a parasite that mainly affects rat heart and pulmonary artery and causes human eosinophilic meningitis or meningoencephalitis in the Far East, Southeast Asia, and the Pacific islands.[1–3] After infected rats, the larvae move through the brain and then become adult worms in the heart and pulmonary artery.[4] However, the larvae remain in the brain of non-permissive hosts, like mice and human, and stop growing[5]. Presently, this parasite infection does not have a very effective treatment.

Central nervous system (CNS) injuries induced by *A. cantonensis* includes hemorrhage, vascular dilatation, focal necrosis with neuronal loss, and inflammatory cell infiltration.[6,7] Cerebral pathogenesis is characterized by eosinophil infiltration that induces significant inflammatory reactions in response to immature worms[7,8]. Eosinophils are recruited to the infected region and try to eliminate the pathogen, but instead causes severe tissue damage. Various eosinophil-secreted molecules, including interleukin (IL)-4, 5, 6, 10, 12, 13, 18, transforming growth factor (TGF)-α/β, leukotrienes, proteases, reactive oxygen species (ROS), and nitric oxide (NO), not only mediate protective immune responses, but also contribute to hypereosinophilic syndrome-associated pathophysiology.[9–12] Although several reported inflammatory mediators may function in infection, the mechanism influence on cerebral angiostrongyliasis pathogenicity and pathophysiology still remain poorly defined.

Typically, IgE plays a vital role in humoral immunity against parasites and its concentration significantly increases in parasite-infected hosts.[13] Many studies have reported that IgE concentrations increased in *A. cantonensis*-infected rat CSF and serum and cooperated with eosinophils to induce immune responses against the parasite.[12,14,15] However, increased IgE antibody concentration influences the immune defense mechanisms and played more critical roles in non-permissive hosts than those in permissive hosts.[16,17] Moreover, different species infected with *A. cantonensis* will trigger different immune response levels.[6] The total and specific IgE present in infected rat and guinea pig serum and CSF were significantly different.[18] The susceptibility of different *A. cantonensis*-infected mouse strains to the parasite will also be different.[19,20] Although specific IgE-dependent eosinophilic cytotoxicity is one of the main host defense mechanisms against parasitic infections, local and systemic immune responses against trichinella are regulated by different mechanisms and have different effects.[21,22] Young adult worms in *A. cantonensis*-infected non-permissive host brain induced eosinophils in the CSF,[15,23,24] and systemic and local Th2 cytokines significantly increased.[6,20,25] In addition, eosinophils increased in only peripheral blood, but not the CSF, of infected rats.[24] Moreover, the anti-parasite antibody concentration in the serum and CSF of *A. cantonensis*-infected eosinophilic meningitis patients were also different.[26–28] Infected rats develop acquired immunity against *A. cantonensis* reinfection.[29] Antigens from different *A. cantonensis* stages, such as L3 or L5, adult, or excreted antigens, induce host immunity, which mitigates and reduces larval size, significantly reducing worm recovery, and impairing female fecundity.[30–33] However, *A. cantonensis* infect different host genotypes and cause diverse local and systemic immune mechanisms, which trigger changes in pathological lesions in the brain, still lacking further research.

Liu et al. revealed that specific IgG in *A. cantonensis*-infected mice sera increased slowly and peaked at day 20 post-infection; however, the specific IgE increased sharply for the first 10 days, then showed a downward trend during days 15 to 25 post-infection.[34] In this study, we first compared the levels of antibodies such as IgM, IgG, and IgE in the serum and CSF of different *A. cantonensis*-infected mouse strains and conform the specific IgE antibody in CSF influences the most of the sensitivity of mice. In addition, the reactive antigen from BALB/c and C57BL/6 mice serum and CSF were also analyzed and suggested the specific antigen expressions in the *A. cantonensis*-infected mice CSF is related to the changes in brain pathological lesions and resistance to parasites.

Materials and method

Animals

Eight-week-old male mice were purchased from the National Laboratory Animal Center and BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). Animal experiments were performed under humane conditions with approval from the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University (license number: LAC-2013-0065), and

conducted in accordance with NIH Guide for the Care and Use of Laboratory Animals (DHHS publication no. NIH 85-23, revised 1996).[35]

Parasite and infection

A. cantonensis L3 were provided by Prof. Lian-Chen Wang, Department of Parasitology, Chang Gung University and maintained by serial passage in *Biomphalaria glabratus* and SD rats in the laboratory. Infective L3 were obtained by 0.6% pepsin digestion (pH 2, 37 °C, 1 h) of snails previously infected with the first-stage larvae. C57BL/6, 10, and CBA mice were orally infected with 50 L3 and BALB/c mice were infected respectively with 25, 40, and 50 L3. The L3-infected mice were grouped respectively and euthanized weekly after infection for 1–5 weeks for worm counting and blood and brain tissue specimen collection. Uninfected mice served as controls.

Worm recovery and sample collection

The experimental and control mice were weighed before euthanizing. Serum samples were collected from mice by heart puncture, stored at 4 °C overnight, and centrifuged at 15,000× *g* for 10 min. The supernatant was collected and stored at −70 °C. After serum collection, the mouse brain was moved to a Petri dish and L5 larvae were counted under a dissecting microscope. The brain of the mouse from another group was removed and cultured in 0.3 mL RPMI 1640 culture medium (Invitrogen; Carlsbad, CA, USA) for 24 h to collect the CSF, which was concentrated 25 times using Amicon Millipore filters (5 kDa MWCO) and centrifuged at 15,000 × *g* for 10 min. The supernatant was collected and stored at −70 °C.

