의학박사 학위논문

Pathogenesis and Immune Reaction in C57BL/6 and CBA/N Mice Infected with *Clonorchis sinensis*

간흡충에 감염된 C57BL/6 마우스와 CBA/N 마우스 모델에서 병리학적 및 면역반응 연구

2012 년 12월

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by

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ABSTRACT

To develop a mouse model for the early immune responses in clonorchiasis, the CBA/N mouse, in which Th1 and Th17 responses dominate, and the C57BL/6, in which the Th2 response is dominant were examined. Two different strains of mice were infected with *Clonorchis sinensis* metacercariae and analyzed 2, 4, and 8 weeks later. CBA/N mice infected with *C. sinensis* developed more severe gross and histopathological changes than C57BL/6 mice, including more pronounced inflammatory cell infiltration and hepatocyte necrosis in CBA/N mice, especially at 2 weeks post-infection. Additionally, serum biomarkers of liver injury (ALT and AST) were increased significantly in CBA/N mice after *C. sinensis* infection, compared with those in infected C57BL/6 mice. Proliferation of splenocytes and cytokine production of the splenocytes was analyzed after stimulation with crude *C. sinensis* antigen. The splenocytes from CBA/N mice were highly proliferative and the IL-17 level was increased. Additionally, an increased IL-4 level was sustained until 8 weeks post-infection in CBA/N mice. The serum levels of *C. sinensis*-specific IgG1 and IgG2a were increased in CBA/N mice, whereas IgE was increased in C57BL/6 mice. To directly ascertain the role of IL-17 in the development of *C. sinensis*-induced liver inflammation, IL-17-neutralizing mAb were treated after infected with *C. sinensis*. Because IL-17 production was increased from 2 weeks p.i., infected C57BL/6 and

CBA/N mice were systemically administrated with anti-IL-17 or rat IgG_{2a} isotype control and the mice were sacrificed on 2 weeks p.i. Anti-IL-17 mAb, but not the control mAb, significantly improved the liver histopathological appearance. Accordingly, anti-IL-17 mAb-treated mice displayed attenuated liver injury, as indicated by considerably decreased levels of serum ALT (from 284 ± 162.8 to 70 ± 16.4) and AST (from 215 ± 65.5 to 121 ± 18.6) in CBA/N mice. Intrahepatic lymphocytes and splenocytes were isolated from each mouse and counted. Their numbers were significantly decreased from *C. sinensis*-infected mice treated with IL-17-neutralizing mAb than the positive control both in CBA/N mice. In conclusion, the CBA/N mouse is a good animal model to study early immune response after infection with *C. sinensis*. The infected CBA/N mice stimulate differentiation of pathogenic Th17 cells as well as to produce humoral immunity by B cells. IL-17 is playing a key role for development of severe immunopathology of the liver in early stage of clonorchiasis.

Keywords: Clonorchiasis, Mouse, CBA/N, C57BL/6, Cytokines, IL-17, Immunoglobulin.

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
AST	Aspartate transanimase
CA	Crude extract of *C. sinensis*
IHL	Intrahepatic lymphocytes
IL	Interleukin
SPL	splenocytes

INTRODUCTION

Clonorchis sinensis is a liver fluke that parasitizes the bile ducts of humans, dogs, cats, pigs, and minks. This liver fluke is mainly distributed in eastern Asia, including Korea, China, Japan, Taiwan, Vietnam, and far-eastern Russia [1]. It is estimated that over 20 million people worldwide are to be infected with this liver fluke [2]. Although clonorchiasis is uncommon in western countries, it is not difficult to find cases as a result of increased immigration [3] and imported freshwater fish containing *C. sinensis* metacercariae from endemic areas [4, 5]. In Korea, the last national survey on the status of intestinal helminthiasis in 2004 recorded a 2.9% egg positive rate of *C. sinensis* in the general population [6]. The data estimate 1.3 million people of clonorchiasis in Korea. Therefore, clonorchiasis is one of major public health issues in Korea.

After infection with *C. sinensis*, the juvenile flukes migrate to the intrahepatic bile duct via the hepatopancreatic ampulla [5]. Then, the adult worms parasitize the bile duct and produce eggs approximately 4 weeks after ingestion of the metacercariae [5]. The clinical pathology of clonorchiasis in humans is mainly caused by adult worms, which are present in the bile duct. Infected intrahepatic or extrahepatic bile ducts undergo severe pathological changes, like adenomatous hyperplasia of the biliary epithelium, mucin-secreting metaplasia, ductal dilatation, periductal inflammation and fibrosis, and dysplasia or neoplasia of biliary cells [7, 8]. Clonorchiasis causes

cholangitis with marked eosinophil infiltration, bile duct obstruction, and subsequent cholangiofibrosis in the liver. Ongoing exposure can predispose to cholangiocarcinoma [7-9].

Clonorchiasis is a significant risk factor of cholangiocarcinoma (CCA) in humans which has been confirmed by several studies. For *in vitro* evidence, excretory-secretory products (ESP) from *C. sinensis* induced cell proliferation of human embryonic kidney 293 cells. It was proliferated in the G2/M phase and cell cycle proteins such as E2F1, p-pRb, and cyclin B were increasingly expressed [10, 11]. Besides that the ESP of *C. sinensis* increased proliferation of HuCCT1 cholangiocarcinoma cells and augmented the expression of cyclooxygenase (COX)-2 and cells pretreated with ESP were resistant to parthenolide-induced apoptosis [12]. The age-standardized incidence of CCA was relatively high in endemic areas of liver flukes including Thailand, China and Korea. Especially the incidence of extrahepatic CCA was the highest in endemic areas in Korea among Asian countries [13]. Based on epidemiological data, the International Agency for Research on Cancer (IARC) classified *C. sinensis* as a group I carcinogen, causing cholangiocarcinoma in humans in 2009 [14]. The effects of *C. sinensis* infection in humans are one way to investigate the mechanism of cholangiocarcinoma and the associated immune response.

Hamsters were frequently used as an animal model for cholangiocarcinoma studies [15, 16]. However, this experimental animal is

not appropriate for evaluating the pathogenesis or immune responses associated with *C. sinensis*, because the commercial reagents for hamsters are very limited. Therefore, mice may serve as a good research model because of plenty of reagents.

A number of mouse models have been tried to investigate the host–parasite interaction of *C. sinensis*. Yoon et al. [17] compared two immunologically competent (BALB/c and FVB/NJ) and two immunodeficient (Nude and SCID) mice for the study of *C. sinensis* pathogenesis. They described FVB/NJ as a suitable model for the host-parasite relationship study, while BABL/c mice were relatively unsusceptible. This susceptibility variation is thought to be associated with Th2 cytokine production, especially IL-4 in FVB mice; which also had significantly higher serum IgE levels than BALB/c mice [19]. However, they suspected that the mouse haplotype could have some relation in regards to susceptibility. The relatively susceptible FVB/NJ mice have the H2q haplotype while BALB/c mice have the H2d haplotype, which is resistant to *C. sinensis* infections. Furthermore, to compare the intraspecific variation in the host–parasite relationship of *C. sinensis*, 6 strains of mice (ICR, BALB/c, C57BL/6, DDY, CBA/N, and C3H/HeN) with 3 different haplotypes were evaluated on their susceptibility [20]. Consequently, mice are insusceptible to infection with *C. sinensis*; however, the C57BL/6, BALB/c and ICR strains are relatively susceptible after 8 weeks of infection. Elevated IgE, IFN-γ, and IL-13 of infected mice suggested both Th1 and Th2 responses

that may be related to the low host susceptibility [20]. An overview of these studies is summarized in Table 1.

The CBA/N mouse strain is more prone to developing Th1 and Th17 responses, and shows severe pathology in response to *Schistosoma mansoni* infection [21-23]. Two distinct pathological syndromes were observed in male CBA/N/J inbred mice with chronic *S. mansoni* infections [24, 25]. Genetically determined, high pathology schistosomiasis linked with a Th1 response is also observed in certain inbred mouse strains, such as C3H and CBA/N (both H-2k), but not in others, such as C57BL/6 (BL/6; H-2b) [26, 27]. In the case of clonorchiasis, the susceptibility of mice to *C. sinensis* infection differs by mouse strain [28].

Helminths are known to skew the immune response towards Th2, characterized by Th2 related cytokines that typically include IL-4, IL-5 and IL-13 that induce B-lymphocytes to switch to IgE antibody production. In addition, helminths are multicellular and long-lived organisms (more than 20 years for *C. sinensis*) and mostly they are not able to replicate within human host. Therefore the use of antigenic variation to escape from the hosts' immune attack is not possible. In addition they are too big to sequestrate in specialized niches away from the immune system. However, helminths are thought to have developed different strategies for survival in the human host.

