

RESEARCH ARTICLE

Clonorchis sinensis infection modulates key cytokines for essential immune response impacted by sex

Shuo Kan^{1,2}✉, Qi Li^{1,2}✉, Hong-Mei Li³, Yan-Hua Yao⁴, Xin-Yue Du², Chen-Yun Wu², Guang-Jie Chen², Xiao-Kui Guo¹, Men-Bao Qian^{1,3*}, Zhao-Jun Wang^{1,2*}

1 NHC Key Laboratory of Parasite and Vector Biology; School of Global Health, Chinese Center for Tropical Diseases Research-Shanghai Jiao Tong University School of Medicine, Shanghai, China, **2** Shanghai Institute of Immunology, Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, Shanghai, China, **3** National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Chinese Center for Tropical Diseases Research); WHO Collaborating Centre for Tropical Diseases; National Center for International Research on Tropical Diseases, Shanghai, China, **4** Department of Biochemistry and Molecular Cell Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

✉ These authors contributed equally to this work.

* qianmb@nipd.chinacdc.cn (MBQ); zjwang@sjtu.edu.cn (ZJW)



OPEN ACCESS

Citation: Kan S, Li Q, Li H-M, Yao Y-H, Du X-Y, Wu C-Y, et al. (2022) *Clonorchis sinensis* infection modulates key cytokines for essential immune response impacted by sex. PLoS Negl Trop Dis 16(9): e0010726. <https://doi.org/10.1371/journal.pntd.0010726>

Editor: Jong-Yil Chai, Seoul National University College of Medicine, REPUBLIC OF KOREA

Received: May 15, 2022

Accepted: August 10, 2022

Published: September 9, 2022

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pntd.0010726>

Copyright: © 2022 Kan et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its [Supporting information](#) files.

Abstract

Infection with helminths can modulate the host immune response, which ultimately shape morbidity and mortality of the associated diseases. We studied key cytokines for essential immune response in sera from 229 southeastern China individuals infected with *Clonorchis sinensis* and 60 individuals without *C. sinensis* infection, and measured serum specific IgG and IgE against worms in these people. Individuals infected with *C. sinensis* had significantly higher antigen-specific IgG and IgE levels, which were positively correlated with egg counts in feces. However, less enhancement of IgE antibody was observed in females when compared to males with similar infection levels. *C. sinensis* infection caused diminished Th1 cytokines (IL-1 β , IL-2, IL-12p70, IFN- γ and TNF- α), Th2 cytokine (IL-4), as well as Th17 cytokine (IL-17A) in sera, which showed decreasing trend by infection intensity. Notably, these phenotypes were more significant in females than those in males. Although *C. sinensis* infection is associated with the development of hepatobiliary diseases, there was no significant correlation between the dampened cytokine profiles and the hepatobiliary morbidities. Our study indicates *C. sinensis* infection is strongly related to the immune suppression in human. Sex differences shape the immune milieu of clonorchiasis. This study provides a better understanding of how worms affect immune responses and cause a long-term immune alternation in humans with *C. sinensis* infection.

Author summary

Clonorchis sinensis, also known as the liver fluke, lives in human bile duct system and is endemic in East Asia. Chronic *C. sinensis* infection without treatment can result in serious

Funding: ZJW was supported by the National Natural Science Foundation of China (81971486), the Natural Science Foundation of Shanghai (19ZR1428500), and the Fifth Round of Three-Year Public Health Action Plan of Shanghai (No. GWV-10.1-XK13). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

illness and predispose the human to bile duct cancer. Helminth infection is able to modulate the host immune response and influence the outcome of infection, but the immune characteristics of *C. sinensis* infection is not yet known. In this study, we analyzed serum samples from individuals living in endemic areas with clonorchiasis in China. We found *C. sinensis* infection caused increased specific IgG and IgE to adult worm antigens, but diminished levels of key cytokines for essential immune response. Th1 cytokines (IL-1 β , IL-2, IL-12p70, IFN- γ and TNF- α), Th2 cytokine (IL-4), as well as Th17 cytokine (IL-17A) showed decreasing trend by infection intensity. Moreover, females exhibited more significant cytokine variation compared to males with similar infection intensity. Our study indicates that *C. sinensis* infection is related to immune suppression in human, which might contribute to the outcome of clonorchiasis. The sexual dimorphism needs to be considered in the clonorchiasis prophylaxis and immune investigation.

Introduction

Parasitic worms coexist with human beings for a very long time. In a life history, host and parasite continually adapt to each other, thus a finely tuned balance between host immunity and chronic parasitism has been developed [1,2]. It has been believed that type-2-cell-mediated immune responses play a critical role in immune responses to parasitic worms [3]. Meanwhile, many parasite species can induce IL-10 production and Treg cell development, then redirect, suppress, and evade host immunity to establish chronic infection [1,4]. Notable progress has been made in understanding helminth immunology, which contributes to disease prophylaxis and immune system investigation. However, most of those advances focus on intestinal helminth or using murine models for intestinal nematodes, such as *Trichuris muris*, *Nippostrongylus brasiliensis*, and *Heligmosomoides polygyrus* [5]. Various parasites with different stages reside in different tissue locations during their life cycle. For example, most trematodes are tissue-dwelling helminths [6]. Their roles in immune regulation may not the same as intestinal helminths.

Clonorchiasis is a food-borne parasitic disease, caused by *Clonorchis sinensis*, which is predominantly endemic in East Asia, including China, Korea and Vietnam [7–9]. About 15 million people are estimated to be affected by this disease. Particularly, about 13 million cases distribute in China [10,11]. The adult worms of *C. sinensis* living in the biliary tree of the liver produce eggs which are passed in feces. *C. sinensis* infection predominantly leads to hepatobiliary abnormalities, such as periductal fibrosis, cholangitis, cholecystitis and cholelithiasis [12–15]. Moreover, it is classified as “carcinogenic to humans” (Group 1) by the International Agency for Research on Cancer, because of the carcinogenesis in fatal cholangiocarcinoma [16]. Up to now, research on the immunology of human clonorchiasis is inadequate, which hinders our understanding on the pathogenesis. In this study, we screened 289 serum samples from individuals living in southeastern China endemic with clonorchiasis, to reveal a physiology long-term immune response profiles of *C. sinensis* infection in human. Our study indicates *C. sinensis* infection is strongly related to the immune suppression, which is influenced by sex.

Methods

Ethics statement

The conducts and procedures were approved by the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai, China

(reference no.2011–005). All individuals or their guardians for those aged <18 years have provided written informed consent. Praziquantel (25 mg/kg, t.i.d, 2 days) and albendazole (400mg, single oral dose) were provided to individuals infected with *C. sinensis* for free.

Human sera

Serum samples were collected from villagers living in Hengxian County, Guangxi, China, where clonorchiasis is highly endemic. Detection of helminth infection, collection of sera, and ultrasound examination for hepatobiliary abnormalities were implemented, as described previously [12,17]. In brief, one stool sample was collected and examined by the Kato-Katz method and washing sedimentation technique. The eggs per gram of feces (EPG) is calculated by multiplying the average of three Kato-Katz smears with a factor of 24. Sera were then collected from the participants and abdominal ultrasound examination was also implemented. A total of 289 participants were included in this study, of which 229 individuals was detected with *C. sinensis* infection (Cs+) and another 60 individuals without *C. sinensis* infection (Cs-). The characteristics of the participants were summarized in [S1 Table](#).

Preparation of adult worm antigen of *C. sinensis* (CsAWA)

Adult worm antigen of *C. sinensis* (CsAWA) were prepared for ELISA. In brief, *C. sinensis* adult worms suspended in PBS were homogenized on ice. The mixture was lysed by sonication in an ice-chilled water bath. Then, the lysed homogenate was centrifuged at 15,000 g for 20 min at 4°C. The supernatant was dialyzed against PBS at 4°C overnight and used as CsAWA. Protein concentration was measured by BCA Protein Assay Kit (Sangon Biotech, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody reactivity of human sera against CsAWA was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 100 μ l 2.5 μ g/ml CsAWA overnight. Human sera were diluted with 1:100 and HRP conjugated goat anti-human IgG (Sigma-Aldrich, USA, 1:5000 dilution) or IgE (Invitrogen, USA, 1:2000 dilution) was used as the secondary antibody. Next, reactions were developed using 3,3',5,5'-Tetramethylbenzidine (TMB) substrates and stopped with 2 N H₂SO₄. The optical densities were read at 450 nm in a microwell reader system (Biotek, USA).