Antigen preparation

L5 were obtained from the infected mouse brain, homogenized in the homogeneous liquid (1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.15 M PBS, pH 7.4) in a glass tissue grinder at 4 °C, and sonicated in an ultrasonic disintegrator (Soniprep 150, MSE Scientific Instruments; Manor Royal, UK). The somatic antigens were prepared from L5 by centrifugation at 12,500× *g* for 45 min at 4 °C. Excretory/secretory (ES) antigens of L5 *A. cantonensis* were prepared following a protocol modified from that reported previously.[32] Briefly, L5 were collected after washing and cultured in vitro in 0.5 mL RPMI 1640 culture medium supplemented with 100 μg mL^{-1} penicillin and 100 U mL^{-1} streptomycin at 37 °C in 5% CO_2. Worms were placed in fresh medium for 24 h. The exhausted medium was centrifuged at 15,000× *g* for 10 min and the supernatants were concentrated 25 times using Amicon Millipore filters (5 kDa MWCO). All supernatants were stored in aliquots at −70 °C. The protein concentration of each antigen extract was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories; Richmond, CA, USA).

Antibody assay and western blotting

The capability of L5 and ES antigens to induce specific antibodies was evaluated using an enzyme-linked immunosorbent assay (ELISA). L5 and ES antigens were incubated overnight at 4 °C (20 μg protein/well). After being emptied, the wells were blocked with 0.5% non-fat skim milk for 30 min at 37 °C and then incubated with 1:2000 diluted sera for 1 h at 37 °C. After washing four times with PBS, 1:500 diluted affinity purified horseradish peroxidase (HRP)-goat anti-rat IgM, IgG, and IgE (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) were added and incubated for 30 min at 37 °C. Then, 100 μL 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) peroxidase substrate solution (Zymed; San Francisco, CA, USA) was added to each well. The optical density 450 nm was read after 30 min at RT (25 °C) using a Dynatech MR5000 microplate reader (Dynatech; Germantown, MD, USA).

Western blot analysis of specific proteins expression

Western blot analysis was conducted to determine the antigen components. Aliquots containing 100 μg/mL antigen was resolved using 12% homologous sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE) and then electrophoretically transferred to polyvinylidene difluoride (PVDF; Millipore; Billerica, MA, USA) membranes. Membrane strips were cut, blocked, and then incubated with rat sera for 60 min at 37 °C. After washing, the strips were incubated with HRP-goat anti-rat IgG and IgE for 35 min at 37 °C. After washing, tetramethylbenzidine (TMB) substrate solution (Thermo-Fisher Scientific; Waltham, MA, USA) was added for staining.

Statistical analysis

Antibody levels were compared using one-way ANOVA of SPSS 18.0 software (SPSS Inc.; Chicago, IL, USA). Other results were analyzed using two-tailed Student's *t*-tests. In all analyses, $p < 0.05$ were considered significant and data were expressed as the means ± standard deviations (SD).

Results

Mortality and worm burdens in different *A. cantonensis*-infected mouse strains

Different mouse strains were infected with L3, grouped, and their mortality and weight changes were recorded. BALB/c mice infected with 50, 40, and 25 L3 died after 19.67 ± 0.88, 26.33 ± 0.88, and 32.33 ± 1.45 d, respectively, while CBA mice infected with 50 L3 were died after 34.67 ± 0.33 d (Table 1). In contrast, both C57BL/6 and C57BL/10 mice infected with 50 L3 did not record any deaths within 5 weeks. The results of body weight change in infected mice are shown in Fig. 1A. The bodyweight of

Table 1 Mortality in different *Angiostrongylus cantonensis*-infected mouse strains.

Strain	Haplotype	Mouse number	Infected larvae numbers	Average survival duration (days)	Number of dead mice
BALB/c	H-2^d	6	50	19.67 ± 0.88	6
		5	40	26.33 ± 0.88	5
		5	25	32.33 ± 1.45	5
CBA	H-2^k	5	50	34.67 ± 0.33	5
C57BL/6	H-2^b	5	50	—	0
C57BL/10	H-2^b	5	50	—	0

Data are expressed as mean ± standard deviation (SD).

