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Immune responses induced by co-infection with *Capillaria hepatica* in *Clonorchis sinensis*-infected rats

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Abstract

Clonorchis sinensis and *Capillaria hepatica* are zoonotic parasites that mainly infect the liver and cause serious liver disorders. However, immunological parameters induced by co-infection with these parasites remain unknown. In this study, for the first time, we investigated immunological profiles induced by co-infection with *C. hepatica* (CH) in *C. sinensis* (CS)-infected rats (Sprague–Dawley). Rats were infected primarily with 50 metacercariae of *C. sinensis*; 4 weeks later, they were subsequently infected with 1000 infective *C. hepatica* eggs. Significantly higher levels of *C. sinensis*- or *C. hepatica*-specific IgG antibodies were found in the sera of rats. Interestingly, no cross-reacting antibody was observed between *C. sinensis* and *C. hepatica* infections. Significantly raised eosinophil levels were found in the blood of *C. sinensis*/*C. hepatica* co-infected rats (CS + CH) compared to the blood of rats infected singly with *C. sinensis*. Co-infected rats showed significantly higher levels of lymphocyte proliferation and cytokine production compared to a single *C. sinensis* infection. The worm burden of *C. sinensis* was significantly reduced in co-infected rats compared to the single *C. sinensis* infection. These results indicate that the eosinophils, lymphocyte proliferation and cytokine production induced by subsequent infection with *C. hepatica* in *C. sinensis*-infected rats might contribute to the observed *C. sinensis* worm reduction.

Introduction

Clonorchis sinensis infection causes clonorchiasis and human cholangitis, and is highly prevalent in China, Korea, Taiwan, Vietnam and Japan (Rim, 2005; Sripa *et al*., 2010). In humans, *C. sinensis* infection causes eosinophilic pulmonary infiltrations, producing 35% eosinophils in a leucocyte count, with a positive skin test and *C. sinensis*-specific IgG antibody response (Lee *et al*., 2003). Humans are infected by consuming undercooked fish containing *C. sinensis* metacercariae. Upon reaching the small intestine, the metacercariae exit and migrate towards the bile duct, causing obstruction of the bile duct and other diseases, including bacterial infections, inflammation, periductal fibrosis, hyperplasia and cholangiocarcinoma (Quan *et al*., 2004; Hong & Fang, 2012). *Capillaria hepatica* is another zoonotic parasite that is found worldwide and causes hepatic capillariasis, a serious liver disorder with remarkable elevation of eosinophil counts (Ferreira & Andrade, 1993; Juncker-Voss *et al*., 2000; Klion, 2015). More than 180 mammalian species (including humans) can be hosts of this pathogen (Fuehrer, 2014; Sharma *et al*., 2015). Humans are infected by ingesting embryonated eggs of *C. hepatica* in food, water or soil contaminated with faeces (Center for Disease Control, 2011). First-stage larvae (L1) hatch from embryonated eggs, and the L1 larvae bore through the intestinal wall and are carried to the liver by the hepatic portal vein. Larvae develop to sexually mature adults, laying eggs in

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the liver parenchyma, causing hepatic capillariasis (Ferreira & Andrade, 1993; Center for Disease Control, 2011).

A number of reports have shown that eosinophils are required for the clearance of primary *Strongyloides stercoralis* infections and secondary *Nippostrongylus brasiliensis* and *Trichinella spiralis* infections (Vallance *et al.*, 2000; Knott *et al.*, 2007; Chu *et al.*, 2016). The deposition of the eosinophil granule proteins major basic protein 1 (MBP-1), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) on helminth surfaces can kill *Brugia* sp. *in vitro* (Hamann *et al.*, 1990).

Since both *C. sinensis* and *C. hepatica* have a wide host range and high infection rates, there is a high possibility that they can co-infect a host. Also, until now, there is no report regarding the basic immunology induced by co-infections by two parasites in the same liver. An understanding of co-infection-induced resistance, antibody response, eosinophil response, lymphocyte proliferation and cytokine production would have a significant impact in providing novel information on the basic immunology of parasitic co-infections.

In this study, we assessed antibody responses, eosinophil counts, lymphocyte proliferation, cytokine production and worm burden reduction of *C. sinensis* that developed in the case of super-infection with *C. hepatica* in rats previously infected with *C. sinensis*. We found that a remarkable eosinophil elevation was induced by co-infection with *C. hepatica* in *C. sinensis*-infected rats. Significantly higher levels of lymphocyte proliferation and cytokines were detected in co-infected rats compared to single *C. sinensis*-infected rats. Interestingly, no cross-reactive antibodies were observed between *C. sinensis* and *C. hepatica*, indicating that eosinophil elevation, lymphocyte proliferation and cytokine production might contribute to the worm reduction during *C. sinensis*/*C. hepatica* co-infection.

