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ORIGINAL ARTICLE

Anti-ST2 monoclonal antibody inhibits eosinophil infiltration in *Angiostrongylus cantonensis*-infected mice

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Background/Purpose: Interleukin-33 (IL-33) could play an important role in the pathogenesis of angiostrongylosis. However, the role of IL-33/ST2 pathway in this parasitic infection is uncertain.
Methods: C57BL/six mice were each infected with 35 *Angiostrongylus cantonensis* larvae. One group of mice received an intraperitoneal injection of anti-ST2 monoclonal antibody (mAb; 50 μg) 3 days postinfection and subsequent booster shots of the same dose at 5-day intervals. Blood samples from each group were collected every week for assays.
Results: The level of IL-5 significantly decreased in the mAb-treated group, and the infiltration of eosinophils in the meninges was also significantly reduced.
Conclusion: The IL-33/ST2 axis may play a crucial role in the pathogenesis of angiostrongylosis and the results of this study could be useful for the development of strategies to reduce the neurological damage caused by this parasitic infection.

Introduction

Angiostrongylus cantonensis, the rat lungworm, is a common causative agent of human eosinophilic meningitis or eosinophilic meningoencephalitis in Taiwan.[1] The parasitic disease is also endemic to mainland China, Japan, some Pacific islands, and Southeast Asia.[2,3]

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Humans contract this parasitic disease by ingesting infective third-stage larvae (L3) that are found in raw or undercooked mollusks, which are the intermediate hosts for *A. cantonensis*. The ingested larvae penetrate into blood vessels in the intestinal tract and are carried to the central nervous system, but are unable to migrate to the lungs, as they do in rats. Most of the larvae develop into young adults (L5) and then die shortly after reaching the subarachnoid space, and hence do not complete the developmental cycle.

When nonpermissive hosts, such as mice, are infected with *A. cantonensis*, the immune response is primarily of the Th2-type, including eosinophilia, increased immunoglobulin E (IgE) antibody levels in the blood and cerebrospinal fluid, and the expression of Th2-type cytokines, especially interleukin-5 (IL-5), IL-4, and IL-33.[4–8]

In *A. cantonensis*-infected mice, eosinophils appear to release some granules that are able to kill the larvae, but some cytotoxic proteins, such as eosinophil protein X and eosinophil cationic protein, can damage the nervous tissues of the host.[9,10] In addition to mechanical injuries caused by the migrating larvae, the proteins produced during eosinophil infiltration and degranulation may be important factors that contribute to the immunopathology of angiostrongylosis.[6,10–12]

Eosinophils act as immunoregulatory cells and are able to produce many types of cytokines;[13,14] however, the mechanisms that regulate the biological function of these cells are uncertain. It has been shown that several factors could enhance the survival and/or the functions of mature eosinophils, such as IL-5, IL-13, and granulocyte-macrophage colony-stimulating factor.[15] IL-5 is an important stimulant for eosinophil, with functions that include the promotion of degranulation and superoxide production. IL-5 also synergises with various stimulating factors to increase eosinophil progenitor production and eosinophil expansion.[12,16,17] Specific inhibitors of IL-5, such as the anti-IL-5 monoclonal antibody (mAb), were able to attenuate eosinophil-associated inflammation.[18,19]

IL-33 belongs to the IL-1 family and has been shown to be a promoter of Th2-type immune response and systemic inflammation both *in vivo* and *in vitro*.[20,21] In a study of patients with asthma, IL-33 induced production of Th2-type cytokines and was associated with mucus overproduction and goblet cell hypertrophy in the lungs and the gastrointestinal tract. Furthermore, the increased expression of this cytokine may be a novel inflammatory marker of asthma.[22] In addition, blockage of IL-33 by anti-IL-33 mAb inhibits airway inflammation in an animal model. It is demonstrated that IL-33 mediated the expressions of IL-5 and IL-13 in *A. cantonensis*-infected mice. Blockage of IL-33 by anti-IL-33 mAb inhibits IL-5 expression in animal models.[23] IL-33 could play an important role in the pathogenesis of angiostrongylosis and also provide a new therapeutic target for this parasitic disease.