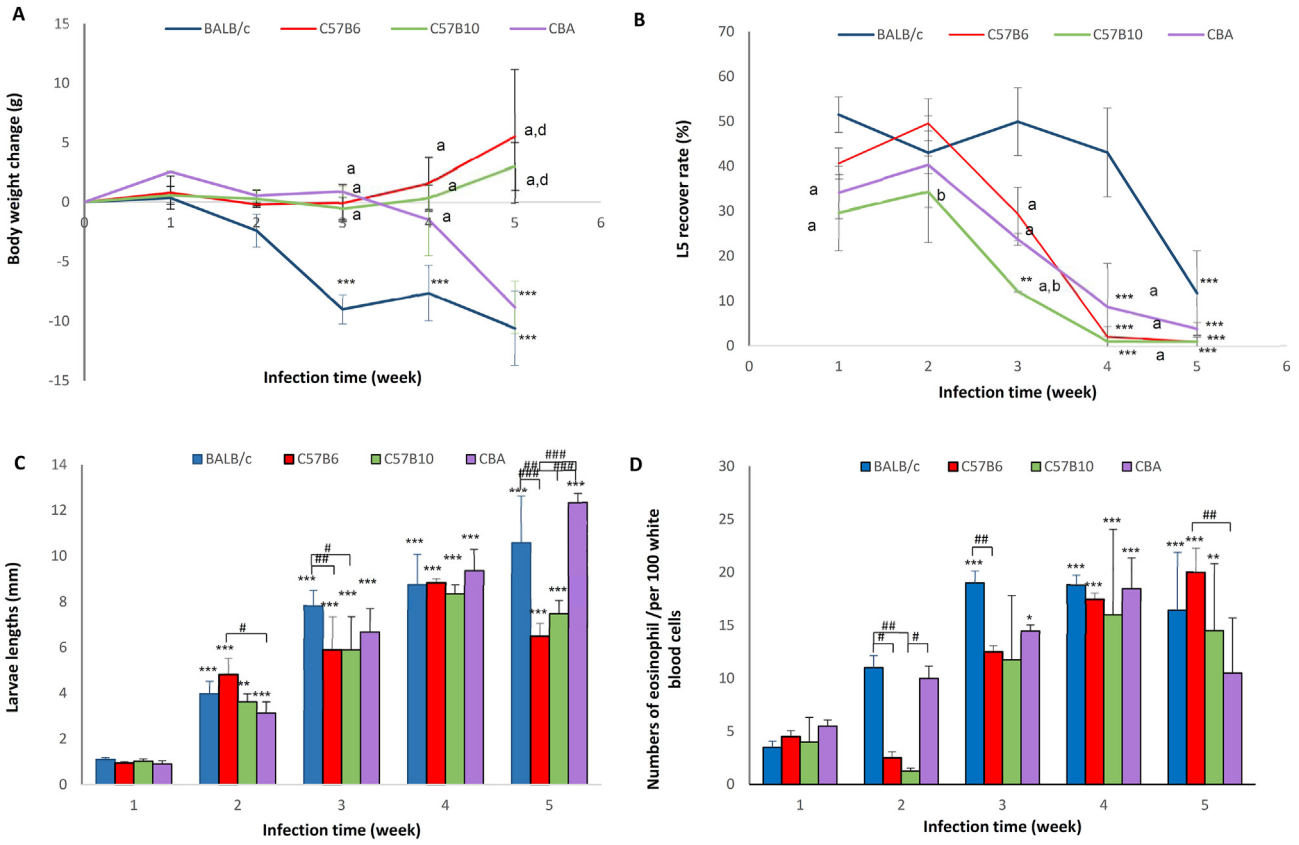

Figure 1. Body weight change, parasite burden rate, parasite length and eosinophil ratio during *Angiostrongylus cantonensis* infection in different mouse strains. Body weight changes (A): All data presented as mean ± standard deviation (SD; n = 5–6). ***p < 0.001, a, and d represent the value is significant difference from those of respective control group or of BALB/c mice (a) and of CBA mice (d), respectively, (a and d, p < 0.05). (B) Fifth-stage larvae (L5) burden rates: Data expressed as mean ± SD (n = 5–6) of L5 recovery percentage in the brains (L5 recovery count in different infection time/L5 recovery count of control group). **p < 0.01 and ***p < 0.001 represent significant differences from respective control group. a and b represent the value is significant difference from the value of BALB/c and of C75BL/6 mice, respectively (a and b, p < 0.05). (C) L5 lengths: Data expressed as mean ± SD (n = 5–6) of L5 length in the brain. **p < 0.01 and ***p < 0.001, represent the value is significant differences from those of post-infection week 1 group; #p < 0.05, ##p < 0.01, and ###p < 0.001 represent the value is significant differences between two indicated groups. (D) Eosinophil ratio: Data expressed as mean ± SD (n = 5–6) eosinophil count per 100 white blood cells. *p < 0.05, **p < 0.01, ***p < 0.001 represent significant differences from respective control group; #p < 0.05, ##p < 0.01, ###p < 0.001 represent significant differences compared between two indicated groups.

infected BALB/c mice continually declined from week 2 to week 5, while that of infected CBA mice declined until week 4 and was near to that of BALB/c mice (p < 0.05). In addition, the body weights of infected C57BL/6 and C57BL/10 mice increased than those of the control groups. For counting worm numbers, another group of different mouse strains was euthanized weekly from week 2 to week 5 post-infection, the L5 were collected from the brains and their