$CD4^+$ T cells play an important role in the initiation of immune responses against an infection by providing help to other cells and by taking on a variety

of effector functions during immune reactions. Upon antigenic stimulation, naive $CD4^+$ T cells activate, expand and differentiate into different effector subsets termed T helper 1 (Th1) and Th2 cells. The appropriate induction and balance between Th1 and Th2 cellular responses to an infectious agent can influence both pathogen growth and immunopathology [29]. Nevertheless, during the past several years, the Th1/Th2 paradigm has been updated to include a third helper subset called Th17. The Th17 cells recently emerged as a third independent effector cell subset differentiated from $CD4^+$ T cells upon antigenic stimulation [30-33].

In the present study, a more appropriate mouse model was evaluated by studying the pathogenesis and host immune response of *C. sinensis* infection. Also, Th17 response was evaluated after *C. sinensis* infection in C57BL/6 and CBA/N mouse strains and analyzed the role of Th17 cells in the host protective responses.

Table 1. Studies that have examined the relationship between *Clonorchis sinensis* and host immune responses

Animals/ mouse strain	Worm recovery rate	Major findings	Th cell skewing	Refer-ences
Hamster	57.9%	Suitable final host		[15]
FVB/NJ	6.7-33.3%	Cystic formation and fibrosis	-	[16]
BALB/cA	6.7%	Fibrosis		
Nude	6.7%	Few pathological change		
SCID	6.7%	Few pathological change		
FVB/NJ BALB/cA	-	IL-4↑↑ ; IgE↑↑ ; IL-5↑ IL-10↑↑; IFN-γ↑↑; IL-2↑↑; IL-5↑	Th2	[17]
Rat/Human	-	Apoptosis of hepatocytes	-	[18]
Rat	83.3%	IgG1↑ ; IL-4↑ ; IgE↑	Th2	[18]
FVB	-	IL-4, IL-5; IL-13; IL-10; TGF-β↑	Th2	[19]
ICR	0	IgE, IgG1, IgG2a, IFN-γ↑	Th1/Th2	[20]
BALB/c	16%	IgE, IgG1, IgG2a↑		
C57BL/6	9.3%	IgE, IgG1, IgG2a, IL-10, IL-13, IFN-γ↑		
DDY	11.3%	IgE, IgG1, IgG2a↑		
CBA/N	8.3%	IgE, IgG1, IgG2a, IFN-γ↑		
C3H/HeN	20.7%	IgE, IgG1, IgG2a, IFN-γ↑		

MATERIALS AND METHODS

1. Preparation of *C. sinensis* crude antigen

(1) Animals

Male Sprague–Dawley rats at 6-8 weeks of age were purchased from the Koatech Co. (Seoul, Korea), and housed in an ABL-2 animal facility in Seoul National University College of Medicine. All rats were bred in filter cages under positive pressure according to institutional-approved guidelines.

(2) Collection of metacercariae of *C. sinensis*

Pseudorasbora parva, the second intermediate hosts of *C. sinensis*, which were naturally infected with *C. sinensis*, were purchased at Shenyang, Liaoning Province, People's Republic of China, which is an endemic area of clonorchiasis. Metacercariae of *C. sinensis* were collected by digesting fish with a pepsin-HCl (0.6%) artificial gastric juice for 1h at 37°C.

(3) Preparation of crude extract (CA) of *C. sinensis*

Sprague–Dawley rats were individually infected orally with 50 metacercariae of *C. sinensis*. Eight weeks post-infection, adult worms were collected from bile ducts and washed 3 times with phosphate buffered saline (PBS). To produce crude extracts, the worms were homogenized in PBS on ice. The homogenate was centrifuged for 30 min at 13000 rpm and 4℃, and

filtered using a syringe-driven 0.2-μm syringe filter. Protein levels in the crude antigen were measured using a Pierce BCA protein assay kit (Thermo, Rockford, IL, USA).

2. Animals and experimental design

(1) Animals

Male C57BL/6 mice and CBA/N mice were obtained from SLC Inc. (Hamamatsu, Kotoh-cho, Japan) and maintained in ABL-2 (animal bio-safety level 2) conditions in accordance with institutional and national guidelines. Five-weeks-old male mice were used in all experiments. All mice were bred in filter cages under positive pressure according to the institutional guidelines.

(2) Experimental design

For the experiments, the mice were divided in four groups: C57BL/6 control, C57BL/6-*C. sinensis* infected, CBA/N control, and CBA/N -*C. sinensis* infected. Five mice were applied for each group. Thirty metacercariae of *C. sinensis* were infected orally to each of the infected groups and maintained for 2, 4, and 8 weeks. All experiments were approved and conducted in accordance with the guidelines established by the institutional animal care and use committee (SNU-100117-1) and institutional biosafety committee (SNUIBC-091127-1).

3. Gross observation, worm recovery and histopathology

To evaluate pathological changes in the livers between experimental groups, the livers of mice were grossly observed with naked eyes. Adult and juvenile worms were recovered from the liver using a combination of stereomicroscopic observations as well as a Baermann worm collection technique. Five livers from each group were fixed in 10% phosphate-buffered formalin for 24 hr and embedded in paraffin wax. Paraffin-embedded liver samples were cut in 5-μm-thick sections on a microtome. Sections were deparaffinized in two changes of xylene for 10 min, rehydrated in 100% ethanol, 90% ethanol, 80% ethanol and 70% ethanol for 5 min each, and stained with hematoxylin and eosin (H&E) for 45 sec. The entire section of the liver was evaluated blindly by a board-certified pathologist.

4. Counting and preparation of supernatants from splenocytes cultures

The spleens from uninfected controls or *C. sinensis*-infected C57BL/6 and CBA/N mice were extracted under anesthesia. Five mice of each strain were used at each time point and the splenocytes were collected by gently grinded with autoclaved glass and flushed 4 times with 0.5 ml of cold PBS. Then the cells were spun down at 1,500 rpm for 5 min at 4^{o}C. Red blood cells were lysed with RBC lysis buffer and washed with cold PBS and splenocytes were counted. A total of 5 x 10^{6} viable nucleated cells in 1 ml of DMEM containing

10% fetal bovine serum were cultured individually in a 24-well tissue culture plate at 37℃ in an atmosphere containing 5% CO_2. Cells were stimulated with 20 μg/ml crude *C. sinensis* antigen or PBS. Cell-free supernatants were harvested after 72 h for cytokine analysis and were stored at -70℃ until use.

5. Cytokine assay

A sandwich ELISA with mouse ELISA kit (eBioscience, San Diego, CA, USA) was used to measure IL-1, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TNF-α, TGF-β and IFN-γ in the culture supernatants of splenocytes. During each step in protocol, plates were washed 5 times. The capture antibodies were coated on a 96-well plate for 2 hr at room temperature. Standard cytokines at several concentrations or supernatants of splenocytes that were not diluted were incubated on the plates for 1 hr at room temperature. After peroxidase-labeled streptavidin conjugates were added and incubated for 1 hr at room temperature, development by substrate (3,3′,5,5′-tetramethylbenzidine) was carried out for 30 min at room temperature. Reactions were stopped by adding 4 N H_2SO_4, and the absorbances were measured at 450 nm using a spectrophotometer (Amersham Bioscience, Cambridge, UK)

6. ELISA for serum antibody titers

Total and CA-specific antibody titers were determined by mouse

immunoglobulin ELISA kit (Bethyl, Montgomery, TX, USA). To determine the optimal condition, serum samples and conjugates were diluted with sample/conjugate diluent solution. During each step in protocol, plates were washed 3 times. The total and CA specific antibody titers were assayed using anti-IgE, IgG1, IgG2a antibodies and CA. Peroxidase-labeled streptavidin conjugates were added and reactions were developed. After termination of the color reaction, the absorbances were measured at 450 nm using a spectrophotometer.

7. Splenocyte proliferation assay

The XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) formazan method was used to measure cell proliferation. XTT (1 mg/ml) was dissolved in warm medium (without phenol red), and 1.25 mM phenazine methosulfate (PMS) was prepared in PBS. The splenocytes were grown in tissue culture grade, 96-well, flat-bottom microtiter plates with 100 μl of culture medium (DMEM-phenol red free) per well. After incubating for the indicated time periods, 50 μl of the XTT-PMS mixture (final XTT concentration, 0.3 mg/ml) was added to each well. The microtiter plates were incubated for 4 h at 37°C, and the formazan product was quantified by measuring the absorbance at 492 and 690 nm on a microtiter plate reader. All experiments were performed in triplicates.