Serum essential immune response cytokine screen

Key cytokines for essential immune response, including IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and free active TGF- β 1 were measured using a LEGENDplex™ HU Essential Immune Response Panel Kit (BioLegend, San Diego, CA, USA). The data were acquired on a BD FACSCanto™ II flow cytometer, analyzed using LEGENDplex™ Data Analysis Software, and calculated by standard curves according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using Graphpad Prism 9 and SPSS 24.0 software. The data were presented as the mean \pm s.e.m. Statistical significance was analyzed by means of Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's multiple comparisons test. Spearman's correlation analysis was used to analyze the association between the EPG and antibodies or cytokines. The correlation coefficient, *r*, ranges from -1 to +1. The significance corresponding to the *r* is: perfect positive correlation *r* = 1, the two variables tend to increase or decrease together $0 < r < 1$, the two variables do not vary together at all *r* = 0, one variable increases as

the other decreases $-1 < r < 0$, perfect negative or inverse correlation $r = -1$. Principal Component Analysis (PCA) was used to extract the main feature components of the data. Results were considered statistically significant difference at $P < 0.05$. The significance corresponding to the asterisk is: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Enhanced levels of IgG and IgE specific antibodies in individuals with *C. sinensis* infection

To determine the immune status of people infected with *C. sinensis*, we firstly measured serum specific antibodies by ELISA. The levels of IgG and IgE antibodies against *C. sinensis* were significantly higher in *Cs+* than in *Cs-* individuals (Fig 1A). Elevated levels of serum IgG and IgE were positively correlated with the intensity of infection expressed as EPG (Fig 1B).

Less enhancement of specific IgE in female individuals with *C. sinensis* infection

It is well known that sex and age differences shape the immune response to infectious diseases [18]. We analyzed the levels of IgG and IgE in females and males respectively. As shown in Fig 2A, there was a significant difference in serum specific IgG and IgE levels between female and male groups in *Cs+* individuals. Positive correlations were observed between EPG and serum specific IgG and IgE in both female and male groups (Fig 2B). However, less enhancement of IgE antibody was observed in females when compared to males with similar EPG (Fig 2B). We also analyzed the levels of specific antibodies in different age groups (10–29 years, 30–44 years, 45–59 years, and 60–86 years). Elevated levels of serum IgG and IgE were positively correlated with the intensity of infection (EPG) in all four age groups (S1 Fig).

Diminished serum levels of key cytokines for essential immune response in *C. sinensis* infected individuals

To further determine the effect of *C. sinensis* infection on host immunity, we measured key cytokines for essential immune response in sera, including IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1. As shown in Fig 3A, *Cs+* individuals had significantly lower levels of IL-1 β , IL-2, IL-4, IL-12p70, IL-17A, IFN- γ and TNF- α in comparison with *Cs-* individuals. In addition, the levels of IL-6, IL-10 and TGF- β 1 also showed a decreasing trend in *Cs+* individuals compared to *Cs-* individuals. Spearman's correlation analysis indicated that the levels of IL-1 β , IL-2, IL-4, IL-10, IL-12p70, IL-17A and TNF- α were significantly decreased with increasing EPG (Fig 3B). Similar trends were found in different age groups (10–29 years, 30–44 years, 45–59 years and 60–86 years groups) (S2 Fig). Overall, *C. sinensis* infection appeared to suppress key cytokines which are associated with innate and adaptive immune responses.

Sex differences shape the cytokine milieu in *C. sinensis* infection

To study the influence of sex on changes in cytokines, we analyzed the cytokine profiles in females and males respectively. No significant difference was observed between females and males without *C. sinensis* infection. The baseline levels of cytokines were comparable in females and males (S3 Fig). As shown in Fig 4, both females and males had suppressed cytokine milieu post *C. sinensis* infection. There were significant negative correlations between EPG and the levels of IL-1 β , IL-4, IL-12p70, IL-17A, TNF- α and TGF- β 1 in females. While in male individuals, only IL-17A level showed a significant correlation with EPG. With the

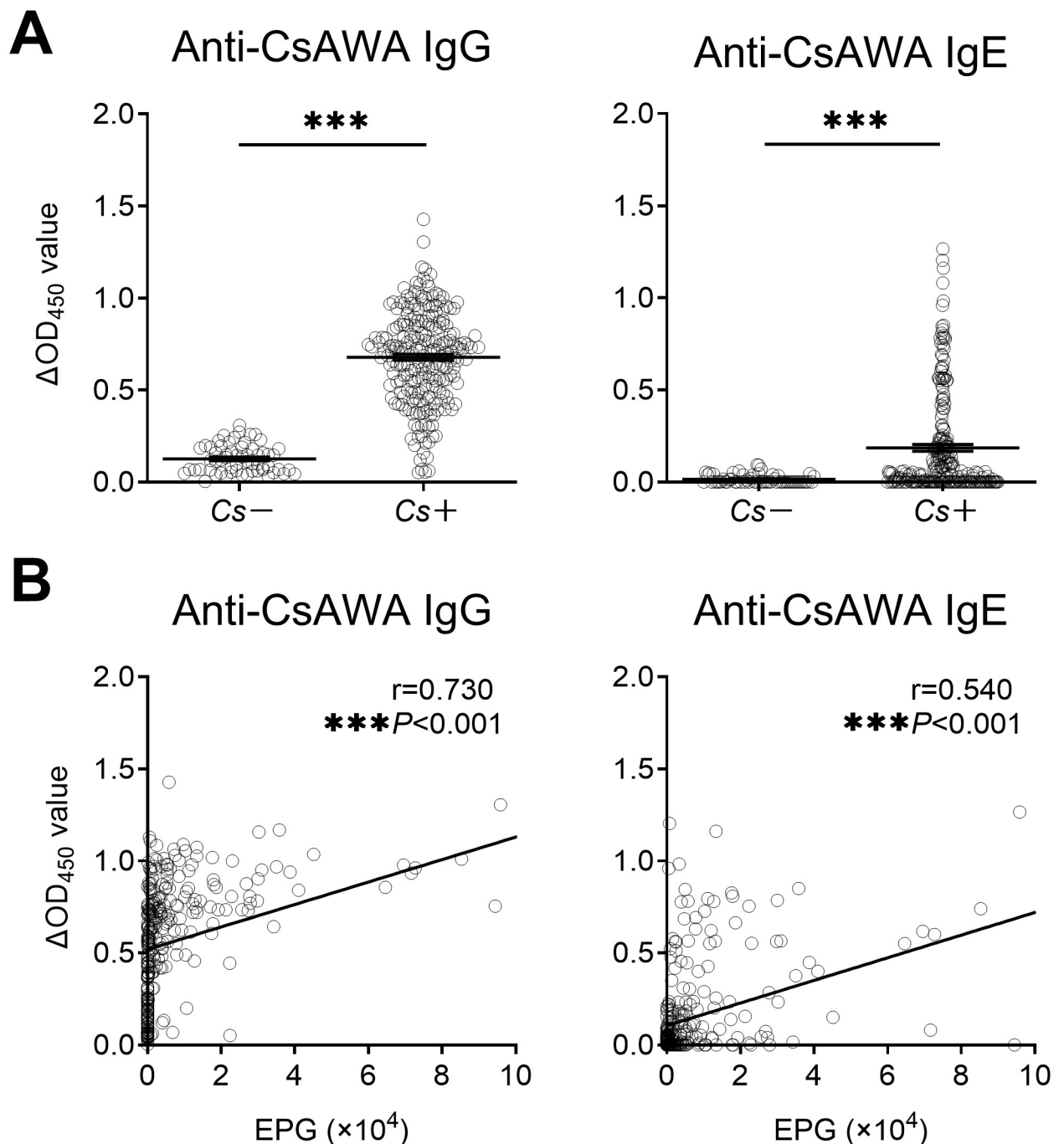


Fig 1. Serum levels of anti-CsAWA IgG and IgE in Cs- and Cs+ individuals. (A) Serum levels of anti-CsAWA IgG and IgE in Cs- individuals (n = 60) and Cs+ individuals (n = 229). (B) Spearman's correlation analysis between EPG and serum levels of anti-CsAWA IgG and IgE (n = 289). CsAWA: *C. sinensis* adult worm antigen, Cs: *C. sinensis* infection, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., *** $P < 0.001$, Mann-Whitney U test in (A), Spearman's correlation test in (B).