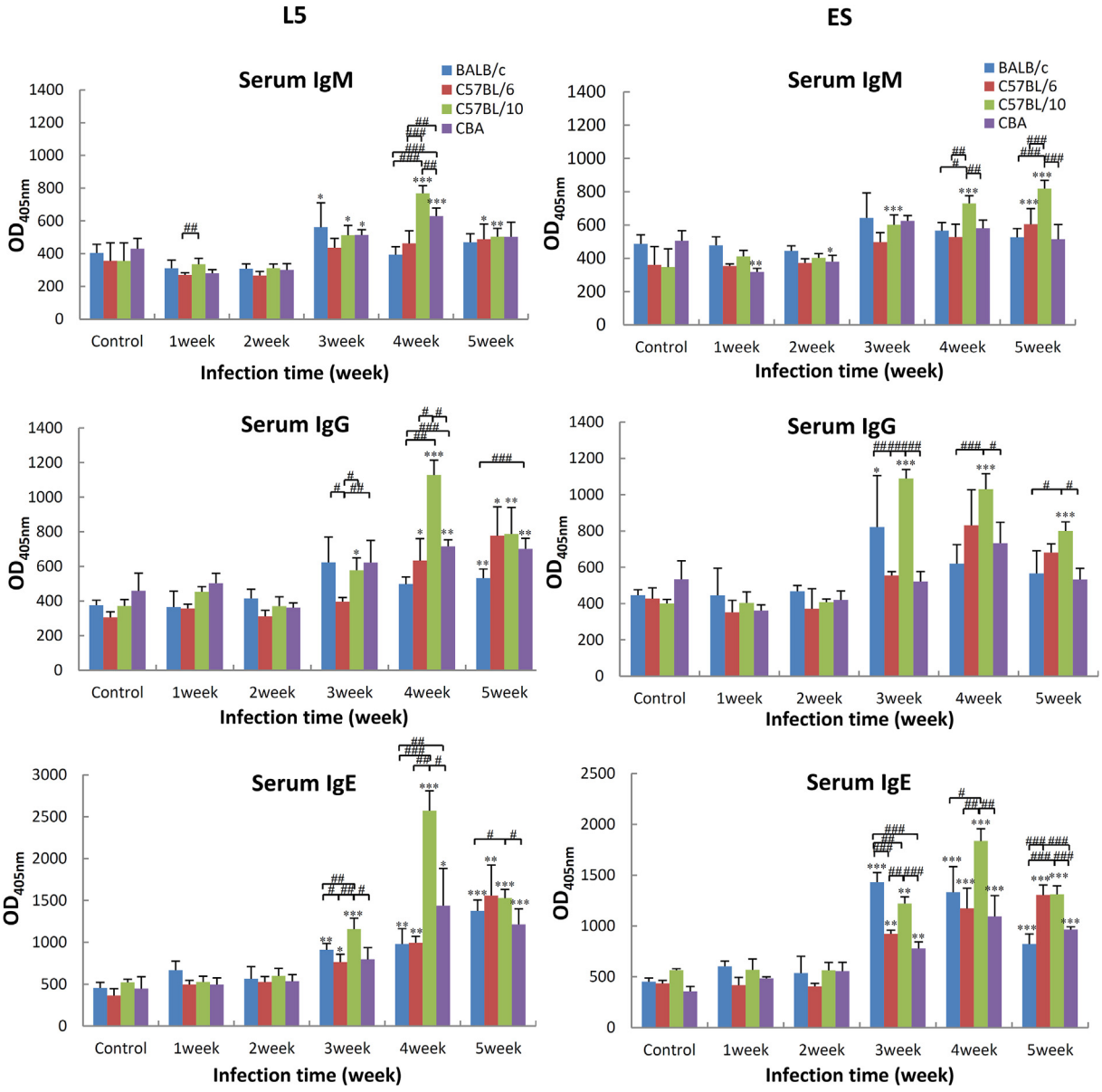

Figure 2. Different antibody subtype titers against fifth-stage larva (L5) and excretory/secretory (ES) antigens of *Angiostrongylus cantonensis* in serum of different mouse strains. Different antibodies (IgM, IgG, and IgE) from 1 to 5 week *A. cantonensis*-infected and control mice serum were examined using ELISA. L5 and ES: antibodies against L5 or ES antigens, respectively, were detected in mice sera. Different colors indicate different mouse strains. The results are shown as the means ± standard deviation (SD; n = 5–6). *p < 0.05, **p < 0.01, ***p < 0.001 indicate comparisons to the control group. #p < 0.05, ##p < 0.01, ###p < 0.001 indicate comparisons between two indicated groups.

lengths were measured. The infection rate of all the mice significantly decreased at week 3, except BALB/c ($p < 0.05$), wherein it was the minimum in C57BL/10 mice (12.24%) (Fig. 1B). However, it decreased to 8.75%, 2.09%, and 0% in CBA, C57BL/6, and C57BL/10 mice at week 4, respectively; while it just declined in BALB/c mice until to week 5 (11.78%). L5 isolated from BALB/c mice were 7.84 ± 0.21 mm and significantly larger than those isolated from C57BL mice at week 3 ($p < 0.05$) (Fig. 1C). Interestingly, at week 5, the larvae isolated from CBA mice were the longest (12.35 ± 0.35 mm), while those isolated from BALB/c mice were 10.14 ± 0.68 mm, both being larger than the 6 and 8 mm worms isolated from C57BL/6 and C57BL/10 mice, respectively ($p < 0.01$).

Eosinophils in the blood of mice infected with *A. cantonensis*

The eosinophil count in the blood of different mouse strains were monitored weekly from week 2 to week 5 (Fig. 1D). The eosinophil count of both BALB/c and CBA mice increased from week 2 and peaked at weeks 3 and 4 (19 ± 1.41 and 18.5 ± 2.88 cells/100 WBC, respectively)

and remained until the fifth week declining. The eosinophil count of C57BL/10 and C57BL/6 mice increased from week 3 and peaked at weeks 4 and 5, respectively.

The fifth-stage larval (L5) and ES antigen-reactive antibody analyses in different *A. cantonensis*-infected mouse strains

The titers of reactive antibodies against L5 and ES antigens in serum and CSF of mouse from different strains were measured using ELISA.

Serum

The anti-L5 antigen IgG increased in infected BALB/c mice serum was higher than that in control and peaked at week 3, then slightly declined (Fig. 2). It also significantly increased in C57BL/10 mice serum from week 3 and peaked to 1128.1 $\pm$ 86.1 at week 4 ($p < 0.05$). The anti-L5 antigen IgG in C57BL/10 mice serum was also significantly higher than that in other strain serum at week 4 ($p < 0.05$). However, it peaked in CBA and C75BL/6 mice at weeks 4 and 5, respectively. The serum IgG level in CBA mice was also sustainably higher than that in BALB/c mice from week 4 to week 5 ($p < 0.001$). Moreover, serum anti-ES antigen IgG significantly increased in C57BL/10 mice from week 3 to week 5 ($p < 0.001$), which was also higher than that in BALB/c and CBA mice ($p < 0.05$). It also increased in BALB/c mice at week 3 ($p < 0.05$) but decreased at week 4; however, there was no significant change in CBA and C75BL/6 mice. The anti-L5 antigen serum IgE increase in all mouse strains was more significant than the IgG increase. It sharply increased in all mouse strains except CBA mice from week 3 ($p < 0.05$) and all strains were significantly higher than that in the controls at weeks 4 and 5 (Fig. 2). However, it peaked in C57BL/10 and CBA mice at week 4 ($p < 0.05$) and BALB/c and C57BL/6 mice at week 5 ($p < 0.05$). Serum anti-L5 antigen IgEs in C57BL/10 mice were significantly higher than that in other strains from week 3 to week 5 ($p < 0.05$). Meanwhile, the anti-ES antigen serum IgE of all mouse strains significantly increased from week 3 to week 5. Moreover, it was the highest in BALB/c and C57BL/10 mice at week 3 ($p < 0.01$) and week 4 ($p < 0.05$), respectively; however, their levels in both C57BL/10 and C57BL/6 mice were higher than those in the other strains at week 5 ($p < 0.001$). In addition, serum IgM levels fluctuated only slightly during infection in all mouse strains except C57BL/10 mice (Fig. 2). The serum anti-L5 antigen IgM at week 4 post-infection and serum anti-ES antigen IgM at weeks 4 and 5 post-infection were significantly higher in C57BL/10 mice than those in other mouse strains ($p < 0.05$).