8. Serum biomarkers for liver injury

Blood samples were obtained from mice of all groups (5 mice per group) for determination of serum biomarkers for liver injury. Serum alanine transaminase (ALT) and aspartate transanimase (AST) were assayed according to by Japan Society of Clinical Chemistry (JSCC) UV method in Clinical Research Institute Seoul National University Hospital (IACUC NO. 10-0138).

9. Flow cytometry

The expression of IL-4, IL-10, IL-17 and IFN-γ in intrahepatic lymphocytes from the liver were assayed after incubation with ionomycin (1.5 μM) and PMA (phorbol 12-myristate 13-acetate) (50 ng/ml) for 2 h in RPMI 1640 supplemented with fetal bovine serum (10%), then treated with Golgi-stop for 4 hour. The cells were washed in cold PBS and samples were incubated for 30 min at 4℃ with 0.5 mg of anti-CD16/CD32 mAb (FC block), followed by the addition of 0.5 mg of PERCP- or FITC-labeled antibodies against TCR, CD4, CD8, or PanNK (all from BD Pharmingen, San Diego, CA) for an additional 30 min at 4℃ in the dark. To detect intracellular IL-4, IL-10, IL-17 and IFN-γ, the cells were fixed with cytofix and cytoperm solution for 15 min at room temperature, washed, and then stained with FITC- or PE-labeled antibodies at 4℃ in the dark and incubated overnight.

Subsequently, the cells were washed twice and suspended in 200 mL of PBS/1% formaldehyde. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Cell Quest Pro software.

10. Inoculation of mice with mAb against IL-17

Mice were treated 12 hour after *C. sinensis* infection with intraperitoneal injections of 100 μg of normal rat IgG or rat anti-mouse IL-17 (IgG2a, clone M210, Amgen, Seattle, WA). The serum samples, livers, and spleens were collected 14 days post infection from 5 infected and treated mice.

11. Statistics analysis

Statistical significance was analyzed by the Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant. And data were represented by the mean ± standard deviation.

RESULTS

1. Gross morphological findings of the livers in each experimental group

In the gross view, the morphological changes differed in CBA/N and C57BL/6 mice. The bile ducts were thickened in both mouse strains after infection with *C. sinensis* metacercariae and showed the dilated extrahepatic bile duct compared with the uninfected control in both strains, while the livers of the infected CBA/N mice showed multiple white nodules representing pyogenic microabsesses scattered throughout the parenchyma, especially 2 and 4 weeks after infection (Fig. 1).

2. Worm recovery

The higher numbers of worms were recovered from CBA/N (34.7%) than from C57BL/6 (8.7%) mice (Table 2). At the 8th week, the worm recovery rate decreased in all of the strains. Among the recovered worms, most of them were juveniles at the 2nd and 4th week of infection. The C57BL/6 strain demonstrated a similar and low recovery at both the 2nd (8.7%) and the 4th (8.7%) week of infection; however, CBA/N revealed high recovery rates at the 2nd (34.7%) and 4th (28%) week (Table 2).

3. Histopathological findings of the livers of C57BL/6 and CBA/N

mice

Hematoxylin and eosin (HE)-stained sections of the infected livers were used to assess the pathological changes caused by *C. sinensis* (Fig. 2). In response to *C. sinensis* infection, large numbers of inflammatory cells aggregated near the bile ducts of the CBA/N and C57BL/6 mice (Fig. 2A). CBA/N mice showed severe infiltration of inflammatory cells that was noticeable at 2 weeks. Adenomatoid hyperplasia developed around dilated bile ducts with extensive infiltration of inflammatory cells in both strains (Fig. 2A).

Large numbers of neutrophils and lymphocytes were infiltrated in the liver parenchyma of CBA/N mice infected with *C. sinensis* compared with the control group, while the livers of infected C57BL/6 mice showed limited inflammatory cell infiltration. Accompanying the inflammatory cell infiltration, there was widespread hepatocyte necrosis in infected CBA/N mice, but not in infected C57BL/6 mice (Fig. 2B).

Inflammation was clearly observed in the liver after 2 weeks of infection and regressed after 8 weeks (data not shown). After 4 weeks of infection, the pathological changes were similar in both CBA/N and C57BL/6 mice. The CBA/N mice had a markedly greater inflammatory response to *C. sinensis* than the C57BL/6 mice at the earlier time point.

4. AST and ALT were increased significantly in CBA/N mice

Generally, levels of biochemical parameters were similar in both normal

C57BL/6 and CBA/N mice. Table 3 shows the alanine transaminase (ALT) and aspartate transaminase (AST) levels, which reflect liver activity. At the 4th week of infection, CBA/N showed an increased amount of ALT (295.8 ± 23.3 U/L) and AST (259.8 ± 17.6 U/L) compared to uninfected control (ALT =35.3 ± 0.8 and AST=89 ± 14.1 U/L), while in the 8th week both of them increased in both of CBA/N and C57BL/6. An increasing tendency was observed in C57BL/6 from the 4th to the 8th week of infection, but in the 4th week infection, those were not significant. In the other hand, a significant increase of ALT was observed in CBA/N ($P < 0.005$) at 2nd post infection, and the level of ALT was increased (354 ± 116.9 U/L) compare with control group (37 ± 5.3 U/L).

5. Splenocyte proliferation on activation with crude *C. sinensis* antigen

Splenocytes were isolated from each mouse and counted. There were significantly more splenocytes from *C. sinensis*-infected CBA/N mice than the negative control at 2, 4, and 8 weeks post-infection. In contrast, in C57BL/6 mice, the number of splenocytes was barely increased until 8 weeks post infection, when the number was increased slightly (Figs. 3 and 4).

We examined whether these splenocytes were able to respond to *C. sinensis* antigens using the XTT assay. Splenocytes from 4-week post-infection and control mice were isolated and activated with crude *C. sinensis*

antigen or phosphate-buffered saline (PBS) in vitro. Fig. 5 shows that the splenocytes from CBA/N mice proliferated much more than those from C57BL/6 mice on adding crude *C. sinensis* antigen .

6. Serum IgG1 and IgG2a levels were increased in infected CBA/N mice

On measuring the serum levels of *C. sinensis*-specific antibody, the production of CA specific IgE was increased slightly in both strains 2 and 4 weeks post-infection (Fig. 6A). At 8 weeks post-infection, the IgE level was greatest in C57BL/6 mice. There was no measurable IgG1 in serum from infected C57BL/6 mice in the control and at 2 weeks post-infection. Conversely, in infected CBA/N mice, the serum IgG1 level increased 2 weeks post-infection and was highest 8 weeks post-infection (Fig. 6B). The production of CA-specific IgG2a began to increase 4 weeks post-infection and the increase was evident at 8 weeks post-infection (Fig. 6C).

7. Cytokine profiles in *C. sinensis*-infected CBA/N and C57BL/6 mice

To examine whether the inflammation in *C. sinensis*-infected mice was associated with altered cytokine production in splenocytes from mice stimulated with crude antigen, cytokine levels were measured in culture supernatants using sandwich ELISAs (Fig. 7). Two weeks post-infection,

production of IL-6 was increased in both C57BL/6 and CBA/N infected mice (Fig. 7C). Although the absolute levels of IL-6 varied, the kinetics of production was similar in the two strains (Fig. 7C).

Four weeks post-infection, the secretion of Th2 type cytokines, such as IL-4, was increased in both CBA/N and C57BL/6 *C. sinensis*-infected mice (Fig. 7A), and IL-6 production was increased persistently in both mouse strains, whereas the production of Th1 type cytokines, such as TNF-α, was not altered in either strain (data not shown). Interestingly, the Th17 type cytokine IL-17 was increased in CBA/N mice compared with the C57BL/6 mice (Fig. 7E). The levels of IL-4 produced by splenocytes increased continuously during infection in CBA/N mice (Fig. 7A). IL-5 production was increased in both infected groups only at 8 weeks post-infection (Fig 7B). At 8 weeks post-infection, IL-13 production by splenocytes reached a peak level in CBA/N mice (Fig. 7D).

8. Enhanced expression of IL-4 and IL-17 in IHL from *C. sinensis* infected mice

IHL(intrahepatic lymphocyte) were isolated from each mouse and counted. There were significantly more IHL from *C. sinensis*-infected CBA/N mice than the negative control at 2, 4, and 8 weeks post-infection. In contrast, in C57BL/6 mice, the number of IHL was barely increased from 4 weeks post infection, when the number was increased slightly (Fig.8).