Materials and methods

Animals and parasites

Sprague–Dawley (SD) rats (females, 8 weeks old) and New Zealand white rabbits (males, 2–4 months old) were purchased from Samyook Animal Center, Osan City, Kyonggi-do, Korea. White rabbits were infected with *C. sinensis* metacercariae to generate *C. sinensis* adult worms. *Clonorchis sinensis* metacercariae were collected from the freshwater fish *Pseudorasbova parva* by digesting muscles with pepsin–HCl, followed by filtration through layers of gauze. To obtain *C. hepatica* eggs, the liver tissues of house rats (*Rattus norvegicus*) infected with *C. hepatica* were digested with pepsin–HCl at 37°C, as described by Kim *et al.* (2007). The eggs were isolated by repeated filtration and cultured in 0.5% formalin solution at 30°C until embryonation. The embryonated eggs were kept at −20°C for egg antigen preparation and infection of rats, as described previously (Lee, 1964; Kim *et al.*, 2007).

Antigen preparation

Adults of *C. sinensis* were collected from rabbit liver, and the excretory–secretory antigen (ES Ag) of *C. sinensis* was obtained as described by Chu *et al.* (2014). Embryonated eggs of *C. hepatica* were homogenized for the preparation of egg antigen (egg Ag). The homogenized eggs were centrifuged at 10,000 rpm for 60 min, and the supernatant was collected. The protein concentration was determined and samples were stored at −70°C until use.

C. sinensis *infection and subsequent infection with* C. hepatica

Sprague–Dawley (SD) rats (females, 8 weeks old) were used. Groups of rats were primarily infected with 50 *C. sinensis* metacercariae (CS group, six rats). After 1 month, six rats with primary infections were subsequently challenge infected with 1000 embryonated eggs of *C. hepatica* (CS + CH group, six rats), at the same time as previously uninfected rats were given a single infection with *C. hepatica* (CH group, six rats). Uninfected rats were used as controls (naïve group, six rats). Thus, at 1 month after *C. hepatica* infection, and at 2 months after *C. sinensis* infection, rats (naïve, CS, CS + CH and CH groups) were sacrificed. Embryonated eggs of *C. hepatica* were isolated as described above (Animals and parasites section) and counted using a haemocytometer. The oral infection route was used for both *C. sinensis* and *C. hepatica*.

Serum antibody responses

Serum samples were collected from the ophthalmic venous plexus of rats 1 month after *C. sinensis* or *C. hepatica* infection (fig. 1). *Clonorchis sinensis* (CS)- or *C. hepatica* (CH)-specific total IgG, IgG1 and IgG2a antibody responses were determined in the naïve, CS and CS + CH rats, using enzyme-linked immunosorbent assay (ELISA). Serially diluted sera were used for ELISA and the data from 1:100 serum dilutions are shown in the fig. 2. Plates (Nunc MaxiSorp flat-bottom 96-well plate; Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 100 µl of *C. sinensis* ES Ag or *C. hepatica* egg Ag (4-µg/ml), and horseradish peroxidase (HRP)-conjugated anti-rat IgG, IgG1 and IgG2a (Bio-Rad, Hercules, California, USA) were used as secondary antibodies, as described previously (Chu *et al.*, 2014).

Eosinophil counts

Whole blood was collected from rats at 1 month after primary infection and subsequent co-infection (fig. 1). Whole blood was stained with Discombe's solution. A 20-µl aliquot of individual blood was immediately added into 180 µl of Discombe's solution (5:5:90 acetone : 1% aqueous eosin : distilled water) and mixed well. Eosinophils in whole blood were counted using a haemocytometer.

Lymphocyte proliferation assay

Spleens were collected from individual rats at week 4 after *C. hepatica* infection, and single-cell suspensions were prepared using 70% and 50% Percoll gradients. Cells were incubated in 96-well flat culture plates (5×10^6

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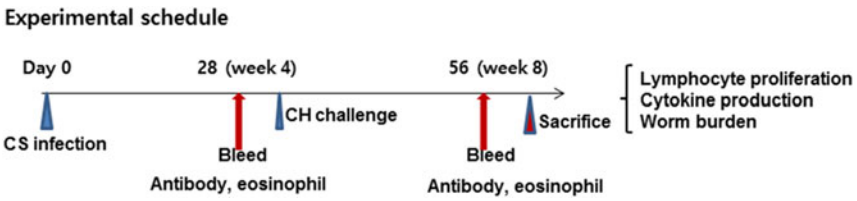