<https://doi.org/10.1371/journal.pntd.0010726.g001>

similar intensity of infection, female individuals showed more reduction in cytokines than male individuals. We further compared the cytokine levels between females and males in different age groups. In general, both females and males had suppressed cytokine milieu post *C. sinensis* infection in 30–44 years, 45–59 years and 60–86 years groups, and female individuals showed more reduction in cytokines than male individuals (S4 Fig).

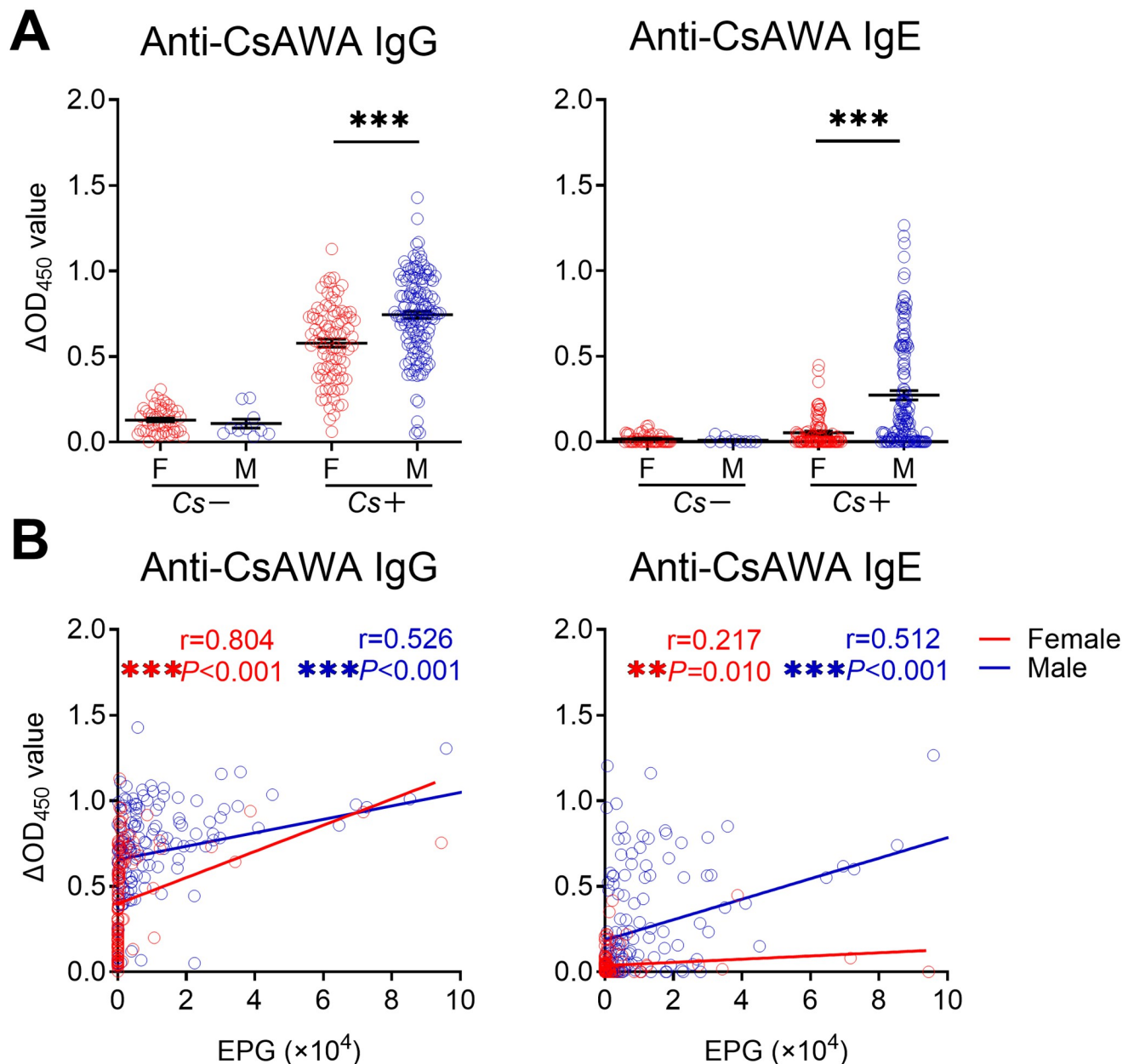


Fig 2. Serum levels of anti-CsAWA IgG and IgE in Cs- and Cs+ individuals with different genders. (A) Serum levels of anti-CsAWA IgG and IgE in Cs- individuals (n = 50 in female and n = 10 in male) and Cs+ individuals (n = 91 in female and n = 138 in male). (B) Spearman's correlation analysis between EPG and serum levels of anti-CsAWA IgG and IgE (n = 141 in female and n = 148 in male). CsAWA: *C. sinensis* adult worm antigen, Cs: *C. sinensis* infection, F: female, M: male, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., ** $P < 0.01$, *** $P < 0.001$, Kruskal-Wallis test followed by Dunn's multiple comparisons test in (A), Spearman's correlation test in (B).

<https://doi.org/10.1371/journal.pntd.0010726.g002>

PCA analysis reveals trends of sex bias in the immune milieu in *C. sinensis* infection

To assess the overall trends in cytokine and antibody discrimination between male and female individuals, we plotted PCA with different inputs. As shown in Fig 5, PCA analysis showed different cytokines and antibodies clusters between Cs+ male and female individuals. In contrast,