IL-17 was predominantly expressed by Th17 and large numbers of lymphocytes were infiltrated into the liver (Fig. 8). The Th17 cells might potentially produce IL-17A during *C. sinensis* infection. Hepatic lymphocytes were isolated from infected mice at 2, 4 and 8 weeks p.i., and stimulated with PMA and ionomycin for the last 4 h, the cells were stained for TCR, CD4, and intracellular IL-4, IL-10 IL-17A and INF-γ. IL-4 and IL-17 were enhanced in the infection groups (Fig. 9).

9. IL-17 neutralization ameliorated liver inflammation and lessened liver damage

To directly ascertain the role of IL-17 in the development of *C. sinensis*-induced liver inflammation, IL-17-neutralizing mAb was treated after infected with *C. sinensis*. Because IL-17 production increased from 2 weeks p.i., infected C57BL/6 and CBA/N mice were systemically administrated with anti-IL-17 or an isotype-matched control mAb and mice were sacrificed on day 14 p.i. Anti-IL-17 mAb, but not the control mAb, significantly improved the liver histopahological findings (Fig. 10). The livers from the *C. sinensis* infected, control mAb treated mice were full of large white patches. This gross appearance was similar to that of the livers of untreated mice that had been infected for the same length of time (data not shown). Accordingly, anti-IL-17 mAb-treated mice displayed

attenuated liver injury, as indicated by considerably decreased levels of serum alanine transaminase (ALT) and aspartate transaminase (AST; Table 4).

IHL (intrahepatic lymphocytes) and splenocytes were isolated from each mouse and counted. There were significantly decreased IHL (Fig. 11) and splenocytes (Fig. 12) from *C. sinensis*-infected mice treated with IL-17-neutralizing mAb than the negative control both in C57BL/6 and CBA/N mice. Since, in C57BL/6 mice, the number of IHL was barely decreased in mice treated with IL-17-neutralizing mAb, when the number was decreased slightlys (Fig. 11). These data indicated that anti-IL-17 treatment significantly suppressed *C. sinensis*-induced liver inflammation and hepatocyte injury.

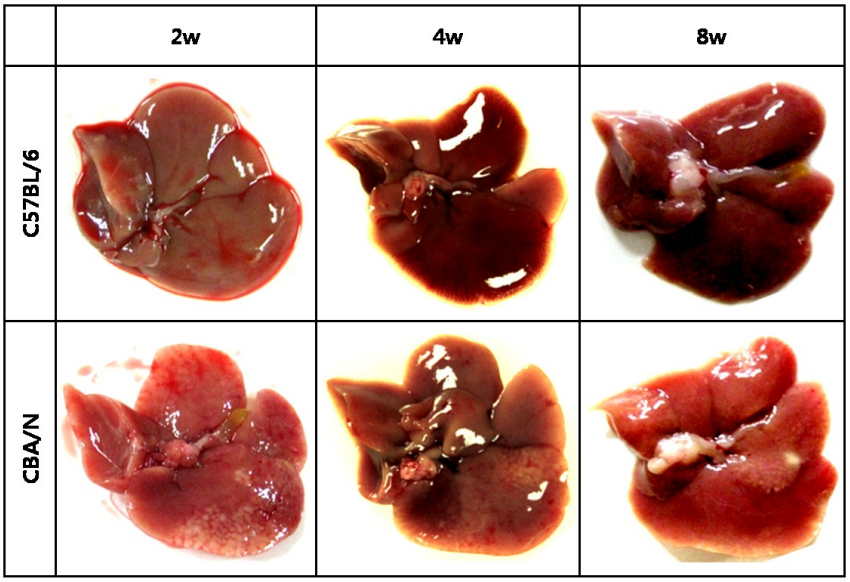


Fig. 1. Gross photographic findings of the infected liver. The livers of CBA/N mice show multiple white nodules representing pyogenic microabsesses scattered throughout the parenchyma. Data are representative of two independent experiments using five mice per group.

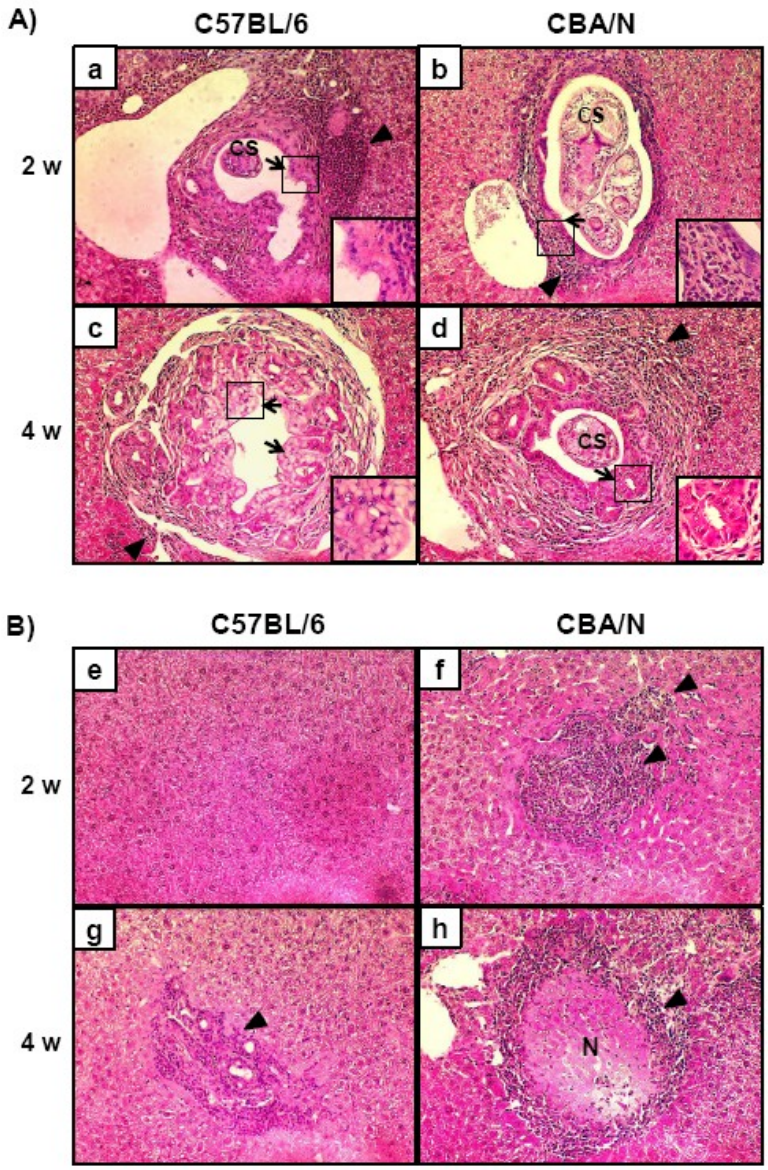
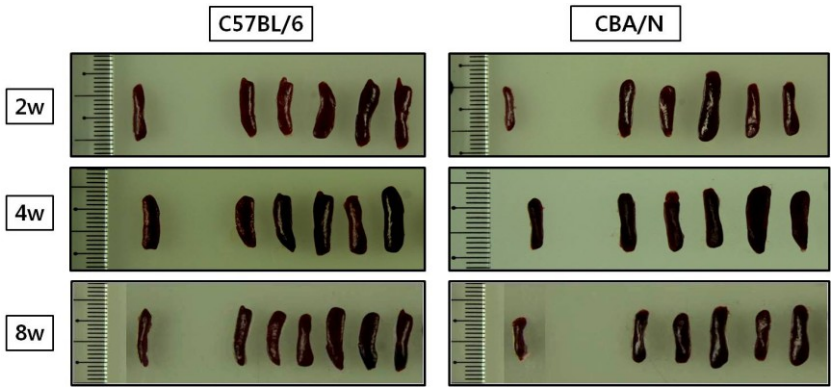


Fig. 2. Histopathology of the bile ducts and livers. (A) The bile ducts of C57BL/6 (a and c) and CBA/N (b and d) mice after 2 (a and b) and 4 (c and d) weeks post infection. Note severe bile duct proliferation (arrows) and severe inflammation (arrowheads) around bile ducts in both strains. Insets represent

higher magnification of proliferation cell populations. (B) The liver parenchyma of a C57BL/6 (e and g) and CBA/N (f and h) mice 2 (e and f) and 4 (g and h) weeks after *C. sinensis* infection. Massive inflammatory cell infiltrations (arrowheads) were observed only in CBA/N mice liver parenchyma after 2 weeks post infection. In C57BL/6 mice, mild inflammatory cell infiltration (arrowheads) were observed in liver parenchyma, whereas in CBA mice massive inflammatory cell infiltration (arrowhead) and severe hepatocytes necrosis (N) were observed after 4 weeks post infection. CS, *C. sinensis*; N, necrosis.