CSF

As shown in Fig. 3, the anti-L5 antigen IgG in CSF from all mouse strains did not increase significantly until week 5 except BALB/c mice, CBA mice at week 3, and C57BL/10 mice at week 4 ($p < 0.01$). The anti-L5 antigen IgG in CSF of infected C57BL/10, C57BL/6, and CBA mice were also higher than that in BALB/c mice at week 5 ($p < 0.001$). The anti-ES antigen IgG in the CSF of all mouse strains fluctuated slightly and were not significantly different ($p < 0.01$) except C57BL/10 mice at week 2, CBA mice at week 3, and BALB/c mice at week 5. However, most importantly, the fluctuations of anti-L5 antigen IgE in CSF from different mouse strains were most correlated with *A. cantonensis* infection morbidity and deworming rates (Fig. 3). Interestingly, it peaked in BALB/c mice at week 3 and remained constant till week 5 ($p < 0.05$), while it increased in CBA, C57BL/10, and C57BL/6 mice from weeks 1, 2, and 3, respectively ($p < 0.05$). The anti-L5 antigen IgE in CSF from three mouse strains were all significantly higher than that from BALB/c mice at week 3 ($p < 0.01$) and 4 ($p < 0.05$), whereas it was maintained in C57BL/6 mice until the week 5 ($p < 0.01$). Among anti-ES antigen IgEs, the IgE in CSF from BALB/c mice were not significantly increased from week 2 to week 5, whereas it began to increase considerably from week 3 in C57BL/10 and CBA mice ($p < 0.001$) and week 4 in C57BL/6 mice ($p < 0.05$) (Fig. 3). It was also higher in CBA mice than that in BALB/c and C57BL/6 mice at weeks 3 and 4 ($p < 0.01$), while that in C57BL/10 and C57BL/6 mice were both higher than that in BALB/c mice at week 4 as well ($p < 0.05$). Furthermore, anti-L5 or -ES antigen IgM antibodies in CSF from all mouse strains were not significantly higher than those in normal controls until weeks 3—5 ($p < 0.05$). However, the anti-L5 or -ES antigen IgM was not significantly elevated in CSF from any mouse strain during infection (Fig. 3).

The L5- and ES-reactive protein analysis in *A. cantonensis*-infected C57BL/6 and BALB/c mice

The IgG- and IgE-recognized L5 and ES antigen components in the CSF of infected BALB/c and C57BL/6 mice from week 2 to week 5 were analyzed using western blotting. Serum responses of normal BALB/c and C57BL/6 mice were provided as reference controls and shown in Supplementary information 1, while these two normal mouse strains were completely devoid of antibody responses in CSF (data not shown).

IgG antibody

No specific protein molecules were detected in C57BL/6 and BALB/c mice CSF at week 2 (Fig. 4). The IgG-recognized antigen was detected more often in C57BL/6 mice CSF than that in BALB/c mice CSF at week 3. No anti-L5 antigen antibodies were detected in BALB/c mice CSF, but antibodies against 105 kDa antigen molecule were detected in C57BL/6 mice. Moreover, the antibodies against 105 and 85 kDa molecules were detected in C57BL/6 mice at week 4; however, it still no antibodies against L5 antigens were detected in BALB/c mice (Fig. 4). In addition, antibodies against 75 kDa molecule in the anti-ES antigen were detected in BALB/c mice. The antibodies against 105 and 85 kDa molecules were detected in C57BL/6 mice and those against 105 kDa molecule in L5 antigen were detected in BALB/c mice at week 5. Moreover, the antibodies against 75 and 105 kDa molecules in the ES antigen were significant at week 5 than those at week 4.

IgE antibody

No specific protein molecules were detected in C57BL/6 and BALB/c mice CSF at week 2 (Fig. 5). The IgE-recognized antigen was detected more often in C57BL/6 mice CSF than