Fig. 1. Experimental schedule. Rats ($n = 12$) were primarily infected with 50 metacercariae of *C. sinensis* (CS) and, after 4 weeks, were challenge infected with 1000 eggs of *C. hepatica* (CH). Blood was collected for antibody and eosinophil responses. Rats were sacrificed and lymphocyte proliferation, cytokine production and worm burden were determined at week 4 after *C. hepatica* challenge. The animal experiment was repeated twice independently.

cells/well) for 3 days at 37°C in the presence of 5% CO_2. Cells in 100 µl of RPMI-1640 were stimulated with 100 µl of 5 µg/ml *C. sinensis* ES Ag or phytohaemagglutinin (PHA). At day 3 after incubation, the cells were pulsed for 16 h with 0.5 µCi/well of [^{3}H]thymidine (Amersham, Piscataway, New Jersey, USA) and then harvested on glass-fibre filters with a semiautomatic cell harvester (Skatron, Norway). Incorporated radioactivity was determined by liquid scintillation counting (LKB 1214 Rackbeta; American Instrument Exchange, Haverhill, Massachusetts, USA). The lymphocyte proliferation was expressed as background-subtracted geometric means (cpm, counts per minute).

Cytokine analysis

For the cytokine assay, supernatants of spleen-cell cultures were collected from each well by separation and stored at −20°C until use. OptEIA sets (BD Bioscience, San Jose, California, USA) were used to determine the concentration of interferon-gamma (IFN-γ), interleukin (IL)-2, IL-6 and IL-10 in culture supernatants, following the manufacturer's procedures.

Parasite burden determination of C. sinensis

Clonorchis sinensis-infected rats were sacrificed at month 1 after secondary infection with *C. hepatica*. Adult worms of *C. sinensis* were collected from the bile ducts and *C. hepatica* eggs were collected from livers; they were counted individually.

Statistical analysis

The antibody levels in sera, eosinophil responses in blood and lymphocyte cellular responses in spleen were recorded for each individual. Every assay was performed using at least three replicate samples, from which the arithmetic mean and standard error (SE) of the mean were calculated. A one-way analysis of variance (ANOVA) was performed. The burdens of *C. sinensis* adult worms were counted individually and non-parametric statistics (Wilcoxon rank sum test) were used for a valid comparison between CS and CS + CH. A value of $P < 0.05$ was considered significant.

Results

Sequential infection with C. hepatica *in* C. sinensis*-infected rats induced CS-specific or CH-specific IgG, IgG1 and IgG2a antibodies*

Rats were primarily infected with *C. sinensis*; 4 weeks later, rats were infected with *C. hepatica* (fig. 1). As shown in fig. 2A, significantly higher levels of *C. sinensis*-specific IgG antibodies were found in *C. sinensis*-infected and *C. sinensis*/*C. hepatica* co-infected rats compared to *C. hepatica*-infected rats or naïve control rats (*$P < 0.01$). *Capillaria hepatica*-infected rats showed no IgG antibody response against *C. sinensis* antigen. Significantly higher levels of *C. hepatica*-specific IgG antibodies were determined in *C. hepatica*-infected and *C. sinensis*/*C. hepatica* co-infected rats compared to *C. sinensis*-infected rats and naïve control rats (fig. 2B, *$P < 0.01$). *Clonorchis sinensis*-infected rats showed no IgG antibody response against *C. hepatica* antigen. *Clonorchis sinensis*/*C. hepatica* co-infected rats showed similar levels of *C. sinensis*-specific IgG antibody and *C. hepatica*-specific IgG antibody, compared to single *C. sinensis* or *C. hepatica* infection. These results indicate that no cross-reacting antibodies were elicited between *C. sinensis*- and *C. hepatica*-infected rats. *Clonorchis sinensis*- or *C. hepatica*-specific IgG isotypes IgG1 and IgG2a were also determined. As shown in fig. 2C, D, significantly higher levels of *C. sinensis*- or *C. hepatica*-specific IgG1 and IgG2a antibodies were found in single *C. sinensis*- or *C. hepatica*-infected and *C. sinensis*/*C. hepatica* co-infected rats than those in naïve rats (fig. 2C, D, **$P < 0.01$, *$P < 0.05$). Compared to IgG2a antibody responses, higher levels of *C. sinensis*- or *C. hepatica*-specific IgG1 antibodies were observed (fig. 2C, D, **$P < 0.01$, *$P < 0.05$). No significant increases of IgG1 and IgG2a antibody responses in *C. sinensis*/*C. hepatica* co-infected rats were observed compared to single *C. sinensis* or *C. hepatica* infection. No cross-reactivities of IgG1 and IgG2a antibodies were found between *C. sinensis*- and *C. hepatica*-infected rats.