IL-33 has a variety of effects on inflammatory cells through the ST2 receptor.[20] The binding of IL-33 to the ST2 receptor activates inflammatory cells through the nuclear factor-κB and mitogen-activated protein kinases pathways and induces the expression of Th2-type cytokines. Blockage of IL-33 signaling by a soluble ST2 protein or anti-ST2 mAb inhibits the immune response of asthma in mice.[24,25]

For elucidating the role of IL-33/ST2 pathway in the pathogenesis of angiostrongylosis, in this study, mice were experimentally infected with larvae of *A. cantonensis* and received injections of anti-ST2 mAb. The effects of anti-ST2 mAb were assessed by pathological examination and by measuring eosinophil percentage and the levels of IgE and cytokines in the peripheral circulation.

Materials and methods

Animals

A. cantonensis was maintained in the laboratory using male Wistar rats (150 g/body weight when first infected) as the final host and the hermaphroditic freshwater snail *Biomphalaria glabrata* as the intermediate host. Male C57BL/six mice (20 g/body weight) were used as the nonpermissive host in this study. The infective larvae (L3) were collected from experimentally infected snails.

The rats and mice were specified as pathogen free and were all purchased from the laboratory of the Animal Centre at the National Taiwan University College of Medicine (Taipei, Taiwan). Rats and mice were housed in accordance with the institutional guidelines. The experiments in this study were previously reviewed by an animal ethics committee. During the experimental period, all mice survived, and the gain in body weight of the mice in the normal control, infected, and treatment groups was similar.

Treatments

The mice were randomly divided into four groups. Each group contained 10 mice for blood collection and 12 mice for pathological examination. The four groups included a noninfected group, an *A. cantonensis*-infected group, and anti-ST2 mAb-injected groups with or without infection.

In the *A. cantonensis*-infected groups, each mouse was orally infected with 35 infective larvae and was later euthanized by anesthesia. In the injected groups, the mice were injected with anti-ST2 mAb (50 μg in 0.1 mL of phosphate-buffered saline; R&D Systems Inc; USA) intraperitoneally 3 days postinfection (dpi) and subsequently injected with the same dose on Day 8, Day 13, Day 18, Day 23, Day 28, and Day 33. Blood samples from all mice were collected from the tail vein every week for thin blood smear and serum collection. The serum samples from each group were pooled for the cytokine and IgE assays. The brains of infected mice were removed for pathological examinations.

Cytokine and IgE assays

The levels of cytokines and IgE in sera were determined with enzyme-linked immunosorbent assay (ELISA) and Activity Assay kits (R&D Systems Inc) and a mouse IgE ELISA kit (Shibayagi Co., Ltd., Japan) according to the manufacturer's instructions by two independent experiments. Approximately 50 μL serum sample was used in each test. The sensitivities of the kits were <2 pg/mL for IL-4 and interferon-γ (IFN-γ), and <7 pg/mL for IL-5.

Pathological examinations

At 14 dpi, 21 dpi, 28 dpi, and 35 dpi, three of the infected mice in each group were sacrificed. The brains of mice were removed and fixed immediately in 10% (vol/vol) neutral formalin. The brain samples were embedded in paraffin wax and cut into 4–5-μm sections. The sections were prepared from the frontal lobe, the central portion of the cerebrum, the mesencephalon, and the medulla–cerebellum, and were stained with hematoxylin and eosin.[6] At least 10 fields were observed from each section under a light microscope at low (100×) and high power (400×).

Statistical analysis

Results were shown as mean ± standard deviation. Statistical significance was assessed by Student *t* test to determine significance. A *p* value <0.01 was taken to be significant.

Results

Eosinophil percentage in differential white blood cell counts

The differential cell count of white blood cells in each group was estimated every week with a thin blood smear after the infection (Fig. 1). In noninfected mice, there was no significant difference between the untreated and anti-ST2 mAb-treated groups. Compared with the noninfected mice, the eosinophil percentage was significantly increased in the *A. cantonensis*-infected group ($p < 0.01$). When the infected mice were injected with anti-ST2 mAb, the eosinophil percentage was significantly decreased at 14 dpi, 21 dpi, and 28 dpi ($p < 0.01$).

The levels of IgE in the sera

The levels of IgE in the sera were analyzed every week in each group after the infection (Fig. 2). In noninfected mice, there was no significant difference between the untreated and anti-ST2 mAb-treated groups.

Mice that were infected with *A. cantonensis* had levels of IgE that were significantly increased with or without anti-ST2 mAb injection from the 1st week after the infection ($p < 0.01$). However, the levels of IgE significantly decreased in the mAb-injected group at the 7 dpi and 14 dpi ($p < 0.01$).

Expression of cytokines

The expression levels of various cytokines were all determined by ELISA. In *A. cantonensis*-infected mice, the levels of IL-5 significantly increased at 14 dpi, 21 dpi, and 28 dpi ($p < 0.01$), and then significantly decreased when the infected mice were injected with anti-ST2 mAb (Fig. 3).