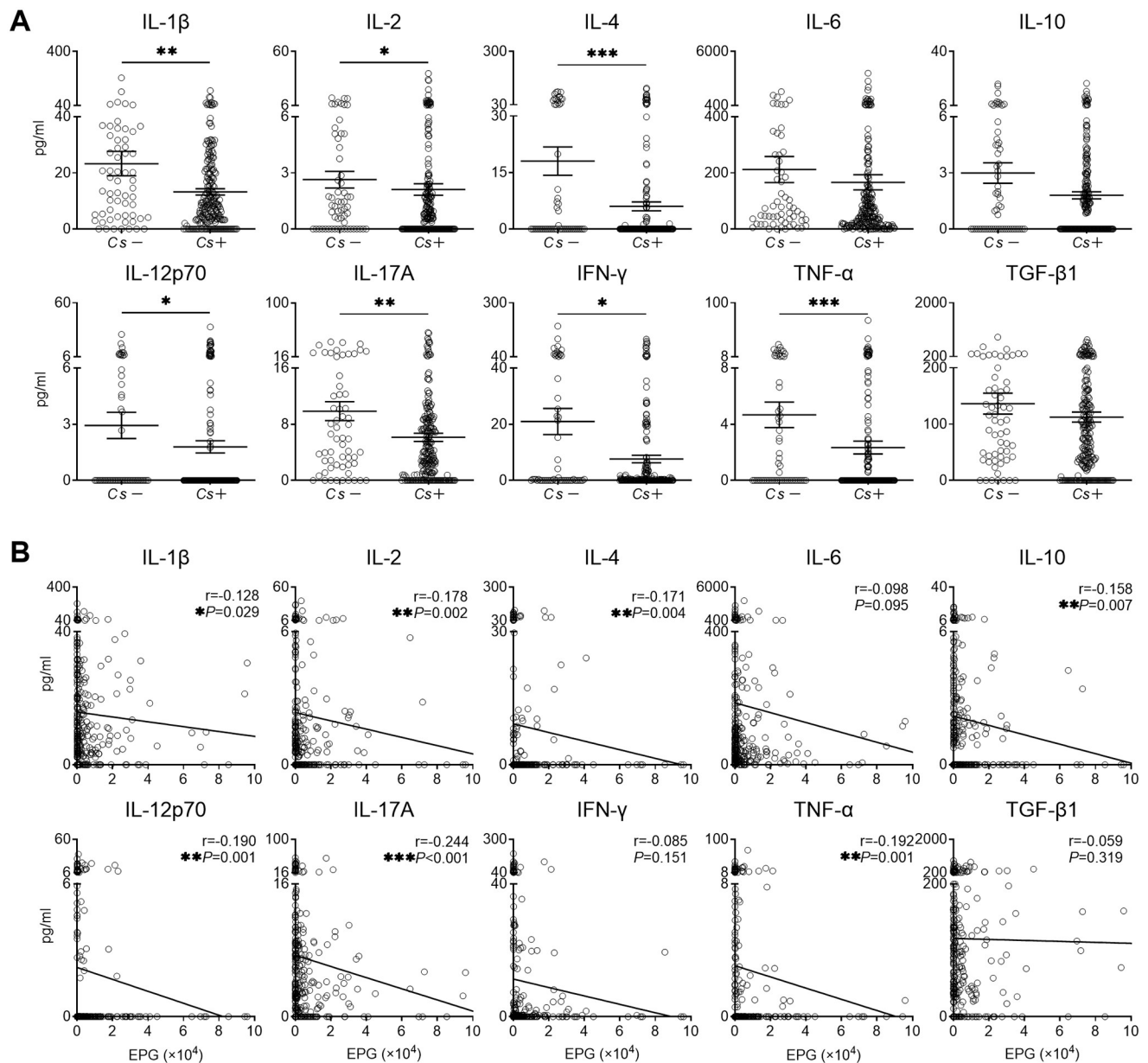


Fig 3. Serum levels of cytokines in Cs- and Cs+ individuals. (A) Serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 in Cs- individuals (n = 60) and Cs+ individuals (n = 229). (B) Spearman's correlation analysis between EPG and serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 (n = 289). Cs: *C. sinensis* infection, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney U test in (A), Spearman's correlation test in (B).

<https://doi.org/10.1371/journal.pntd.0010726.g003>

PCA analysis showed very little clustering between Cs- male and female population. These findings suggested that host immune status was influenced by sex in *C. sinensis* infection.

Diminished cytokines in *C. sinensis* infection are not related to hepatobiliary morbidities or soil-transmitted helminth co-infection

C. sinensis infection may result in various complications in the liver and biliary systems, which might dampen immune responses. Therefore, we asked whether diminished cytokine profiles

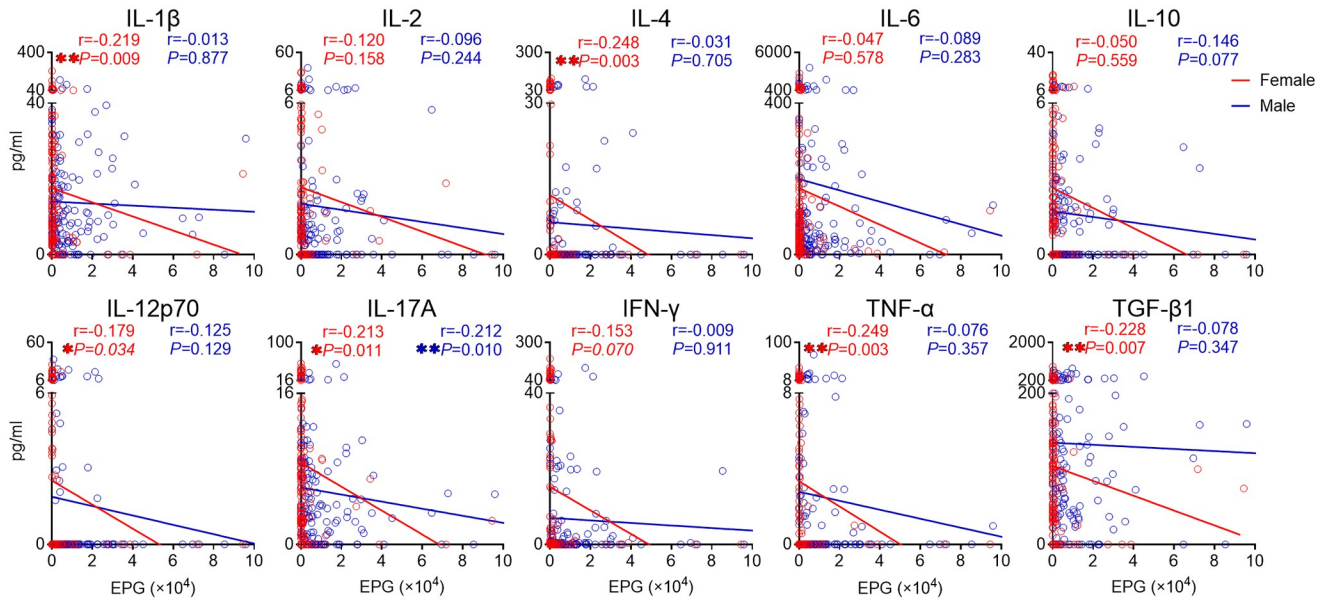


Fig 4. Spearman's correlation analysis between EPG and serum levels of cytokines in female and male groups. Spearman's correlation analysis between EPG and serum levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN-γ, TNF-α and TGF-β1 in female (n = 141) and male (n = 148). EPG: eggs per gram of feces. The data were shown as the mean ± s.e.m., *P < 0.05, **P < 0.01, Spearman's correlation test.

<https://doi.org/10.1371/journal.pntd.0010726.g004>

in *C. sinensis* infection is associated with hepatobiliary morbidities. Excluding individuals with hepatobiliary diseases detected by ultrasound, the remaining Cs+ individuals also had significant lower levels of IL-1β, IL-2, IL-4, IL-12p70, IL-17A, IFN-γ and TNF-α in comparison with Cs- individuals (Fig 6A). Spearman's correlation analysis indicated that the levels of IL-1β, IL-

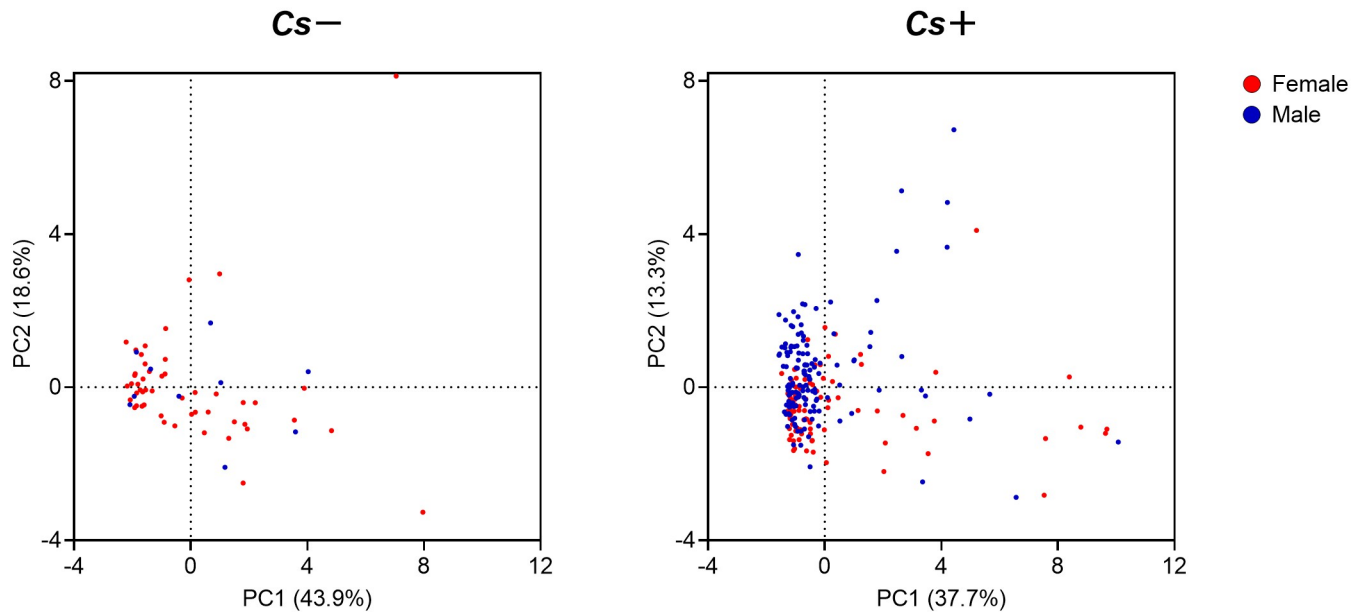


Fig 5. Principle component analysis (PCA) plots of anti-CsAWA specific antibodies and cytokines from Cs- and Cs+ individuals. The PCA stands for the two principal components of variation. Left panel, Cs- individuals (n = 50 in female and n = 10 in male). Right panel, Cs+ individuals (n = 91 in female and n = 138 in male). Cs: *C. sinensis* infection.