Sequential infection with C. hepatica *in* C. sinensis*-infected rats induced remarkable increases of eosinophil levels in peripheral blood*

Whole-blood samples were collected as indicated in the Materials and methods section, and eosinophil profiles were counted. As shown in fig. 3, single infection with *C. hepatica* (CH) or *C. sinensis* (CS) in rats produced

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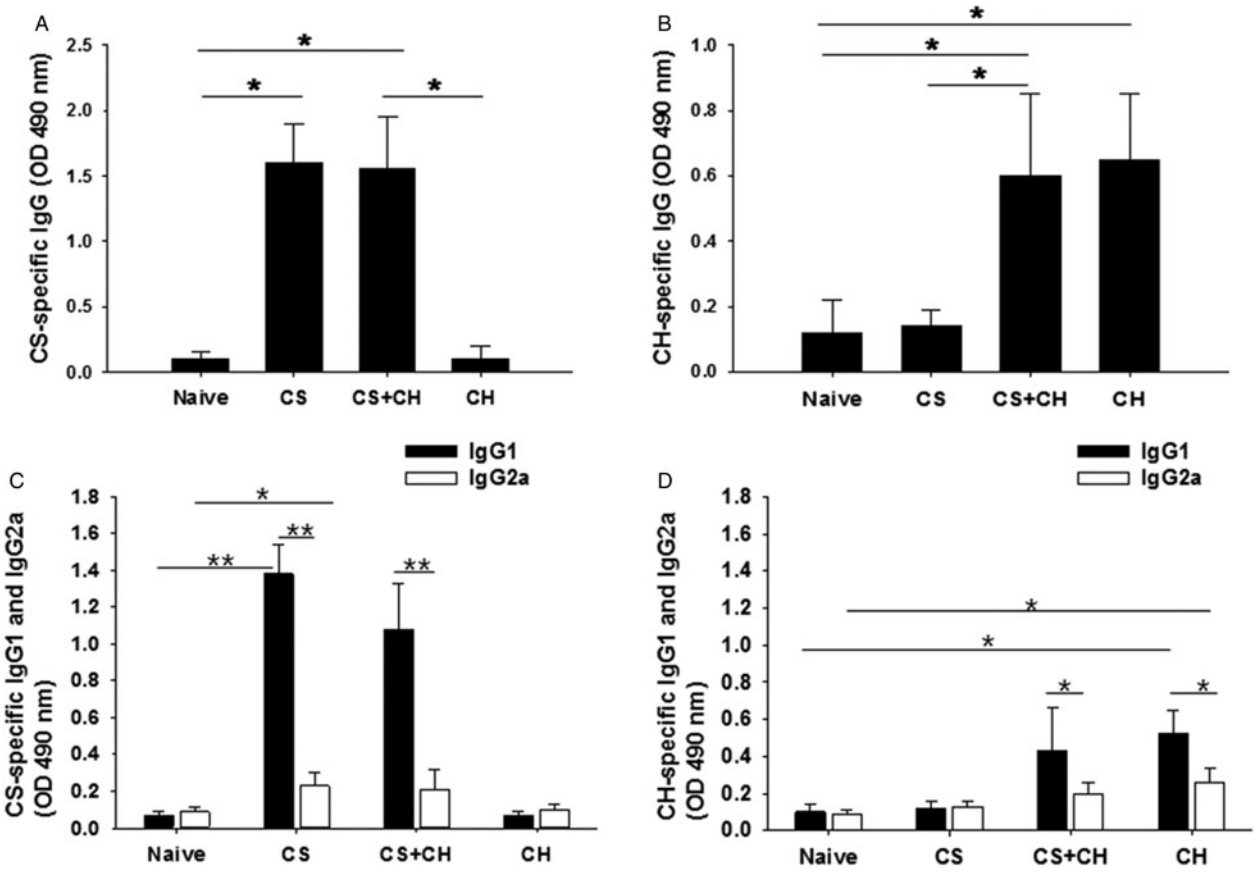

Fig. 2. *Clonorchis sinensis*- or *C. hepatica*-specific IgG, IgG1 and IgG2a antibody responses. *Clonorchis sinensis*-specific IgG (A) and *C. hepatica*-specific IgG levels (B), from rats at week 4 after primary (CS, CH rats) and post-challenge infection (CS + CH rats), were determined by ELISA. Significantly higher levels of *C. sinensis*- specific IgG antibodies were found in the sera of *C. sinensis*-infected and co-infected rats (A). Significantly higher levels of *C. hepatica*-specific IgG antibodies were found in the sera of *C. hepatica*-infected and co-infected rats (B). Importantly, no cross-reacting antibody was observed between *C. sinensis* and *C. hepatica* infections. *Clonorchis sinensis*-specific and *C. hepatica*-specific IgG1 and IgG2a antibody responses were also determined (C, D). *Clonorchis sinensis*-specific IgG1 and IgG2a (C) and *C. hepatica*-specific IgG1 and IgG2a (D), from rats at week 4 after primary (CS, CH rats) and post-challenge infection (CS + CH rats), were determined by ELISA. Significantly higher levels of *C. sinensis*-specific IgG1 antibodies were found in the sera of *C. sinensis*-infected and co-infected rats compared to IgG2a (C). Significantly higher levels of *C. hepatica*-specific IgG1 antibodies were found in the sera of *C. hepatica*-infected and co-infected rats compared to IgG2a (D). No cross-reacting antibody was observed between *C. sinensis* and *C. hepatica* infections. The error bars represent SE.