A)



B)

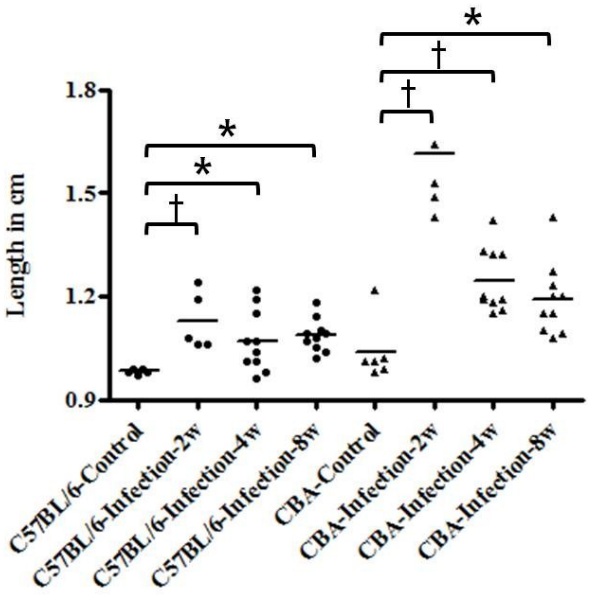


Fig. 3. The spleens of control and *C. sinensis* infected mice at the 2nd , the 4th and 8th week of infection between C57BL/6 mice and CBA/N mice. (A) Gross findings of the spleens. (B) Measurements of spleens from mice. *$P <$ 0.05 and †$P < 0.01$ (*t*-test) were considered statistically significant.

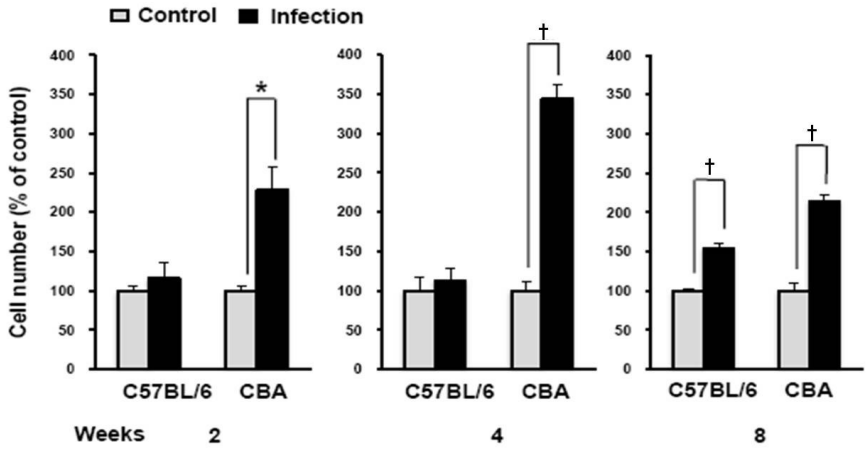


Fig. 4. Number of splenocytes in C57BL/6 and CBA/N mice after *C. sinensis* infection. Splenocytes were harvested and counted from the whole spleen. The bars represent means±SD (n=5). *$P < 0.05$ and †$P < 0.01$ (*t*-test) were considered statistically significant.

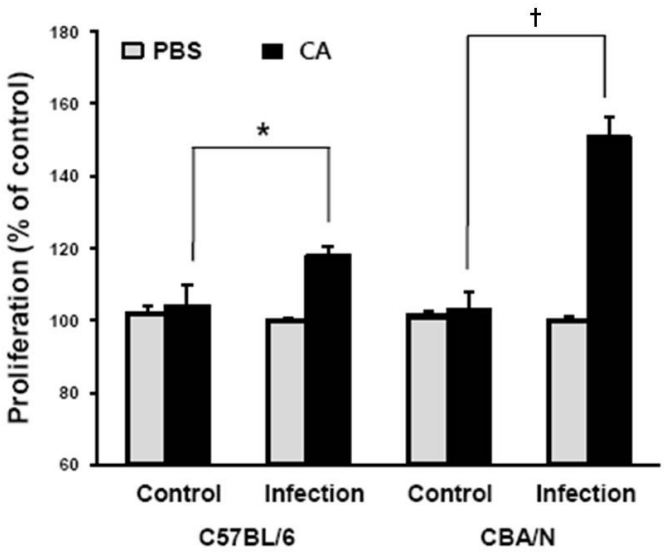


Fig. 5. Effect of CA (crude antigen) on splenocyte proliferation. Splenocytes were harvested from C57B/L and CBA/N mice 4 weeks post infection. Cells were plated in 96 well plates ($5x10^5$ cells/well). The cells were treated with PBS (vehicle) or CA (crude antigen) from *C. sinensis*. After a 72-h incubation, cells were treated with XTT reagent and incubated for an additional 4h. Cell proliferation in each group was determined using the XTT assay. The histograms represent cell proliferation as a percentage of the control ± S.D. (n=5). *$P < 0.05$ and †$P < 0.01$ (t-test) were considered statistically significant.

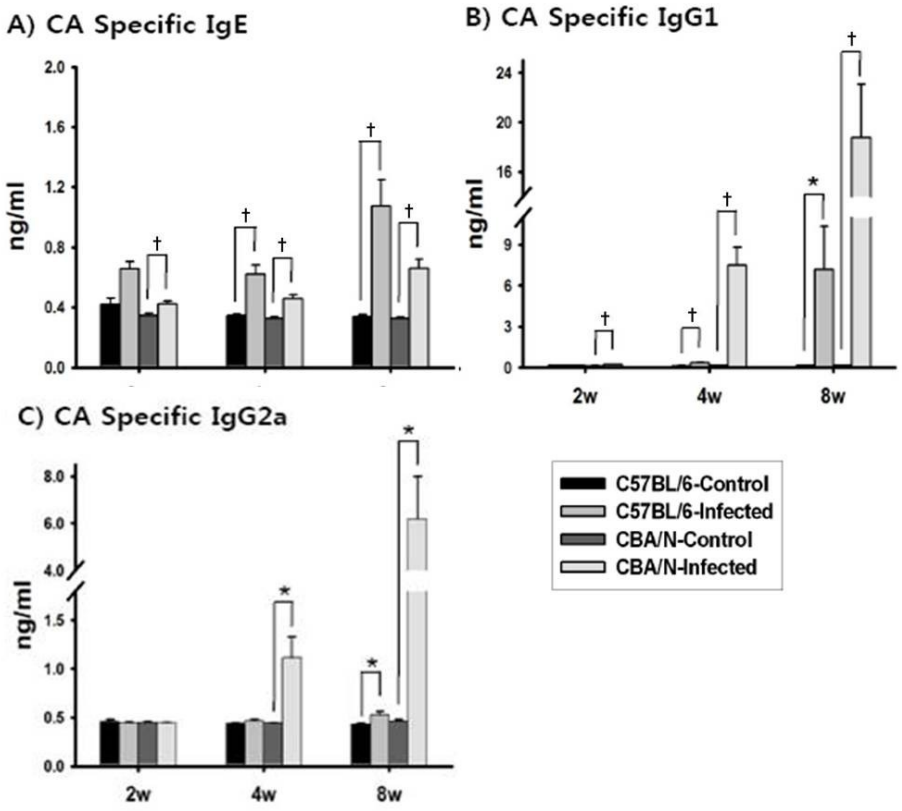


Fig. 6. Serum immunoglobulin levels in C57BL/6 and CBA/N mice after *C. sinensis* infection. *C. sinenisis* crude antigen specific serum IgE (A), IgG1(B) and IgG2a (C) quantification were performed after 2, 4 and 8 weeks post infection in C57BL/6 and CBA/N mice. The bars represent means±SD (n=5). *$P < 0.05$ and †$P < 0.01$ (t-test) were considered statistically significant.