<https://doi.org/10.1371/journal.pntd.0010726.g005>

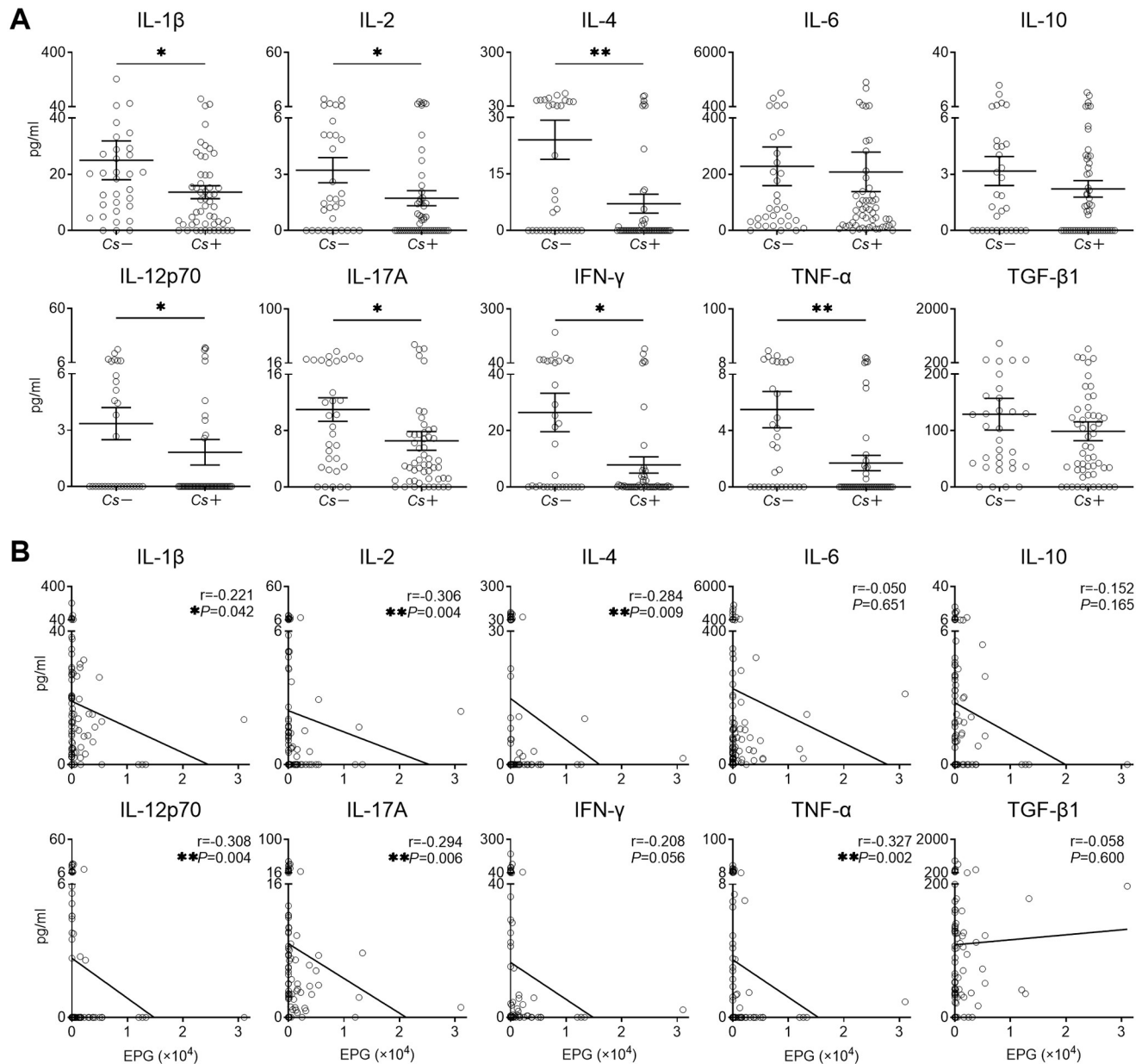


Fig 6. Serum levels of cytokines in Cs- and Cs+ individuals without hepatobiliary diseases. (A) Serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 in Cs- individuals (n = 32) and Cs+ individuals (n = 53) without hepatobiliary diseases. (B) Spearman's correlation analysis between EPG and serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α , and TGF- β 1 (n = 85). Cs: *C. sinensis* infection, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney U test in (A), Spearman's correlation test in (B).

<https://doi.org/10.1371/journal.pntd.0010726.g006>

2, IL-4, IL-12p70, IL-17A and TNF- α were significantly decreased with increasing EPG in Cs+ individuals without hepatobiliary morbidities (Fig 6B). In addition, we analyzed the effects of different hepatobiliary diseases on cytokines in Cs+ individuals respectively. Levels of most cytokines were similar between individuals with and without periductal fibrosis, fatty liver, or bile duct dilatation (S5 Fig). Therefore, diminished serum levels of key cytokines in *C. sinensis* infection were due to *C. sinensis* infection other than the morbidities caused by *C. sinensis* infection.