significantly higher levels of eosinophil counts compared to naïve controls, and *C. hepatica* (CH) infection produced much higher levels than did *C. sinensis* (CS) infection (*$P <$ 0.05). Importantly, subsequent infection with *C. hepatica* in *C. sinensis*-infected rats (CS + CH) showed a remarkable increase in eosinophil counts ($271 \pm 81/\mu l$) compared to single infections ($154 \pm 100/\mu l$) (fig. 3, *$P < 0.05$, **$P <$ 0.01). These results indicate that *C. hepatica* infection in helminthic co-infections is critically effective in increasing eosinophil counts in peripheral blood.

Sequential infection with C. hepatica *in* C. sinensis*-infected rats induced higher levels of lymphocyte proliferative response*

Lymphocyte proliferation is the process whereby lymphocytes begin to replicate after their antigen receptors recognize an antigen. As seen in fig. 4, a higher degree of lymphocyte proliferation was found in spleens from CS and CS + CH rats when stimulated with *C. sinensis* ES Ag or PHA, compared to unstimulated cells or those from naïve rats (*$P < 0.05$). Interestingly, cells from CS + CH rats showed remarkably higher levels of lymphocyte proliferation compared to cells from CS rats (*$P < 0.05$). These results indicate that a significantly higher level of lymphocyte proliferation is induced by subsequent infection with *C. hepatica*.

Cytokine production

Cytokine production is an indicator of cellular immune responses. To compare cytokine production levels in states of single infection (CS) and co-infection (CS + CH) and its relationship with worm burden, spleens were collected at week 4 post-challenge and cytokine

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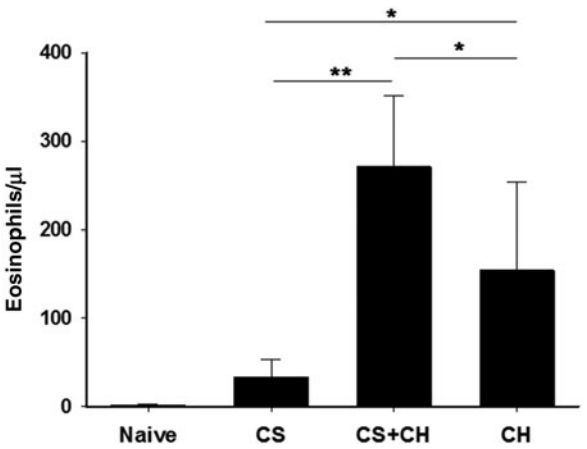

Fig. 3. Eosinophil responses. Eosinophils were counted from blood samples of rats ($n = 12$) at week 4 after infection with *C. sinensis* or *C. hepatica* in a single infection (CS, CH) or co-infection (CS + CH). Eosinophil levels were raised significantly in CS, CH and CS + CH rats compared to naïve controls (*$P < 0.05$). Significantly raised eosinophil levels were detected in CS + CH co-infected rats compared to CS or CH rats (**$P < 0.01$; *$P < 0.05$). The error bars represent SE.

production levels (IFN-γ, IL-2, IL-4 and IL-10) were determined in response to stimulation with *C. sinensis* ES Ag. As shown in fig. 5, significantly higher levels of the cytokines IFN-γ, IL-4 and IL-10 were produced in the CS + CH rats following *C. sinensis* ES Ag stimulation compared to the CS rats or naïve control rats, indicating that cytokine responses are stimulated upon subsequent infection.

Co-infection with Capillaria hepatica *induced resistance against* Clonorchis sinensis *infection*

Adult *C. sinensis* worms in livers of co-infected CS + CH rats were counted, and reduced parasite loads were observed compared to those in singly infected CS rats. As shown in fig. 6A, a significant reduction of *C. sinensis* was found in CS + CH rats (18.2 ± 10) compared to CS rats (35.6 ± 6) (*$P < 0.05$). *Capillaria hepatica* infection was counted by determining *C. hepatica* eggs in the livers (fig. 6B). These results indicate that subsequent infection with *C. hepatica* in *C. sinensis*-infected rats reduced the *C. sinensis* worm burden.