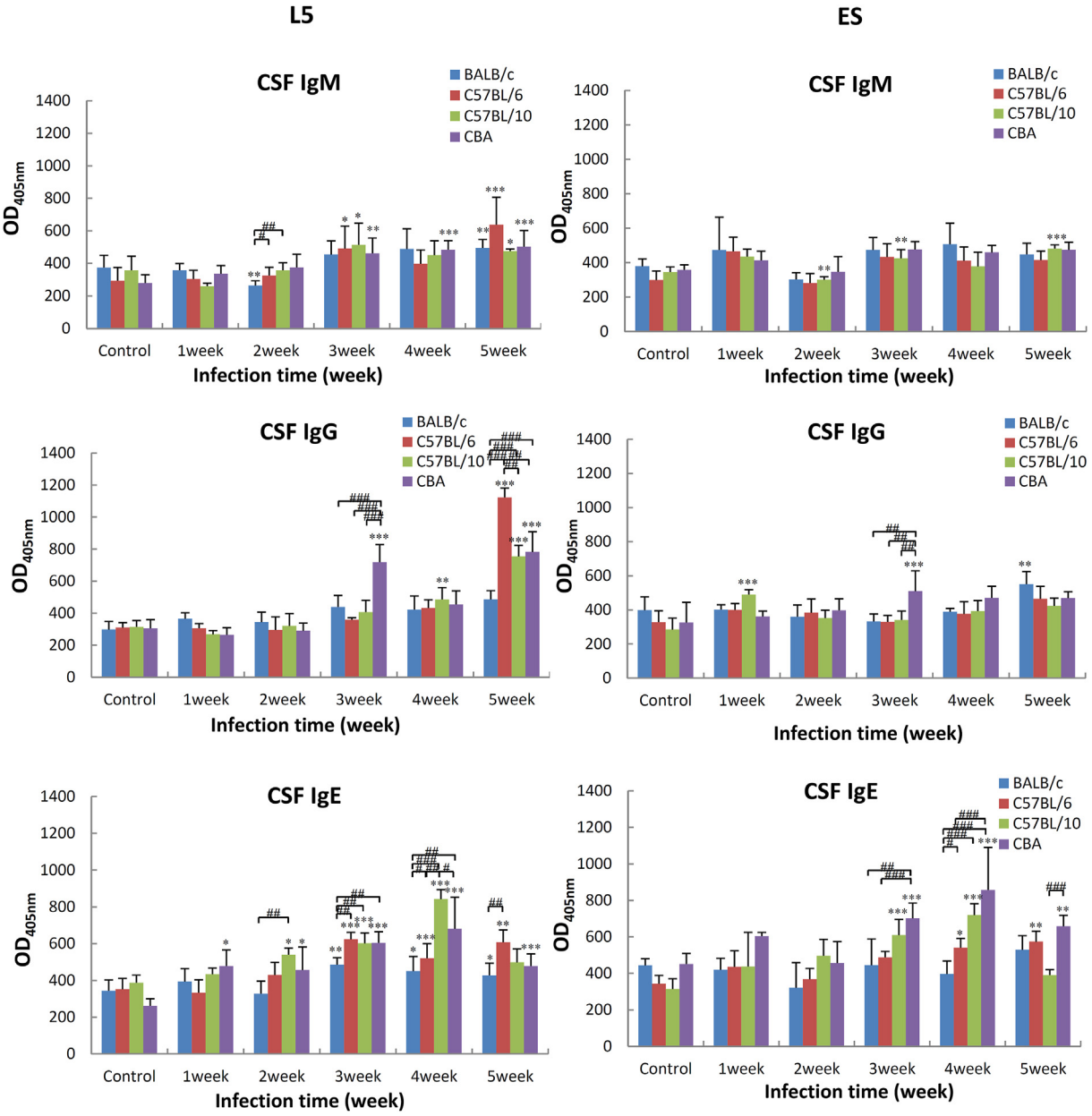

Figure 3. Different antibody subtype titers against fifth-stage larva (L5) and excretory/secretory (ES) antigens of *Angiostrongylus cantonensis* in cerebrospinal fluid (CSF) of different mouse strains. Different antibodies (IgM, IgG, and IgE) from 1 to 5 week *A. cantonensis*-infected and control mice CSF were examined using ELISA. L5 and ES: antibodies against L5 or ES antigens, respectively, were detected in mice CSF. Different color bars indicate different strains of mice. The results are shown as the means ± standard deviation (SD; n = 5–6). *p < 0.05, **p < 0.01, ***p < 0.001 indicate comparisons to the control group. #p < 0.05, ##p < 0.01, ###p < 0.001 indicate comparisons between two indicated groups.

that in BALB/c mice CSF at week 3. Antibodies against 105 kDa molecule in L5 antigen were detected in C57BL/6, but not BALB/c mice, similar to the IgG antibodies. Moreover, the antibodies against 85 kDa molecules were also detected in C57BL/6 mice in addition to those against 105 kDa molecules at week 4 (Fig. 5). In addition, the antibodies against 75 kDa molecule in ES antigens were detected in BALB/c mice like the IgG, which was not very evident in C57BL/6 mice. However, the antibodies against 105 kDa molecule were evidently detected in the C57BL/6 at week 5 and the antibodies against the 85 kDa molecule reduced, which are different from results of IgG distribution. Meanwhile, antibodies against 105 kDa molecule and 75 kDa molecule in the ES antigen were also detected in BALB/c mice at week 4.

Discussion

The host total IgE and specific IgE against L3 of *A. cantonensis* increase earlier in the non-permissive hosts than those in the permissive hosts.[18] Previous study reported the