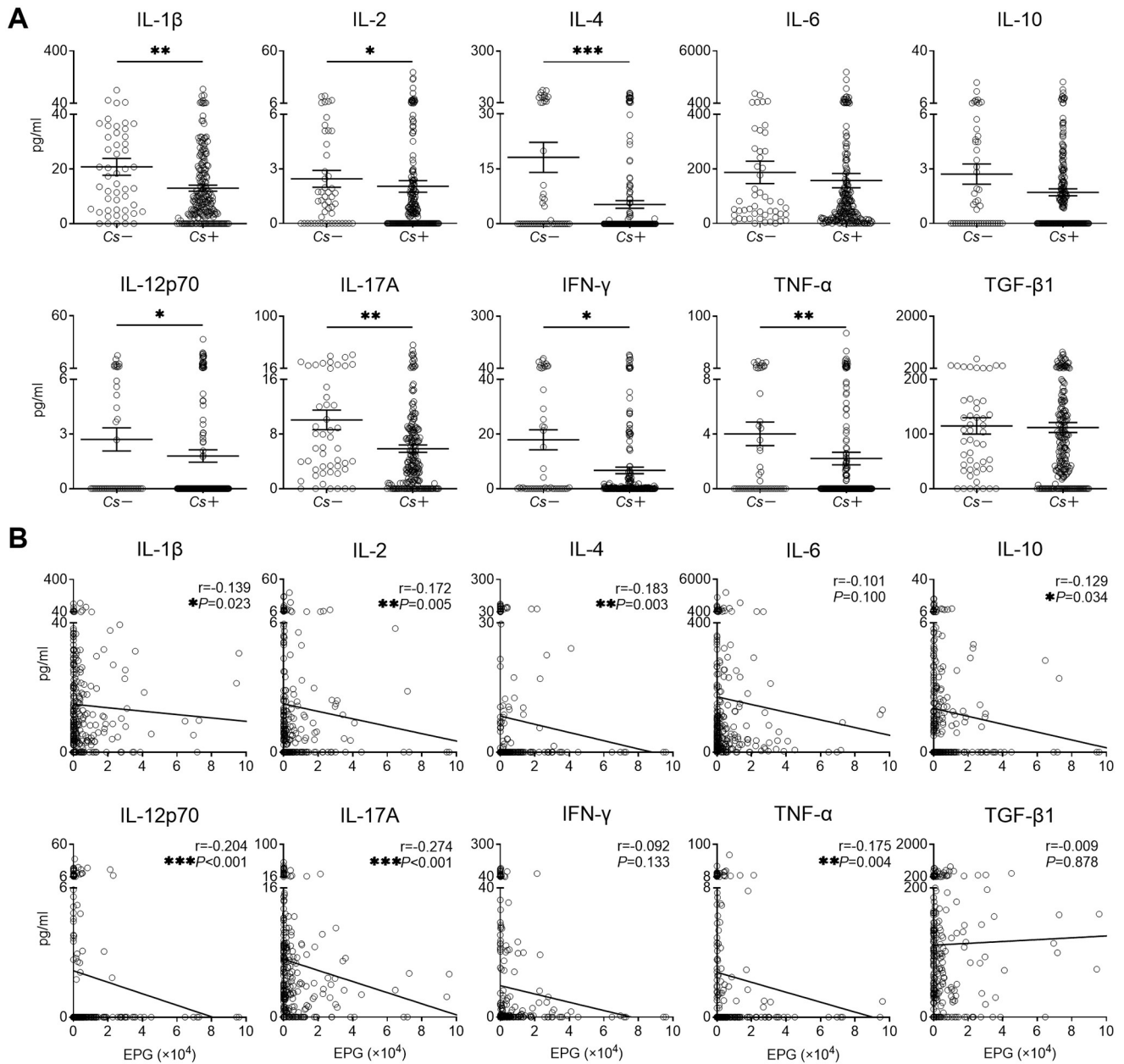


Fig 7. Serum levels of antibodies and cytokines in C- and C+ individuals excluding co-infection with soil-transmitted helminths. (A) Serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 in C- individuals (n = 50) and C+ individuals (n = 218) excluding co-infection with soil-transmitted helminths. (B) Spearman's correlation analysis between EPG and serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 (n = 268). C-: *C. sinensis* infection, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., * P < 0.05, ** P < 0.01, *** P < 0.001, Mann-Whitney U test in (A), Spearman's correlation test in (B).

<https://doi.org/10.1371/journal.pntd.0010726.g007>

Infections with soil-transmitted helminths (hookworms, roundworms, and whipworms) are widespread in tropical and subtropical regions. We collected the information about whether participants have co-infection with soil-transmitted helminths (S1 Table). Excluding co-infection with soil-transmitted helminths, individuals with *C. sinensis* still had significantly higher antigen-specific IgG and IgE levels, and diminished serum levels of IL-1 β , IL-2, IL-4, IL-12p70, IL-17A, IFN- γ and TNF- α (Fig 7).

Discussion

Helminths are extraordinarily successful parasites due to their ability to modulate the host immune response [1,2,5]. Th2 type immune responses are characteristic features of human infection with multicellular parasites [3,19]. In laboratory animals, clonorchiasis, like other helminth infections, is a potent inducer of Th2 responses [8,20,21]. Choi et al. studied antibody and cytokine responses in mice infected with *C. sinensis* and found that susceptibility to *C. sinensis* infection was associated with Th2 cytokine production, especially IL-4 [22]. Wang and colleagues carried out an experimental model in rats and found that immune response presented a tendency to Th2 type by expressing transient high levels of IgG, IgE and IL-4 [23]. Our present study described the immune responses of clonorchiasis in humans. As expected, anti-CsAWA IgG and IgE antibody levels were elevated in individuals infected with *C. sinensis*. Serum IgG and IgE levels were correlated with egg output in the stool, suggesting anti-*C. sinensis* specific immune responses were induced in infected people. However, systemic cytokines represented a general decline in human clonorchiasis. Not only Th1 cytokines (IL-1 β , IL-2, IL-12p70, IFN- γ and TNF- α), but also Th2 cytokine (IL-4) and Th17 cytokine (IL-17A) reduced markedly in *C. sinensis* infected people. Negative correlation between cytokine levels and egg counts furtherly supported the characteristics of general immune suppression in *C. sinensis* infection. In this study, *C. sinensis* infection didn't show increased Th2 immune response. It might due to the duration of infection. Acuteness and chronicity of infection drive distinct immune profiles. According to literatures, in *C. sinensis* infected mice, IL-4 production by splenocytes increased (> threefold) until 2–4 weeks post-infection, but declined thereafter [22]. In rat models, compared with control, IFN- γ and IL-4 levels were elevated post infection, and both decreased to lower levels at week 16 after primary infection [24]. In human beings, *C. sinensis* usually causes long-term infection. Untreated, infection may persist for up to 25–30 years [7]. With long-standing chronic infection, immune suppression might be the dominant phenotype in human clonorchiasis. It is interesting to establish a long-standing, persistent infection model (e.g., more than 24 weeks) to track the dynamic changes of cytokines and investigate the intrinsic mechanism. Helminth infections and their components have been shown to have the potential to modulate and attenuate immune responses [25]. *C. sinensis* infection is carcinogenic to human [26–28]. The pathogenic mechanisms include mechanical injury of biliary epithelia by the flukes, immunopathological changes caused by infection-related inflammation, and direct effects of the excretory-secretory products (ESPs) [8,29–31]. Here we propose, besides above 3 mechanisms, *C. sinensis* infection caused immune suppression might also facilitate the transformation and proliferation of the tumor cells.

Numerous investigations have revealed a bias toward males in the susceptibility to and severity of a variety of infectious diseases, especially parasitic diseases [32,33]. In clonorchiasis, the infection rate and intensity in males is usually higher than that in females [7,34]. The provincial level survey in Guangxi demonstrated a prevalence of 14.0% in male and 7.2% in female in 2019. Consistently, the prevalence in the male was around 2 times than that in the female in local survey in Republic of Korea and Vietnam [35,36]. It was believed that the difference between sexes in *C. sinensis* infection is mainly related to dietary customs namely raw-fishing eating behavior [7,37,38]. We focused on the immune alternations in people with *C. sinensis* infection, and demonstrated that *C. sinensis* infection induced immune suppression was influenced by sex. It would be interesting to explore whether this sex-based immune suppression contributes to the concomitant immunity in *C. sinensis* infection as well as its outcomes.

It has been known that sex broadly influences the host immune response [18]. Both genetic and hormonal factors may result in the sex difference of cytokine milieu [39,40]. For example, genes on the X chromosome code for numerous proteins involved in immune processes,

including pattern recognition receptors (PRRs, e.g., *TLR7* and *TLR8*), transcriptional factors (e.g., *FOXP3*) and main members in nuclear factor- κ B pathway (e.g., *IRAK-1* and *NEMO*), which are important in immune cell activation and cytokine production [41,42]. Sex hormones can influence the function of host immune cells by binding to specific receptors that are expressed in most immune cells, such as lymphocytes, macrophages and dendritic cells [39]. Moreover, hormone response elements are present in the promoters of several immune genes, thus sex hormones may directly alter gene expression and immune response [40]. It was reported that the expression of PRRs (e.g., *TLR4* and *TLR9*) could be regulated by sex hormone [43]. Innate immune cells from males express higher levels of *TLR4* and produce more pro-inflammatory cytokine *TNF α* and chemokine *CXCL10* than female cells both constitutively and following activation [39,44]. Activation of *TLR9* in PBMCs from human males results in more *IL-10* production compared with cells from females, which is positively correlated with androgen concentration in males [39]. In this study, we found that female individuals developed less specific IgE and had more reduction of systemic cytokines compared to males with similar infection intensity. We focused on the immune profiling and reported a new characteristic of host immune response in clonorchiasis. Due to the problem of insufficient specimen, we have not analyzed sex hormone levels. To further explore the underlying mechanisms of immune suppression in clonorchiasis, the roles of sex hormones are worth to be investigated.

In conclusion, our study demonstrated *C. sinensis* infection is strongly related to the immune suppression in human being and it is influenced by sex. It provides a better understanding of how worms affect immune responses and cause a long-term immune alternation in humans with *C. sinensis* infection. This finding may benefit to the prevention of clonorchiasis and subsequent morbidity. Moreover, the influence of sexual dimorphism is worth to be further explored in clonorchiasis.

Supporting information

S1 Table. Characteristics of individuals with or without *C. sinensis* infection.

(XLSX)

S1 Fig. Serum levels of antibodies in Cs- and Cs+ individuals with different ages. Spearman's correlation analysis between EPG and serum levels of anti-CsAWA IgG and IgE (n = 37 in 10–29 years, n = 73 in 30–44 years, n = 97 in 45–59 years, and n = 82 in 60–86 years).

CsAWA: *C. sinensis* adult worm antigen, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., ** $P < 0.01$, *** $P < 0.001$, Spearman's correlation test.