Discussion

In the present study, we investigated IgG antibody responses, eosinophil levels, lymphocyte proliferation, cytokine production and worm burden induced by co-infection with *C. sinensis* and *C. hepatica*, which have not been investigated previously. We found that cross-reactive *C. sinensis*-specific IgG antibody was not detected in *C. hepatica*-infected rats, while high levels of *C. sinensis*-specific IgG antibody were detected in CS and CS + CH co-infected rats, indicating no IgG cross-reactivity between *C. sinensis* and *C. hepatica*. However, eosinophils, lymphocytes and cytokine responses were increased significantly in CS + CH co-infected rats, indicating that these responses might contribute to *C. sinensis* worm reduction in CS + CH co-infected rats.

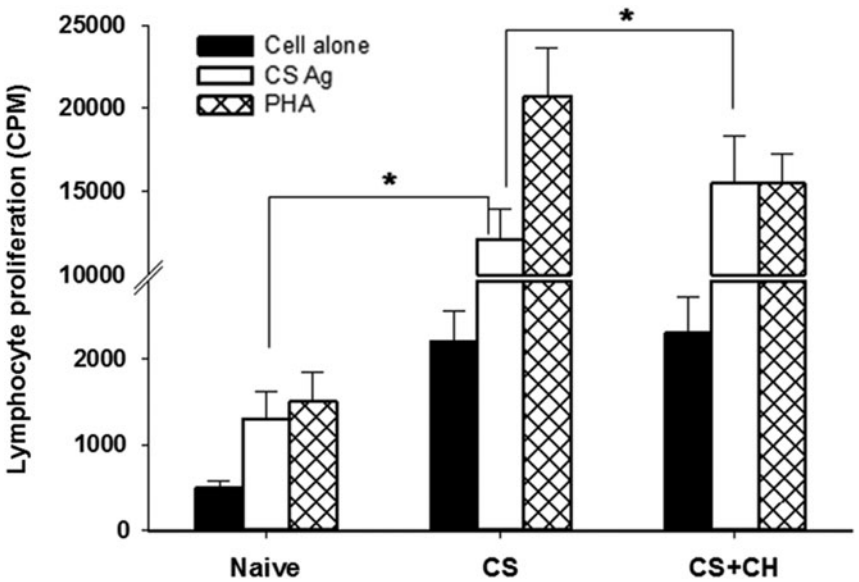

Fig. 4. Lymphocyte proliferation. Rats ($n = 12$) were primarily infected with 50 metacercariae of *C. sinensis* and, after 4 weeks, rats were challenge infected with 1000 eggs of *C. hepatica*. Four weeks after post-challenge infection, rats were sacrificed and lymphocytes were collected from spleens. Each group was stimulated by the *C. sinensis* ES Ag (CS Ag) and the positive control, phytohaemagglutinin (PHA). No stimulator was added to the cell-alone group. The experiment was carried out in triplicate. Significant increases of lymphocytes (cpm) were determined in CS and CS + CH rats (*$P < 0.05$). The error bars represent SE