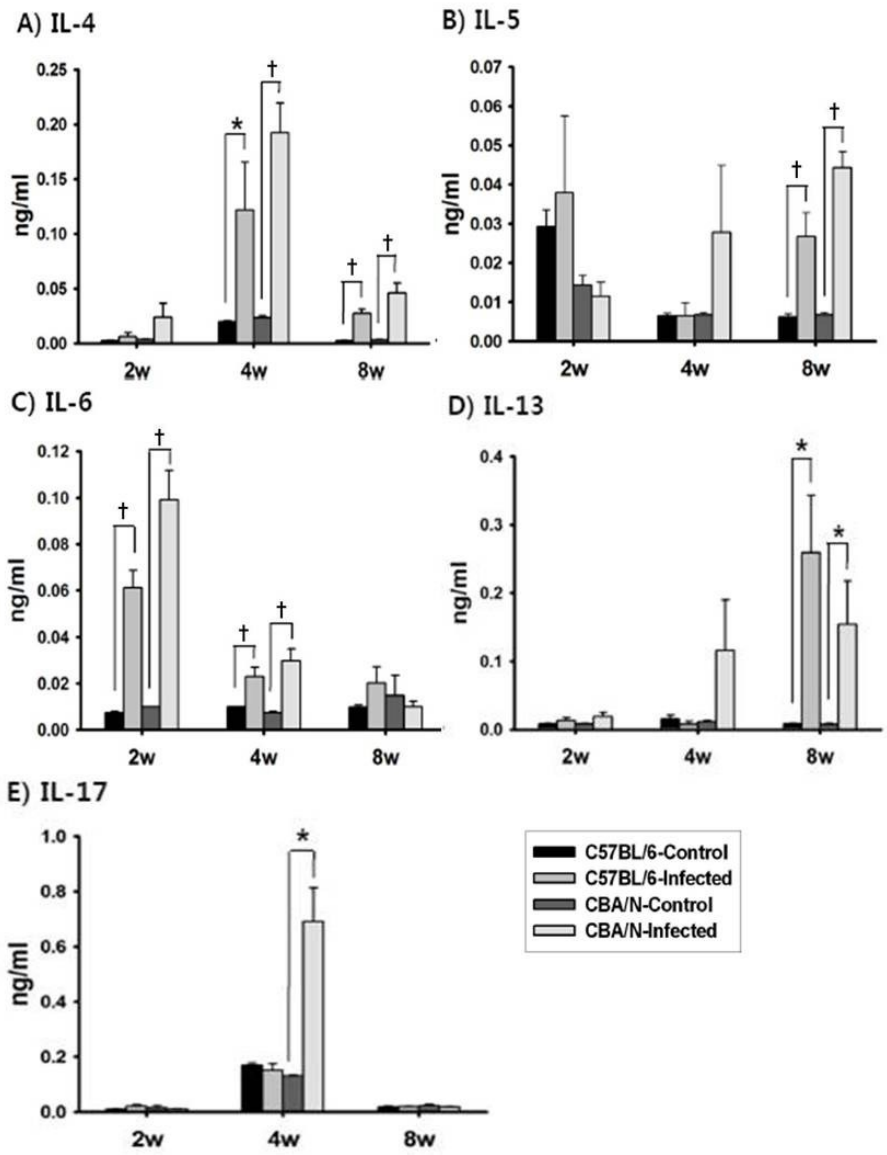


Fig. 7. Cytokine production by the splenocytes from C57B/L and CBA mice stimulated with *C. sinensis* crude antigen. Splenocytes from each mouse were cultured with 20μg/ml of *C. sinensis* crude antigen. Cell-free supernatants were harvested after 72 h for cytokine analysis. Cytokines measured are IL-4

(A), IL-5 (B), IL-6 (C), IL-13 (D) and IL-17 (E). The bars represent means±SD (n=5). *$P < 0.05$ and †$P < 0.01$ (t-test) were considered statistically significant.

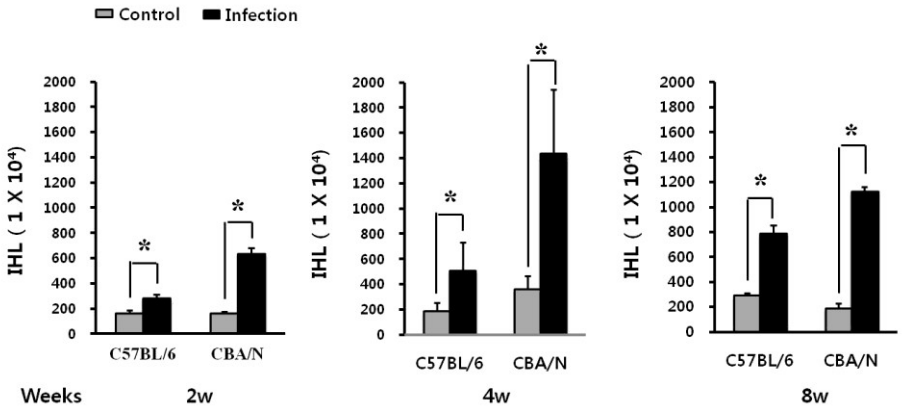


Fig. 8. Number of IHL in C57BL/6 and CBA/N mice after *C. sinensis* infection. IHL were harvested from the whole liver and counted. The bars represent means±SD (n=5).

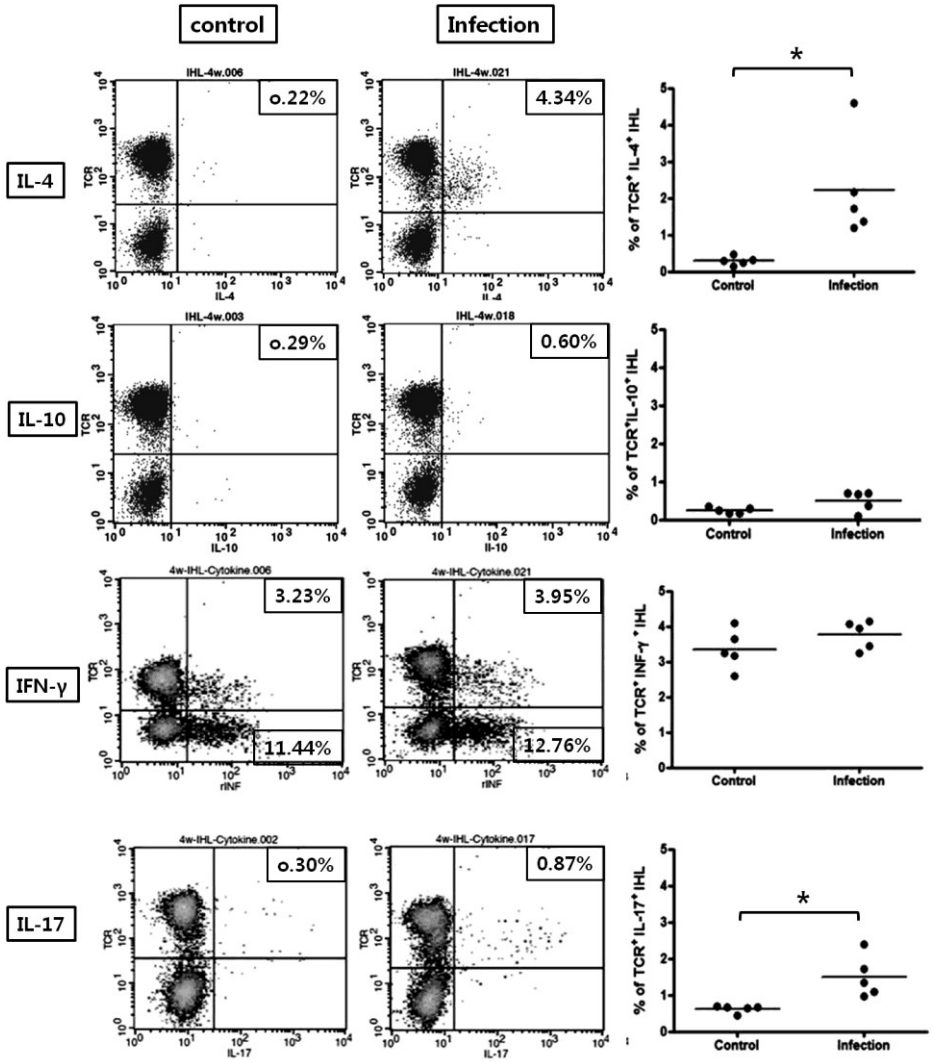


Fig. 9. FACS analysis with IHL in CBA/N mice after *C. sinensis* infection. The percentage of cytokine producing T cells in the liver in mice. Number in upper right boxes showed percentages of double positive cells in TCR populations. Each group consisted of 5 mice.