(TIF)

S2 Fig. Serum levels of cytokines in Cs- and Cs+ individuals with different ages. Spearman's correlation analysis between EPG and serum levels of *IL-1 β* , *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12p70*, *IL-17A*, *IFN- γ* , *TNF- α* and *TGF- β 1* in different age groups (n = 37 in 10–29 years, n = 73 in 30–44 years, n = 97 in 45–59 years, and n = 82 in 60–86 years). CsAWA: *C. sinensis* adult worm antigen, EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m.,

* $P < 0.05$, ** $P < 0.01$, Spearman's correlation test.

(TIF)

S3 Fig. Serum levels of cytokines in females and males without *C. sinensis* infection. Serum levels of *IL-1 β* , *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12p70*, *IL-17A*, *IFN- γ* , *TNF- α* and *TGF- β 1* in Cs- individuals (n = 50 in female and n = 10 in male). Cs: *C. sinensis* infection, F: female, M: male. The data were shown as the mean \pm s.e.m., Mann-Whitney U test.

(TIF)

S4 Fig. Spearman's correlation analysis between EPG and serum levels of cytokines in female and male groups with different ages. Spearman's correlation analysis between EPG and serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 in 30–44 y (n = 38 in female and n = 35 in male), 45–59 y (n = 46 in female and n = 51 in male) and 60–86 y (n = 44 in female and n = 38 in male). EPG: eggs per gram of feces. The data were shown as the mean \pm s.e.m., * P < 0.05, ** P < 0.01, Spearman's correlation test. (TIF)

S5 Fig. Serum levels of cytokines in Cs+ individuals with or without hepatobiliary morbidities. (A) Serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IFN- γ , TNF- α and TGF- β 1 in Cs+ individuals with (n = 159) or without periductal fibrosis (n = 70). (B) Serum levels of cytokines in Cs+ individuals with (n = 39) or without fatty liver (n = 190). (C) Serum levels of cytokines in Cs+ individuals with (n = 55) or without bile duct dilatation (n = 174). PF: periductal fibrosis, FL: fatty liver, BDD: bile duct dilatation. The data were shown as the mean \pm s.e.m., * p < 0.05, Mann-Whitney U test. (TIF)

Acknowledgments

Thanks Dr. Deng-Yu Liu from Guangxi Medical University for providing *C. sinensis* adult worm.

Author Contributions

Conceptualization: Men-Bao Qian, Zhao-Jun Wang.

Data curation: Shuo Kan, Qi Li, Yan-Hua Yao.

Formal analysis: Qi Li.

Funding acquisition: Zhao-Jun Wang.

Investigation: Shuo Kan, Qi Li, Xin-Yue Du.

Methodology: Shuo Kan, Yan-Hua Yao, Chen-Yun Wu.

Project administration: Zhao-Jun Wang.

Resources: Hong-Mei Li, Xiao-Kui Guo, Men-Bao Qian, Zhao-Jun Wang.

Supervision: Guang-Jie Chen, Xiao-Kui Guo, Zhao-Jun Wang.

Writing – original draft: Shuo Kan, Qi Li, Men-Bao Qian.

Writing – review & editing: Qi Li, Xin-Yue Du, Guang-Jie Chen, Men-Bao Qian, Zhao-Jun Wang.

References

1. Gazzinelli-Guimaraes PH, Nutman TB. Helminth parasites and immune regulation. *F1000Res*. 2018; 7. Epub 2018/11/13. <https://doi.org/10.12688/f1000research.15596.1> PMID: 30416709.
2. Oyesola OO, Fruh SP, Webb LM, Tait Wojno ED. Cytokines and beyond: Regulation of innate immune responses during helminth infection. *Cytokine*. 2020; 133:154527. Epub 2018/09/23. <https://doi.org/10.1016/j.cyto.2018.08.021> PMID: 30241895.
3. Harris NL, Loke P. Recent Advances in Type-2-Cell-Mediated Immunity: Insights from Helminth Infection. *Immunity*. 2017; 47(6):1024–36. Epub 2017/12/21. <https://doi.org/10.1016/j.immuni.2017.11.015> PMID: 29262347.

4. Zhang BB, Yan C, Fang F, Du Y, Ma R, Li XY, et al. Increased hepatic Th2 and Treg subsets are associated with biliary fibrosis in different strains of mice caused by *Clonorchis sinensis*. *Plos One*. 2017; 12(2). ARTN e0171005 <https://doi.org/10.1371/journal.pone.0171005> PMID: 28151995
5. Douglas B, Oyesola O, Cooper MM, Posey A, Tait Wojno E, Giacomini PR, et al. Immune System Investigation Using Parasitic Helminths. *Annual review of immunology*. 2021; 39:639–65. Epub 2021/03/02. <https://doi.org/10.1146/annurev-immunol-093019-122827> PMID: 33646858.
6. Casacuberta-Partal M, Janse JJ, van Schuijlenburg R, de Vries JJC, Erkens MAA, Suijk K, et al. Antigen-based diagnosis of *Schistosoma* infection in travellers: a prospective study. *J Travel Med*. 2020; 27(4). Epub 2020/04/21. <https://doi.org/10.1093/jtm/taaa055> PMID: 32307517.
7. Qian M-B, Utzinger J, Keiser J, Zhou X-N. Clonorchiasis. *The Lancet*. 2016; 387(10020):800–10. [https://doi.org/10.1016/s0140-6736\(15\)60313-0](https://doi.org/10.1016/s0140-6736(15)60313-0)
8. Na BK, Pak JH, Hong SJ. *Clonorchis sinensis* and clonorchiasis. *Acta tropica*. 2020; 203:105309. Epub 2019/12/22. <https://doi.org/10.1016/j.actatropica.2019.105309> PMID: 31862466.
9. Sripa B, Suwantrai AT, Sayasone S, Do DT, Khieu V, Yang YC. Current status of human liver fluke infections in the Greater Mekong Subregion. *Acta Tropica*. 2021;224. ARTN 10613310. <https://doi.org/10.1016/j.actatropica.2021.106133> PMID: 34509453
10. Lai DH, Hong XK, Su BX, Liang C, Hide G, Zhang XL, et al. Current status of *Clonorchis sinensis* and clonorchiasis in China. *T Roy Soc Trop Med H*. 2016; 110(1):21–7. <https://doi.org/10.1093/trstmh/trv100> PMID: 26740359
11. Qian MB, Zhou XN. *Clonorchis sinensis*. *Trends Parasitol*. 2021; 37(11):1014–5. Epub 2021/07/08. <https://doi.org/10.1016/j.pt.2021.05.011> PMID: 34229953.
12. Qian MB, Li HM, Jiang ZH, Yang YC, Lu MF, Wei K, et al. Severe hepatobiliary morbidity is associated with *Clonorchis sinensis* infection: The evidence from a cross-sectional community study. *PLoS neglected tropical diseases*. 2021; 15(1):e0009116. Epub 2021/01/29. <https://doi.org/10.1371/journal.pntd.0009116> PMID: 33507969.
13. Yan C, Zhou QY, Wu J, Xu N, Du Y, Li J, et al. Csi-let-7a-5p delivered by extracellular vesicles from a liver fluke activates M1-like macrophages and exacerbates biliary injuries. *P Natl Acad Sci USA*. 2021; 118(46). ARTN e2102206118 <https://doi.org/10.1073/pnas.2102206118> PMID: 34772807
14. Jeong JH, Yi J, Hwang MK, Hong SJ, Sohn WM, Kim TS, et al. The Overactivation of NADPH Oxidase during *Clonorchis sinensis* Infection and the Exposure to N-Nitroso Compounds Promote Periductal Fibrosis. *Antioxidants-Basel*. 2021; 10(6). ARTN 869 <https://doi.org/10.3390/antiox10060869> PMID: 34071467
15. Wu YJ, He Q, Shang M, Yin YX, Li Y, Du X, et al. The NF-kappaB signalling pathway and TM7SF3 contribute to liver fibrosis caused by secreted phospholipase A2 of *Clonorchis sinensis*. *Parasit Vectors*. 2021; 14(1):152. Epub 2021/03/12. <https://doi.org/10.1186/s13071-021-04663-z> PMID: 33691755.
16. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. A review of human carcinogens—Part B: biological agents. *Lancet Oncol*. 2009; 10(04):321–2. Epub 2009/04/08. [https://doi.org/10.1016/s1470-2045\(09\)70096-8](https://doi.org/10.1016/s1470-2045(09)70096-8) PMID: 19350698.
17. Qian MB, Zhuang SF, Zhu SQ, Deng XM, Li ZX, Zhou XN. Improving diagnostic performance of the Kato-Katz method for *Clonorchis sinensis* infection through multiple samples. *Parasites & vectors*. 2019; 12(1):336. Epub 2019/07/10. <https://doi.org/10.1186/s13071-019-3594-5> PMID: 31287026.
18. Ingersoll MA. Sex differences shape the response to infectious diseases. *PLoS pathogens*. 2017; 13(12):e1006688. Epub 2017/12/29. <https://doi.org/10.1371/journal.ppat.1006688> PMID: 29284060.
19. Babu S, Nutman TB. Helminth-Tuberculosis Co-infection: An Immunologic Perspective. *Trends Immunol*. 2016; 37(9):597–607. <https://doi.org/10.1016/j.it.2016.07.005> PMID: 27501916
20. Koda S, Zhu XQ, Zheng KY, Yan C. Molecular Mechanisms of *Clonorchis sinensis*-Host Interactions and Implications for Vaccine Development. *Front Cell Dev Biol*. 2021; 9:781768. Epub 2022/02/05. <https://doi.org/10.3389/fcell.2021.781768> PMID: 35118069.
21. Zhao L, Shi MC, Zhou LN, Sun HC, Zhang XN, He L, et al. *Clonorchis sinensis* adult-derived proteins elicit Th2 immune responses by regulating dendritic cells via mannose receptor. *Plos Neglect Trop D*. 2018; 12(3). ARTN e0006251 <https://doi.org/10.1371/journal.pntd.0006251> PMID: 29505573
22. Choi YK, Yoon BI, Won YS, Lee CH, Hyun BH, Kim HC, et al. Cytokine responses in mice infected with *Clonorchis sinensis*. *Parasitology research*. 2003; 91(2):87–93. Epub 2003/08/05. <https://doi.org/10.1007/s00436-003-0934-2> PMID: 12898229.
23. Sripa B, Mairiang E, Thinkhamrop B, Laha T, Kaewkes S, Sithithaworn P, et al. Advanced periductal fibrosis from infection with the carcinogenic human liver fluke *Opisthorchis viverrini* correlates with elevated levels of interleukin-6. *Hepatology*. 2009; 50(4):1273–81. Epub 2009/08/14. <https://doi.org/10.1002/hep.23134> PMID: 19676135.