The levels of IFN-γ and IL-4 in all groups were very low and nearly undetectable.

Pathological examinations

Dilatation of the skull and softening of the cranial bone were observed macroscopically in *A. cantonensis*-infected mice at 14 dpi, 21 dpi, 28 dpi, and 35 dpi with or without treatment. The sections of all infected groups revealed

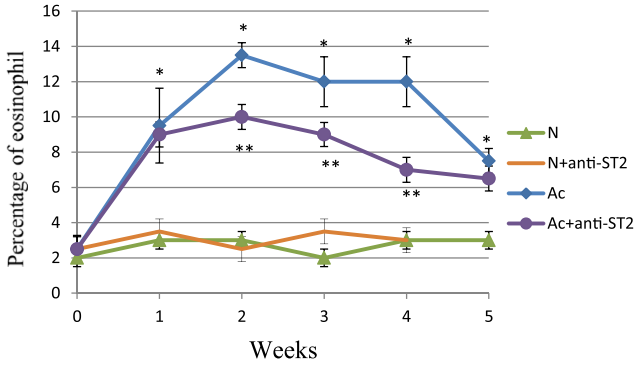

Figure 1. Eosinophil percentage in differential white blood cell counts was decreased when *Angiostrongylus cantonensis*-infected mice received the injections of anti-ST2 monoclonal antibody (mAb). The blood samples were collected every week for thin blood smear. N = noninfected group; Ac = *A. cantonensis*-infected group; each mouse was orally infected with 35 infectious larvae. +anti-ST2 = mice received injections of anti-ST2 mAb (50 μg) 3 days postinfection and subsequent booster injections of the same dose at 5-day intervals. Data are presented as mean ± standard deviation; $n = 10$. * Significantly increased, related to the N group, $p < 0.01$. ** Significantly decreased, related to the Ac group, $p < 0.01$.

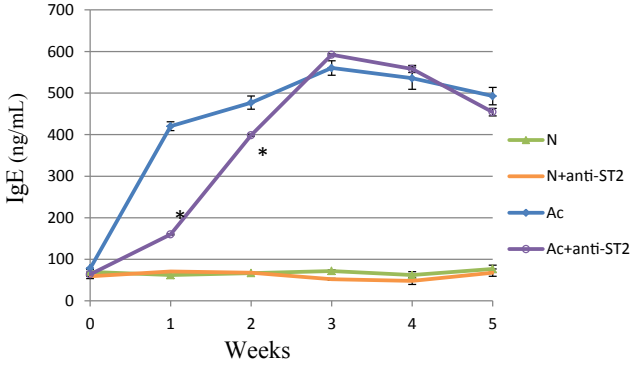

Figure 2. The levels of immunoglobulin E (IgE) were significantly increased in *Angiostrongylus cantonensis*-infected and anti-ST2 monoclonal antibody (mAb)-injected mice. The serum samples from each group (10 mice) were collected every week and pooled for enzyme-linked immunosorbent assay. N = noninfected group; Ac = *A. cantonensis*-infected group; each mouse was orally infected with 35 infectious larvae. +anti-ST2 = infected mice received injections of anti-ST2 mAb (50 μg) at 3 days postinfection and subsequent booster injections of the same dose at 5-day intervals. Data are presented as mean ± standard deviation and are representative of two independent experiments. * Significantly decreased, related to the Ac group, $p < 0.01$.

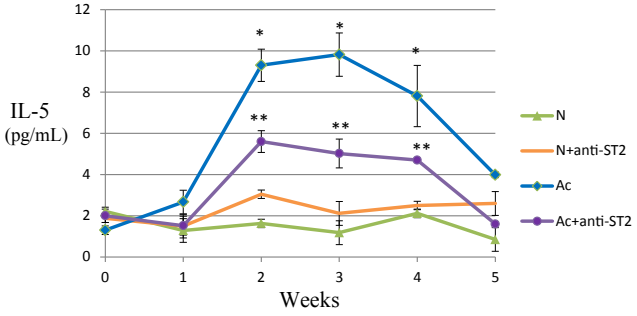

Figure 3. Anti-ST2 monoclonal antibody (mAb) reduces the expressions of interleukin-5 (IL-5) in the blood of *Angiostrongylus cantonensis*-infected mice. The serum samples from each group (10 mice) were collected every week and pooled for enzyme-linked immunosorbent assay. N = noninfected group; Ac = *A. cantonensis*-infected group; each mouse was orally infected with 35 infectious larvae. +anti-ST2 = infected mice received injections of anti-ST2 mAb (50 μg) at 3 days post-infection and subsequent booster injections of the same dose at 5-day intervals. Data are presented as mean ± standard deviation and are representative of two independent experiments. * Significantly increased, related to the N group, $p < 0.01$. ** Significantly decreased, related to the Ac group, $p < 0.01$.

hemorrhage and traumatic lesions caused by migrating larvae in the parenchyma of the brains.