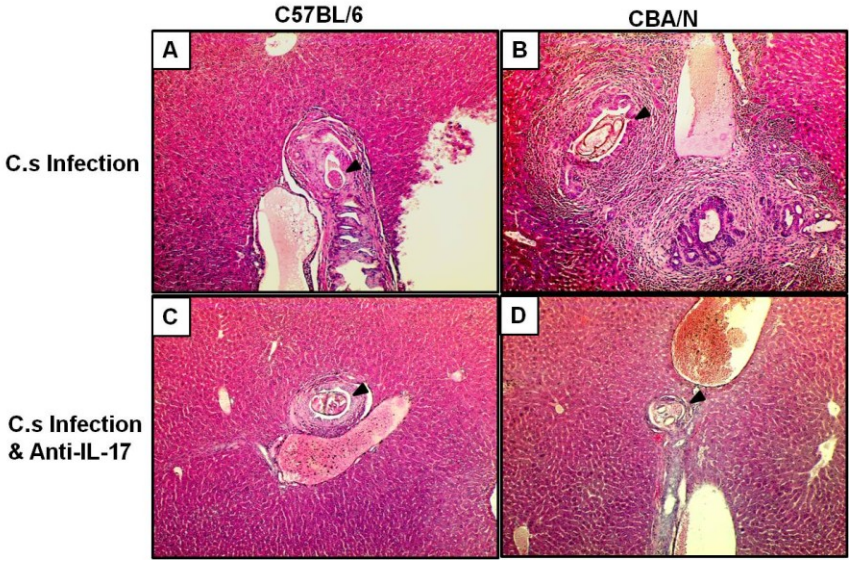


Fig. 10. Histopathology of bile ducts and livers after *C. sinensis* infection and anti-IL-17 injection. The bile ducts of C57BL/6 (A and C) and CBA/N (B and D) mice.

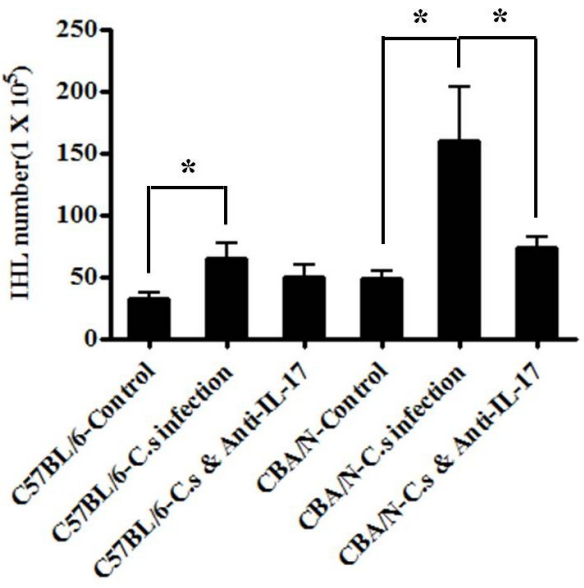


Fig. 11. Number of IHL in C57BL/6 and CBA/N mice after *C. sinensis* infection and anti-IL-17 injection. IHL were harvested from the whole liver and counted. The bars represent means±SD (n=5).

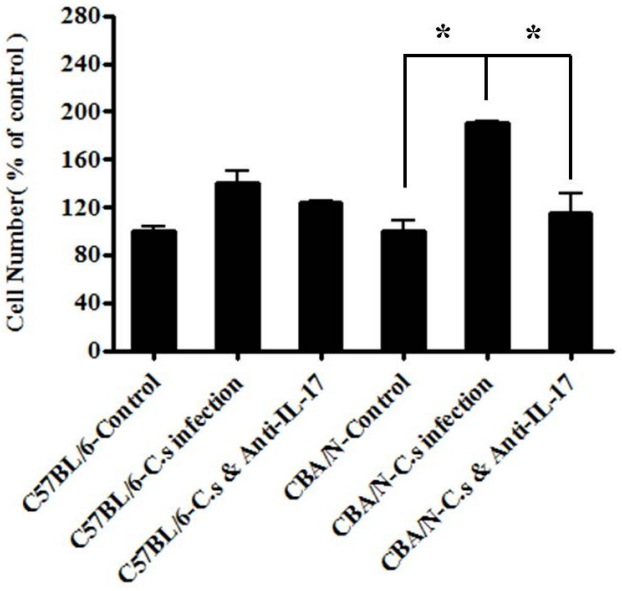


Fig. 12. Number of splenocytes in C57BL/6 and CBA/N mice after *C. sinensis* infection and anti-IL-17 injection. SPL were harvested from whole spleen and counted. The bars represent means±SD (n=5).

Table 2. The worm recovery of *C. sinensis* from 30 mice (in 6 experimental groups) infected with 30 metacercariae respectively

Duration of infection (weeks)	No. (%) of worms recovered from					
	C57BL/6			CBA/N		
	Juvenile	Adult	Total	Juvenile	Adult	Total
2	13	0	13 (8.7)	52	0	52 (34.7)
4	11	2	13 (8.7)	36	6	42 (28)
8	4	2	6 (4)	22	3	25 (16.7)

Table 3. Biochemical parameters measured in the liver of *C. sinensis*-infected and non-infected C57BL/6 and CBA/N mice

Biochemical parameter	weeks	C57BL/6		CBA/N	
		Control	Infected	Control	Infected
AST[a] (U/L)	2w	74.3±13.3	83.3±15.3	76.5±19.5	263±127.1
	4w	92.3±10.7	151.2±23.9	89±14.1	259.8±17.6*
	8w	49.2±7.6	97.3±8.1*	54±11.2	180.7±11.0*
ALT[b] (U/L)	2w	38.3±8.3	60±10.2	37±5.3	354±116.9*
	4w	58.2±5.5	70.8±7.5	35.3±0.8	295.8±23.3*
	8w	27.6±3.1	73±6.5*	28.4±2.6	144.8±14.1*

[a] aspartate transaminase; [b] alanine transaminase; *$P < 0.05$ (*t*-test) was considered statistically significant compared with non-infected mice.

Table 4. Biochemical parameters measured in the liver of *C. sinensis*-infected and non-infected C57BL/6 and CBA/N mice after injected with anti-IL-17

Biochemical parameter	Groups	Mouse strain	
		C57BL/6	CBA/N
AST[a] (U/L)	Control	74.8±33.3	80.8±26.2
	Infected	95.6±26.8	215±65.5
	Infection & Anti-IL-17	72.8±20.2 *	121±18.6 *
ALT[b] (U/L)	Control	24±3.7	24±2.5
	Infected	31.2±7.3	284±162.8
	Infection & Anti-IL-17	28.8±3.9 *	70±16.4 *

[a] aspartate transaminase; [b] alanine transaminase; *$P < 0.05$ (*t*-test) was considered statistically significant compared with non-infected mice.

DISCUSSION

In this study, two different strains of mice, C57BL/6 and CBA/N, were infected with *C. sinensis*, and investigated the relationship between cytokine production and the pathological changes in the liver. The gross and microscopic findings of the livers revealed that CBA/N and C57BL/6 mouse livers differed significantly at 2 and 4 weeks post-infection because of the different immunological responses. Dilated bile ducts and the thickened hepatic bile duct were marked in both strains, but the CBA/N mouse livers had many gross white nodules on the liver surface. Those nodules were composed of severe hepatocyte necrosis with many inflammatory cells. This was likely related to the immune response following *C. sinensis* infection, which plays a role in the pathogenesis of the infection.

At the early stage of infection, the metacercariae excyst and migrate to the liver [34]. The infection triggered infiltration of inflammatory cells, hyperplasia of bile duct epithelial cells, and hepatocyte necrosis in CBA/N mice from the 2nd to the 4th week after infection. Compared to this, infected C57BL/6 mice showed less cellular infiltration in the liver and no hepatocyte necrosis. These findings were consistent with the intrahepatic lymphocyte counts. The CBA/N mice were more sensitive to the *C. sinensis* crude antigen than the C57B/L mice, especially in the early response. The histopathological findings were well corrleated with worm recovery or gross appearance. According to the results, it is necessary to examine further the immune

response in future.

The worm recovery rate of *C. sinensis* in mice varies greatly by their strains. The worm recovery rate (8.3%) of C57BL/6 mice strain in the present study was similar with the previous rate of 9.3% [20]. Furthermore, CBA/N mice show a higher recovery rate (16.7% to 34.7%) than in C57BL/6 mice. It is different with the previous reported rate (8.3%) [20]. However, the recovery rate in mice is not as high as that in rats, rabbits, and other experimental mammals. The same phenomenon was demonstrated in the resistant rats that were re-infected or super-infected [35, 36]. The data of low recovery rates in both mouse strains suggest that it is hard for *C. sinensis* to survive and develop in mice regardless of strains, which is similar with that in the resistant re-infected or super-infected rats. .