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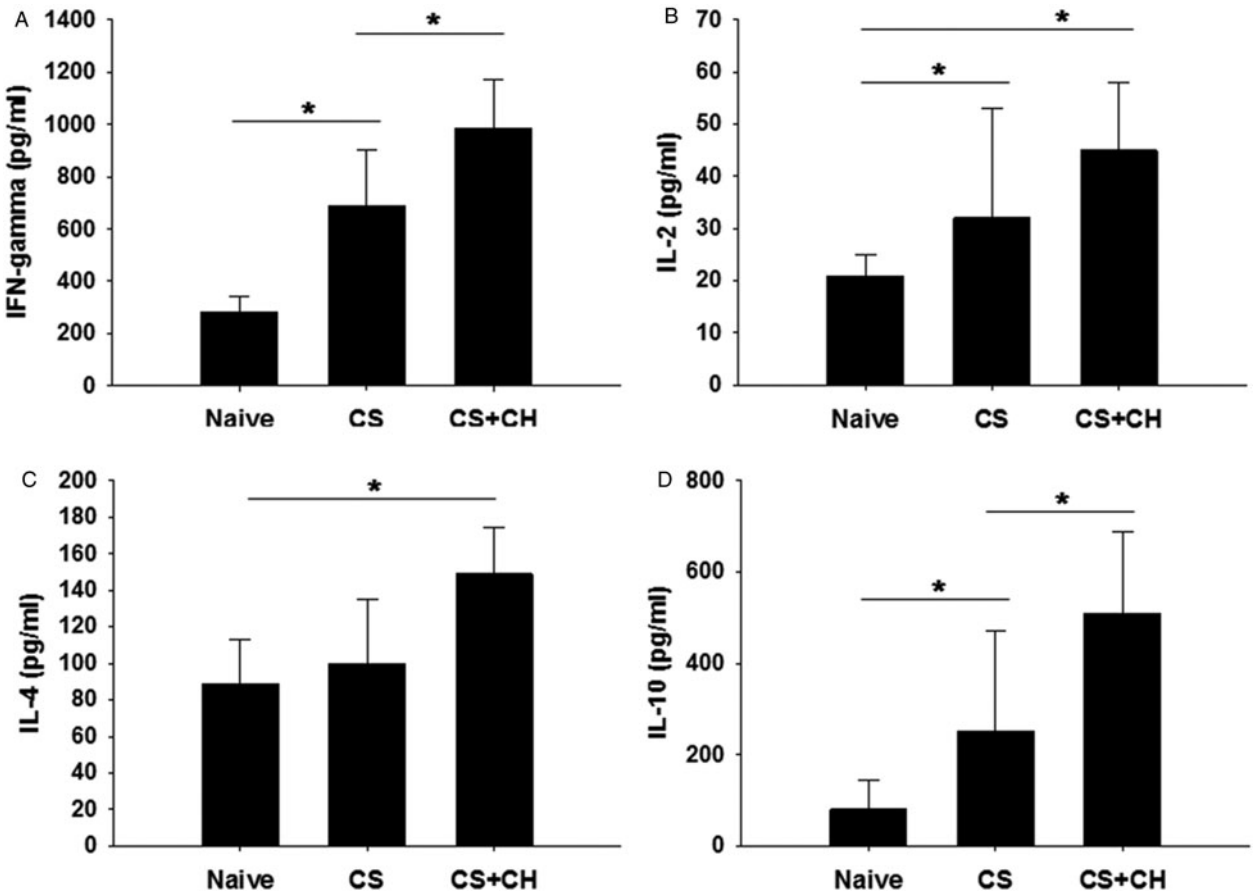

Fig. 5. Cytokine responses. Rats ($n = 12$) were primarily infected with 50 metacercariae of *C. sinensis* and, after 4 weeks, rats were challenge infected with 1000 eggs of *C. hepatica*. At week 4 post-challenge infection, rats were sacrificed and cytokines IFN-γ, IL-2, IL-4 and IL-10 were determined in lymphocytes of spleens from naïve, CS and CS + CH rats. Levels of IFN-γ, IL-4 and IL-10 were found to be significantly higher in CS + CH compared to CS rats (A, C, D, $^*P < 0.05$). The error bars represent SE.

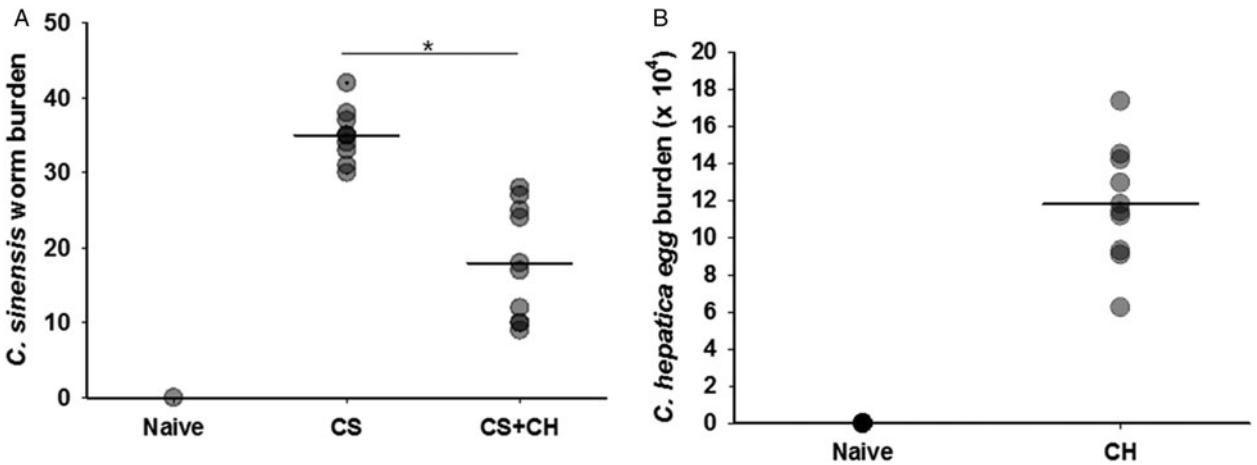

Fig. 6. *Clonorchis sinensis* worm burden. Rats ($n = 12$) were primarily infected with 50 metacercariae of *C. sinensis* and, after 4 weeks, rats were challenge infected with 1000 eggs of *C. hepatica*. At week 4 post-challenge infection, rats were sacrificed and *C. sinensis* adults were collected in livers and counted from CS and CS + CH rats. Significantly reduced levels of *C. sinensis* adults were found in CS + CH rats compared to CS rats (A, $^*P < 0.05$). (B) *Capillaria hepatica* infection was determined in the rat livers. The error bars represent SE.