The average width of the meninges in normal mice was approximately 3 μm (Fig. 4A). There was no infiltration of inflammatory cells in noninfected mice that were treated with anti-ST2 mAb (Fig. 4B).

In *A. cantonensis*-infected mice, meningitis with the infiltration of a large number of inflammatory cells was observed in untreated (Fig. 4C and D) and mAb-injected mice (Fig. 4E and F) at 21 dpi. The average width of the meninges in 10 fields was 51.22 ± 6.61 μm in the untreated group and 53.33 ± 4.71 μm in the mAb-injected group. The percentage of eosinophil in infiltrated cells was 63.33% ± 4.78% in the untreated group and 39.0% ± 3.56% in the mAb-injected group. Meningeal infiltration of eosinophils was significantly reduced ($p < 0.01$).

Discussion

The accumulation and activation of eosinophils in the brain is an important hallmark of *A. cantonensis* infection in both humans and mice.[6,26] IL-5, a Th2-type cytokine, enhances the maturation and survival of eosinophil,[15,27] and is essential in the orchestration of eosinophil-associated inflammation. Exogenous IL-33 enhances the expression of IL-5 in noninfected and *A. cantonensis*-infected mice.[23] It is believed that both IL-5 and IL-33 play crucial roles in the pathogenesis of angiostrongylosis. Reducing the expressions of IL-5 and IL-33 is also a useful approach to treat angiostrongylosis.[6,23]

According to our study, the expression of IL-5 obviously increased in *A. cantonensis*-infected C57BL/six mice. However, it decreased in anti-ST2 mAb-injected mice. IL-33 could mediate the expression of IL-5 by signaling through ST2 receptor in angiostrongylosis.

IL-33 enhances the production of IgE in allergic diseases and is a requisite for IL-13-driven Type 2 responses during hookworm infection.[28,29] The level of IgE increased in mice when mice were infected with *A. cantonensis*. Thus, the role of IgE in angiostrongylosis is uncertain. In *A. cantonensis*-infected mice that were treated with anti-ST2 mAb, the expression of IgE may still be enhanced even though the level of IL-5 was significantly lower than that in untreated mice. IgE seems to be enhanced through a mechanism mediated by IL-5 rather than by IL-33. The expression of IgE at the 1st week and 2nd week after the infection significantly decreased. Blocking of IL-33 by anti-ST2 mAb could delay the production of IgE.

ST2 is a transmembrane glycoprotein in the IL-1 receptor family. This receptor is expressed on various types of cells, including epithelial cells, mast cells, fibroblasts, eosinophils, and Th2 lymphocytes.[30,31] Eosinophils seem to be the direct target leukocyte for IL-33 but not neutrophils. IL-33 induces adhesion and CD11b expression and enhances survival of eosinophils.[32,33] *In vivo* treatment with IL-33 leads to eosinophilia in *A. cantonensis*-infected mice.[8] It has been demonstrated that anti-IL-33 mAb could prevent the pathological development of asthma in a mouse model.[34]

In our study, the percentage of eosinophil in white blood cells increased in *A. cantonensis*-infected mice. However, the percentage significantly decreased when the infected mice received anti-ST2 mAb injections. According to the pathological examinations, eosinophilic meningitis was noticed in *A. cantonensis*-infected mice. The injection of anti-ST2 mAb did not alter the severity of meningitis. This could be because the amount of mAb injected in our study is not enough to reduce the immunopathology, or because the antibody is not functional. More tests are necessary to determine the function and appropriate dose for the injected antibody. However, the proportions of cell types in the treated and untreated groups were different. Meningeal infiltration of eosinophils in anti-ST2 mAb-treated mice was markedly reduced. The IL-33/ST2 axis may play an important role in eosinophil expansion in *A. cantonensis* infection.

In addition to eosinophils, lymphocytes, plasma cells, and other inflammatory cells are also infiltrated into the meninges, but more detailed studies are needed to determine the types and ratios of these infiltrated cells in untreated and mAb-treated mice.