In terms of host responses to a foreign pathogen, the mice require IL-6 for resistance against the bacterium *Streptococcus pneumonia* [37]. Thus, it is expected that greater IL-6 production in CBA/N mice, because IL-6 is an important mediator of fever and acute responses [38]. The production of IL-6 was detected in the supernatant of splenocytes from both mouse strains stimulated with *C. sinensis* crude antigen. The level of IL-6 was higher in CBA/N and C57BL/6 compared with control group at the 2nd and 4th week of infection. However, after 8 weeks, both mice showed moderate level of IL-6. This finding indicated the same trend in both strains after infected with *C. sinensis*. It is contradicted the relation between IL-6 production and the

susceptibility of the mice as well as the resistance against the worms.

Production of IL-4 was detected in the supernatant of stimulated splenocytes at 4 weeks post-infection in both CBA/N and C57B/L mice, but the level was sustained until 8 weeks post-infection only in CBA/N mice. IL-4 is an important inducer of Th2 responses in vitro [39] and influences the differentiation of Th0 cells into Th2 cells in vivo [40, 41]. Additionally, IL-4 enhances the ability of B cells to produce IgG1 and IgE antibody production [42, 43]. The serum level of *C. sinensis* CA-specific IgE was increased in both infected strains at 4 and 8 weeks post-infection. Levels of CA specific IgG1 were significantly ($P < 0.001$) greater in CBA/N mice than in C57B/L mice from 4 to 8 weeks post-infection. This was consistent with the amount of IL-4, which was associated with serum IgE and IgG1 production. To further investigate the mechanism of IL-4 and IgG1 in murine clonorchiasis, future studies should examine IL-4-receptor-deficient mice or use an anti-IL-4 antibody to neutralize the cytokine, and then evaluate immunoglobulin levels.

Clonorchiasis in the CBA/N mice was characterized by an increased number of neutrophils in the liver parenchyma. There is evidence that IL-17 released from T-lymphocytes of the CD45RO+ subset is linked to neutrophil recruitment and activation [44]. Marked production of IL-17 was observed only in CBA/N mice infected with *C. sinensis* at 4 weeks post-infection. The production of IL-17 could be responsible for the early stage hepatocyte necrosis and inflammation in the liver. However, further studies are needed to

determine whether IL-17 plays a direct role in the neutrophil infiltration in *C. sinensis*-infected mice by inhibition of IL-17 production or activation [45, 46].

To confirm the direct involvement of IL-17 in the development of granulomatous inflammation, IL-17-specific neutralizing antibody was injected into *C. sinensis* infected mice. It resulted in markedly downregulated inflammation and mitigated hepatocyte damage. Nevertheless, these effects were not observed in mice treated with an isotype-matched control IgG, indicating that the decreased liver inflammation was associated with specific neutralization of IL-17. Besides that, ALT and AST were significantly decreased in the infected mice after anti-IL-17 treatment.

Th17 cells are recently known as a third independent effector cell subset differentiated from $CD4^+$ T cells upon antigenic stimulation. Although the functions of these cell subtypes are not completely understood, emerging data suggest that by producing their defining cytokine IL-17, Th17 cells play an important role in host defenses against extracellular pathogens, such as *Klebsiella pneumoniae* [47], *Pseudomonas aeruginosa* [48], *Porphyromonas gingivalis* [49] and *Bacteroides fragilis* [50], which are not efficiently cleared by Th1-type and Th2-type immunity. Meanwhile, several studies have shown that Th17 cells and IL-17 also play important roles in immunopathology in some infectious diseases, such as pulmonary tuberculosis [51], toxoplasmosis [52] and schistosomiasis [53-58]. According to the present results, IL-17 is related with immunopathologic damage to the host in the early stage of

infection with *C. sinensis* in CBA/N mice.

ALT is found in the serum and various tissues, but is most commonly associated with the damaged liver cells, also lesser quantities are found in the kidneys, heart, and skeletal muscle [59]. Similarly, AST is also increased with damaged liver parenchymal cells. ALT is a more specific indicator of the liver inflammation than AST, because AST can be elevated in diseases affecting other organs [60, 61]. In the present experiment, accompanying the inflammatory cell infiltration in the liver, the most noticeable phenomenon was the widespread hepatocyte necrosis in *C. sinensis*-infected CBA/N mice at 2 and 4 weeks post-infection. Both ALT and AST levels were elevated significantly in *C. sinensis*-infected CBA/N mice at 2, 4, and 8 weeks post-infection compared with the uninfected control group. These peaked levels 2 weeks post-infection suggested that *C. sinensis* infection affected the liver pathology in the initial stage after infection. In the C57BL/6 mice, the ALT and AST were increased slightly and reached at the highest levels at 8 and 4 weeks post-infection, respectively. The ALT and AST levels matched the histopathology results and were similar to the pathological changes in *C. sinensis*-infected humans. A previous report indicated a role for the Fas/FasL-mediated pathway in the apoptosis that occurs in response to *C. sinensis* infection [18]. In the liver samples from Wistar rats and patients with *C. sinensis* infection, most hepatocytes exhibited hydropic degeneration, and some hepatocytes showed densely condensed nuclei, suggesting apoptosis

[18]. ALT and AST are suggested as good indicators of the liver damage in the initial stage of clonorchiasis. The mechanism of the damage should a topic of further study.

In conclusion, the CBA/N mouse is a good animal model to study early immune response after infection with *C. sinensis*. The infected CBA/N mice stimulate differentiation of pathogenic Th17 cells as well as to produce humoral immunity by B cells. IL-17 is playing a key role for development of severe immunopathology of the liver in early stage of clonorchiasis.

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국문 초록

이 연구는 간흡충 감염 초기 면역반응을 연구하고자 Th1 과 Th17 면역반응이 우세한 CBA/N 마우스와 Th2 면역반응이 우세한 C57BL/6 마우스를 대상으로 실험하였다. 이 두 가지 계통의 마우스를 간흡충 피낭유충으로 감염시킨 뒤 각각 감염 2 주, 4 주, 8 주간 숙주 조직반응과 면역반응을 관찰하였다. CBA/N 마우스는 간흡충에 감염된 뒤 C57BL/6 마우스에 비하여 간조직의 병리학적인 변화로 염증세포의 침윤이 유의하게 많았으며 특히 감염 2 주차에는 간세포의 괴사가 관찰되었다. 더욱 간세포 손상의 지표효소인 ALT 와 AST 농도가 CBA/N 마우스에서 유의하게 증가되었다. 간흡충 조항원으로 자극한 지라세포를 배양한 뒤 세포분열과 상청액의 싸이토카인을 측정하였다. 그 결과 CBA/N 마우스에서 지라세포의 증식이 유의하게 증가하였고 싸이토카인 IL-17 분비가 증가하였다. 또한 같은 마우스에서 IL-4 가 감염 8 주차까지 증가하여 유지되었다. 혈청내 간흡충 조항원 특이 IgG1 과 IgG2a 의 항체가가 CBA/N 마우스에서 증가한 반면 C57BL/6 마우스에서는 IgE 의 항체가가 증가하였다. 또한 간흡충 감염시 발생하는 간

염증반응에서 IL-17 의 역할을 알아보기 위하여 항 IL-17 단클론항체를 간흡충이 감염된 마우스의 복강에 주입하고 14 일 후에 마우스를 희생하였다. 항 IL-17 단클론항체를 투여한 마우스에서 간조직 염증과 간세포 괴사가 완화되었다. 동시에 혈청 내 효소 ALT (284±162.8 에서 70±16.4 로 감소)와 AST (215±65.5 에서 121±18.6 로 감소)의 수준도 유의하게 감소된 것을 확인하였다. 간 내 림프구와 지라세포를 각각의 마우스에서 분리하여 계수한 결과 항 IL-17 단일 클론 항체가 처리된 마우스에서 유의하게 감소한 것이 관찰되었다. 이러한 결과는 IL-17 이 간흡충증 초기에 유발되는 간조직 염증반응과 간세포의 손상을 일으키는 핵심물질임을 확인하였다. 결론적으로 간흡충증의 초기 면역반응을 연구하는 데에 CBA/N 마우스가 좋은 동물 모델임을 확인하였다. 또한 간흡충에 감염된 숙주에서 간조직의 염증과 간세포 괴사를 일으키는 과정에 IL-17 이 관여하고 있음을 확인하였다.

주요어: 간흡충, 마우스, CBA/N, C57BL/6, 싸이토카인, IL-17

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