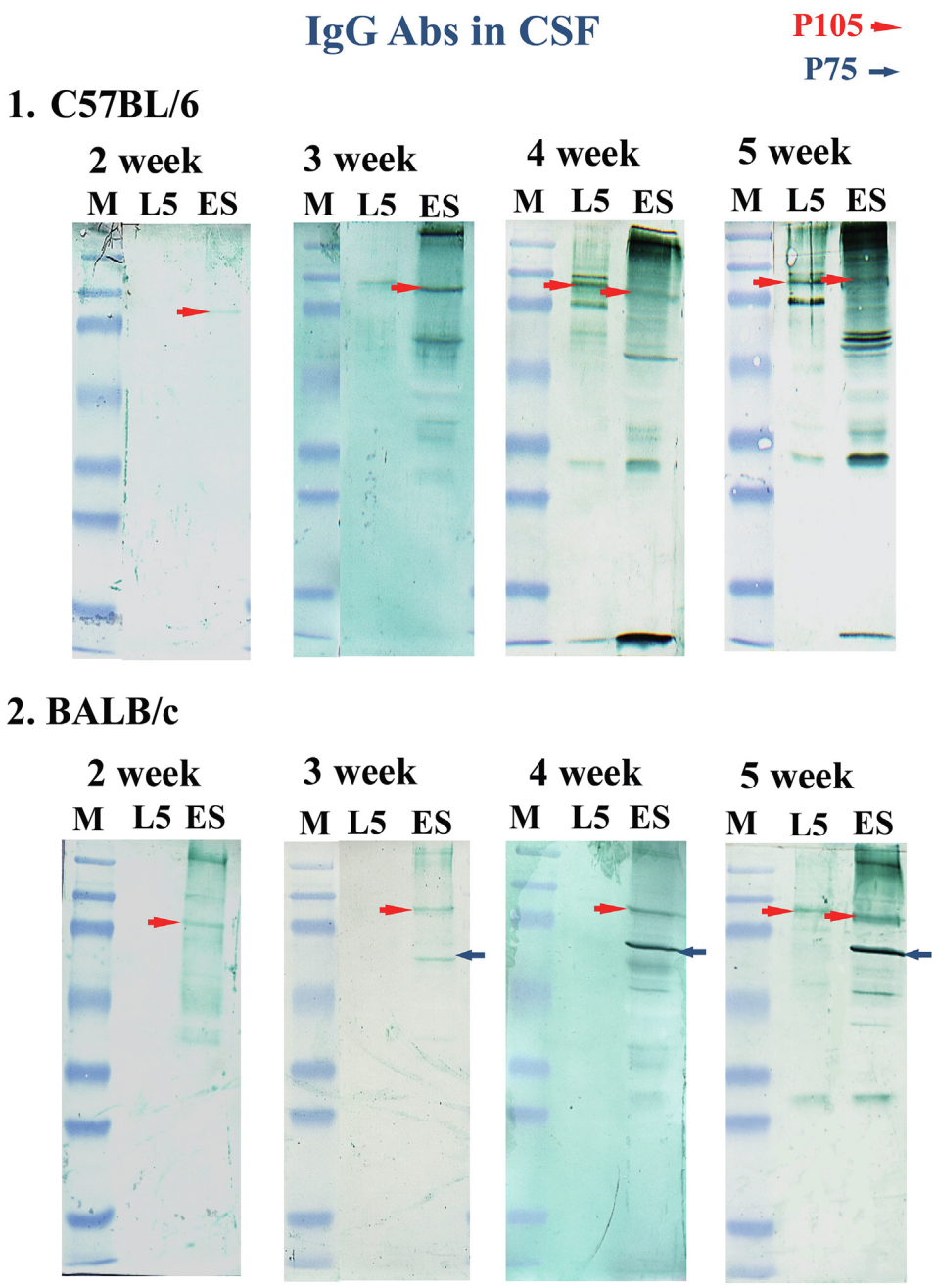

Figure 4. Western blotting analyses of the specific IgG responses against L5 and ES proteins in C57BL/6 and BALB/c mice cerebrospinal fluid (CSF). The L5 and ES antigens recognized by IgG in the CSF of the 2—5-week infected BALB/c and C57BL/6 mice. ES, Excretory/secretory antigens of L5 *Angiostrongylus cantonensis*; L5, the fifth-stage larvae antigens. A standard protein marker is shown to the left with 7.4, 21.0, 30.2, 36.3, 52.2, 84.0, 120.0, and 205.0 kDa molecular weights (lane M). Red arrow indicates specific 105 kDa protein; blue arrow indicates specific 75 kDa protein.

degrees of IgE antibody in different strains of rat serum with *A. cantonensis* infection are different and related the protection.[16,17] IgE antibodies in serum also increased after infection, but the specific IgE antibodies against the L5 antigens in serum did not increase significantly.[14] This study investigated the antibody-reactivity of L5 and its effect on the antibody responses in serum and CSF of different mouse strains.

Infecting different mouse strains with *A. cantonensis* resulted in varying levels of lethality and susceptibility due to different major histocompatibility complex genotypes.[36] Among the four strains used in this study, BALB/c mice had the shortest survival period after infection, which was negatively correlated with worm count. L5 recovery rate from the brain was the highest and the weight loss was the earliest and also the highest, indicating that BALB/c mice

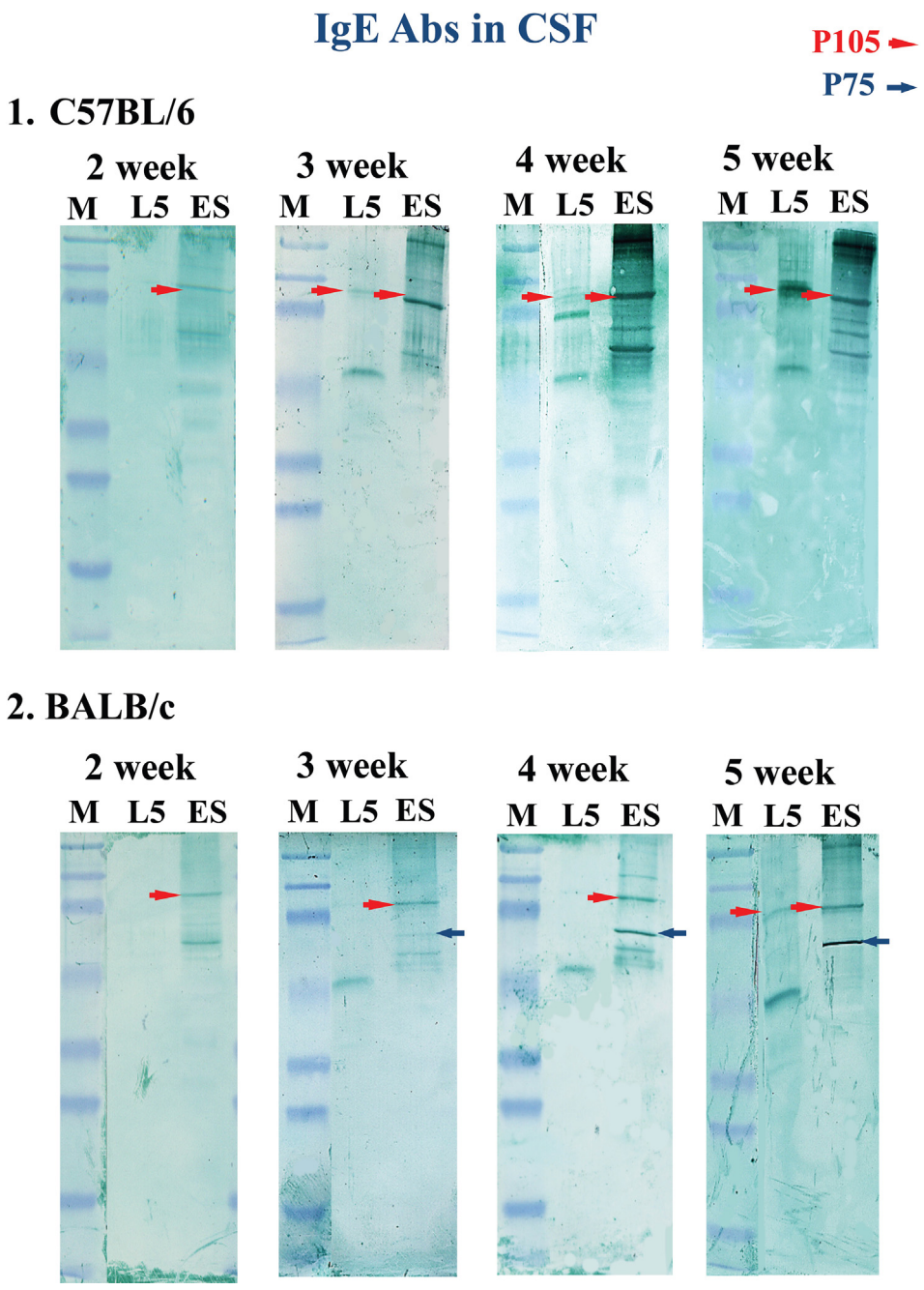

Figure 5. Western blotting analyses of the specific IgE responses against L5 and ES proteins in C57BL/6 and BALB/c mice cerebrospinal fluid (CSF). The L5 and ES antigens recognized by IgE in the CSF of the 2–5-week infected BALB/c and C57BL/6 mice. ES, Excretory/secretory antigens of L5 *Angiostrongylus cantonensis*; L5, the fifth-stage larvae antigens. A standard protein marker is shown to the left with 7.4, 21.0, 30.2, 36.3, 52.2, 84.0, 120.0, and 205.0 kDa molecular weights (lane M). Red arrow indicates specific 105 kDa protein; blue arrow indicates specific 75 kDa protein.