IL-33 has been shown to modulate intestinal nematode expulsion by inducing the Th2 adaptive response.[35] Eosinophils are able to release some granules for killing the larvae, and have been thought to be responsible for innate resistance in nonpermissive hosts in angiostrongylosis.[6] More tests are necessary to determine the larvae recovery and prognosis of *A. cantonensis*-infected mice that are treated with anti-ST2 mAb.

In conclusion, the results of this study show that blockage of IL-33 signaling by anti-ST2 mAb could decrease the expression of IL-5, delay the production of IgE, and reduce the infiltration eosinophils. The IL-33/ST2 axis may play a crucial role in the pathogenesis of angiostrongylosis, and could be useful for the development of strategies to reduce the neurological damage caused by this infection.

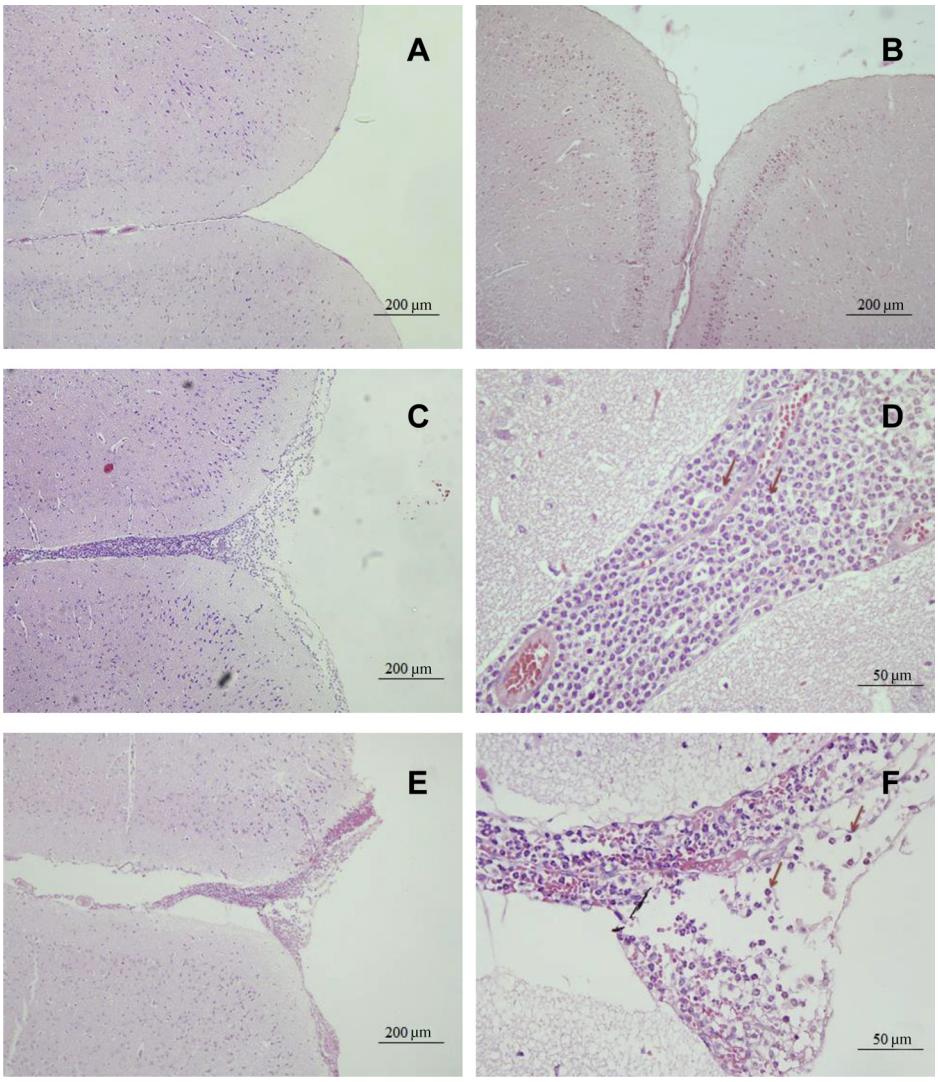

Figure 4. Histopathological examinations in the brains of mice. All the mice were sacrificed at 21 days postinfection. There was no inflammatory response in (A) noninfected mice and (B) those treated with anti-ST2 monoclonal antibody (mAb). (C,D) Sections showing meningitis with eosinophilic infiltration (arrows) in *Angiostrongylus cantonensis*-infected mice and (E,F) those treated with anti-ST2 mAb.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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