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It has been reported that single infection with *C. hepatica* or *C. sinensis* induces eosinophilic liver infiltration or peripheral blood eosinophilia (Ewing & Tilden, 1956; Lee, 1964). Eosinophil elevation in parasitic helminth infection is beneficial to the host via the antibody-dependent cellular cytotoxicity system (Kazura, 1981; Klion & Nutman, 2004; Ganley-Leal *et al*., 2006; Bruschi *et al*., 2008; Cadman & Lawrence, 2010; Cadman *et al*., 2014). In a study of co-infections with *C. sinensis* and *T. spiralis*, single infection with *T. spiralis* was found to significantly increase eosinophil levels compared to co-infection, in which resistance against subsequent *T. spiralis* infection was seen. Intestinal pathological changes and immune responses elicited by prior infection with *C. sinensis* contributed to protection (Chen *et al*., 2013). In the current study, we observed that eosinophil levels were significantly increased upon subsequent infection with *C. hepatica* in *C. sinensis*-infected rats, and the pre-existing *C. sinensis* worm burden was reduced upon subsequent infection, indicating that the increase in eosinophil levels may contribute to the resistance against pre-existing *C. sinensis*. The mechanism of resistance involved in co-infection is likely to be very complicated, and more studies are needed for this mechanism to be elucidated.

Cross-reactive IgG and IgA antibody responses induced by co-infection with *C. sinensis* and *T. spiralis* were previously shown to protect against *C. sinensis* in co-infection (Chu *et al*., 2014). Shared antigens between *C. sinensis* and *T. spiralis* were reported to elicit cross-reactivity, thereby producing resistance against pre-existing *C. sinensis* infection (Chu *et al*., 2014). However, in our present co-infection study, *C. sinensis*-specific antibody responses were not detected in *C. hepatica*-infected rats. Also, *C. hepatica*-specific antibody responses were not found in *C. sinensis*-infected rats, indicating there are no cross-reactive antibody responses between *C. sinensis* and *C. hepatica*. Consistently, it has also been observed that *Capillaria philippinensis* antigen is not cross-reactive with sera from patients with schistosomiasis mansoni and fascioliasis (El Dib *et al*., 2004). In the present study, similar levels of *C. sinensis*-specific IgG antibody responses were observed in CS and CS + CH rats, indicating that a subsequent infection with *C. hepatica* does not enhance *C. sinensis*-specific antibody responses. This indicates that cross-reactive antibodies in co-infection might not be involved in *C. sinensis* worm reduction upon subsequent infection with *C. hepatica*. In the present study, higher levels of *C. sinensis*- or *C. hepatica*-specific IgG1 and IgG2a antibodies were found in single *C. sinensis*- or *C. hepatica*-infected and *C. sinensis*/*C. hepatica* co-infected rats compared to naïve rats. It is well known that IgG2a and IgG1 are, respectively, induced by T-helper cells Th1 and Th2. Our results suggest that *C. sinensis*, *C. hepatica* and co-infection of these two helminths induced a combined Th1/Th2 immune response, which is consistent with the cytokine production seen in the present study.

Our data indicate that a high degree of lymphocyte proliferation was detectable in CS and CS + CH rats. We also found that higher levels of cytokines IFN-γ, IL-4 and IL-10 were detected in co-infection compared to single infection with *C. sinensis*, indicating that Th1 and Th2 cytokines may be involved in the immune response changes induced by *C. hepatica* infection. In murine schistosomiasis, the presence of any eggs in the liver induced a marked Th2 response that developed into granulomatous lesions (Cheever *et al*., 2000). A mixed Th1- and Th2-type immune response is induced in humans concurrently infected with *Necator americanus* and *Oesophagostomum bifurcum* (Pit *et al*., 2001). The Th1 response is induced to prevent alternative macrophage activation and to limit the fibrosis-enhancing effects of the protective Th2 response (Hoffmann *et al*., 1998; Hesse *et al*., 2000). Future studies will focus on detailed cytokine-related immune responses in *C. sinensis*/*C. hepatica* co-infected rats.

The present findings demonstrated that the worm burden of *C. sinensis* was significantly reduced in *C. sinensis*-infected rats upon subsequent infection with *C. hepatica*. Cross-reactive antibody responses against *C. sinensis* were not involved in worm reduction.

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Conflict of interest

None.

Ethical standards

All animal experiments and husbandry involved in the studies presented in this manuscript were conducted under the guidelines of the Kyung Hee University IACUC (permit number: KHUASP (SE) - 17 - 009).

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