was the most susceptible to *A. cantonensis*. Interestingly, C57BL/6 and C57BL/10 mice have revealed strong resistance to *A. cantonensis* and their worm recovery rate approached zero even after week 4; in addition, their body weight did not decrease and even increased. The results were similar to those reported previously.[19] The eosinophil count increases after infecting the mice with *A. cantonensis*. Moreover, the appearance and decrease of eosinophils in BALB/c and CBA mice are earlier than those in C57BL/6 and C57BL/10 mice, showing that the eosinophil count in the blood and the worms infecting the brain may not be directly correlated. Previous study has demonstrated that eosinophils in blood are less effective in killing *A. cantonensis* than those in the CSF.[37] Sugaya and Yoshimura have also confirmed that generating eosinophils in the CSF is more consistent with the effect of parasite killing in the brain.[24] Therefore, the immune responses against *A. cantonensis* in brain may be more important than that in blood.

The levels of various antibodies in CSF and serum increased in humans infected with *A. cantonensis*.[26] In terms of correlation with the susceptibility of each mouse strain in this study, reactive IgE in CSF was most consistent with L5 recovery rate in the brain, followed by IgG, which may be related to IL-4-induced IgE and IgG1.[38] The increased degree of IgE antibodies against both L5 and ES antigens in BALB/c mice brain is significantly lower than that in other mouse strains; suggesting the effect of anti-L5 IgE in CSF may be more important than that in serum. Deborah et al. have shown that have shown that local IgE antibody in the parasitic tissue regions are more effective than systemic IgE by rejection study of gastrointestinal nematodes.[21,22] The combined action of IgG1, IgE, and eosinophils may be the main mechanism for killing *A. cantonensis* L5 in mice brains; particularly eosinophils are significantly involved in antibody-dependent cell-mediated cytotoxicity against parasites.[25,39,40] Moreover, reactive anti-ES antigen IgE antibodies increased the most in the brain of CBA mice with intermediate susceptibility, suggesting that *A. cantonensis* may have an immune mechanism to evade the host by interfering with antibody attack through ES antigens. In addition, serum anti-ES antigen IgG and IgE increased sharply in BALB/c mice after week 3 and continued until week 5. Eosinophils in BALB/c mice also increased after week 3, which co-occurred with death onset. Eosinophils contribute to inflammation initiation and modulation, including antibody-dependent cell-mediated cytotoxicity induction and cytotoxic granular protein degranulation, which are common eosinophil-mediated pathogenic mechanisms.[34,41] Whether the non-specific antibody-dependent cell-mediated cytotoxicity caused by ES antigen-induced antibody binding to eosinophils is one of the main causes of severe brain inflammation in BALB/c mice should be confirmed in further studies.

Deborah et al. studied the immune response induced by gastrointestinal nematodes using immunoprecipitation to show that the resistant host has an earlier and stronger appearance of antigen-specific IgE in the intestine than the susceptible host.[42] In this study, we detected IgE and IgG against L5 antigens earlier and more frequently in resistant C57BL/6 mice than that in susceptible BALB/c mice in CSF. An anti-105 kDa protein molecule was detected in C57BL/6 mice brain at week 3, which was consistent with the declined *A. cantonensis* recovery rate in C57BL/6 mice. This protein was also expressed in the BALB/c mice brain at week 5, which also coincided with the drop in L5 recovery rate of BALB/c mice. Therefore, the 105 kDa protein in the worm antigen may be one of the main antigens that cause the mice antibody responses against L5 *A. cantonensis*. Previous studies have found that the similar protein of the L5 antigens in the mouse brain may be an eosinophil chemotactic molecule to attract eosinophils to kill *A. cantonensis*.[37] The antigen that mainly reacts to the antibody appears earlier and more strongly in resistant mice, may be the target antigen against this parasite.[43] BALB/c mice contain an anti-75 kDa antibody against the ES antigen, which is not present in C57BL/6 mice. In addition, the antibodies against ES antigens are more and stronger than those against L5 antigens in both mouse strains, indicating that many antibodies produced by the host may be avoided by *A. cantonensis* via excreting ES antigens. Yen et al. have developed monoclonal antibodies against the L5 antigens of *A. cantonensis* that can react specifically with the 204 kDa L5 antigen.[44] Previous mass spectrometric experiment conducted a comparative analysis using sera from individuals with angiostrongyliasis to identify three antigenic proteins from ESP: ES-7, Lec-5, and 14-3-3, which represented promising diagnostic molecules for specific identification of *A. cantonensis* infection and all belong glycoproteins. Glycosylation is a very important post-translational modification for the recognition, and seem to be essential for efficient biding by the antibody.[45,46] However, few related reports have analyzed the effects of antibodies against *A. cantonensis* L5, particularly of IgE antibodies in the CSF. The 105 kDa antigen in the CSF detected here may have a considerable correlation to immunity against *A. cantonensis*.

Conclusion

Taken together, this study first analyzes antibody responses in serum and brains of different mouse strains due to angiostrongyliasis. We found that antibodies in mice CSF against L5 antigens related to worm rejection in mice brains. On the side correlated with susceptibility of different mouse strains, level of CSF IgE of susceptible BALB/c mice is lower than in others, suggesting IgE response in the CSF is more important than in serum. In addition, the antibody-dependent eosinophil-mediated cytotoxicity induced by anti-ES antibodies may be the reason of severe brain inflammation in infected BALB/c mice. We also suggest that 105 kDa protein of L5 antigen is related to the antibody responses of mice against *A. cantonensis*. This study may help to develop specific marker or vaccine for angiostrongyliasis that can be applied to the clinical diagnosis[47–49] and treatment in the future.

Declaration of competing interest

The authors do not have a commercial or other association that might pose a conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.08.012.