24. Wang X, Liang C, Chen W, Fan Y, Hu X, Xu J, et al. Experimental model in rats for study on transmission dynamics and evaluation of *Clonorchis sinensis* infection immunologically, morphologically, and pathologically. *Parasitology research*. 2009; 106(1):15–21. Epub 2009/09/17. <https://doi.org/10.1007/s00436-009-1622-7> PMID: 19756744.
25. Lee YJ, Kim MJ, Jo S, Jin SH, Park PR, Park K, et al. *Clonorchis sinensis*-Derived Protein Attenuates Inflammation and New Bone Formation in Ankylosing Spondylitis. *Front Immunol*. 2021; 12:615369. Epub 2021/03/16. <https://doi.org/10.3389/fimmu.2021.615369> PMID: 33717104.
26. Florio AA, Ferlay J, Znaor A, Ruggieri D, Alvarez CS, Laversanne M, et al. Global trends in intrahepatic and extrahepatic cholangiocarcinoma incidence from 1993 to 2012. *Cancer*. 2020; 126(11):2666–78. Epub 2020/03/05. <https://doi.org/10.1002/cncr.32803> PMID: 32129902.
27. Chang JI, Lee K, Kim D, Yang JI, Park JK, Choi K, et al. Clinical Characteristics of *Clonorchis sinensis*-Associated Cholangiocarcinoma: A Large-Scale, Single-Center Study. *Front Med (Lausanne)*. 2021; 8:675207. Epub 2021/06/15. <https://doi.org/10.3389/fmed.2021.675207> PMID: 34124104.
28. Wang CQ, He Q, Yin YX, Wu YJ, Li XR. *Clonorchis sinensis* Granulin Promotes Malignant Transformation of Hepatocyte Through EGFR-Mediated RAS/MAPK/ERK and PI3K/Akt Signaling Pathways. *Front Cell Infect Mi*. 2021; 11. ARTN 734750 <https://doi.org/10.3389/fcimb.2021.734750> PMID: 34858869
29. Ma XX, Qiu YY, Chang ZG, Gao JF, Jiang RR, Li CL, et al. Identification of Myoferlin, a Potential Serodiagnostic Antigen of Clonorchiasis, via Immunoproteomic Analysis of Sera From Different Infection Periods and Excretory-Secretory Products of *Clonorchis sinensis*. *Front Cell Infect Mi*. 2021; 11. ARTN 779259 <https://doi.org/10.3389/fcimb.2021.779259> PMID: 34733798
30. Shi Y, Yu K, Liang A, Huang Y, Ou F, Wei H, et al. Identification and Analysis of the Tegument Protein and Excretory-Secretory Products of the Carcinogenic Liver Fluke *Clonorchis sinensis*. *Front Microbiol*. 2020; 11:555730. Epub 2020/10/20. <https://doi.org/10.3389/fmicb.2020.555730> PMID: 33072014.
31. Won J, Cho Y, Lee D, Jeon BY, Ju JW, Chung S, et al. *Clonorchis sinensis* excretory-secretory products increase malignant characteristics of cholangiocarcinoma cells in three-dimensional co-culture with biliary ductal plates. *PLoS Pathog*. 2019; 15(5):e1007818. Epub 2019/05/24. <https://doi.org/10.1371/journal.ppat.1007818> PMID: 31121000.
32. Bernin H, Lotter H. Sex bias in the outcome of human tropical infectious diseases: influence of steroid hormones. *J Infect Dis*. 2014; 209 Suppl 3:S107–13. Epub 2014/06/27. <https://doi.org/10.1093/infdis/jit610> PMID: 24966190.
33. Liu XH, Wu MQ, Liu Y, Li J, Yang DQ, Jiang LP. Foodborne Parasites Dominate Current Parasitic Infections in Hunan Province, China. *Front Cell Infect Mi*. 2021; 11. ARTN 774980 <https://doi.org/10.3389/fcimb.2021.774980> PMID: 34722349
34. Li ZJ, Xin HL, Qian MB, Sun JL, Yang YC, Chen YD, et al. *Clonorchis sinensis* Reinfection Rate and Reinfection Determinants: A Prospective Cohort Study in Hengxian County, Guangxi, China. *J Infect Dis*. 2022; 225(3):481–91. <https://doi.org/10.1093/infdis/jiab403> PMID: 34375427
35. Lee SE, Shin HE, Lee MR, Kim YH, Cho SH, Ju JW. Risk Factors of *Clonorchis sinensis* Human Infections in Endemic Areas, Haman-Gun, Republic of Korea: A Case-Control Study. *The Korean journal of parasitology*. 2020; 58(6):647–52. Epub 2021/01/09. <https://doi.org/10.3347/kjp.2020.58.6.647> PMID: 33412768.
36. Nguyen TTB, Dermauw V, Dahma H, Bui DT, Le TTH, Phi NTT, et al. Prevalence and risk factors associated with *Clonorchis sinensis* infections in rural communities in northern Vietnam. *PLoS neglected tropical diseases*. 2020; 14(8):e0008483. Epub 2020/08/04. <https://doi.org/10.1371/journal.pntd.0008483> PMID: 32745095.
37. Gao Y, Li Y, Liu X, Zhang T, Yu G, Wang Y, et al. High prevalence of *Clonorchis sinensis* infections and coinfection with hepatitis virus in riverside villages in northeast China. *Sci Rep*. 2020; 10(1):11749. Epub 2020/07/18. <https://doi.org/10.1038/s41598-020-68684-x> PMID: 32678224.
38. Deng ZH, Fang YY, Zhang QM, Mao Q, Pei FQ, Liu MR. The control of clonorchiasis in Guangdong province, southern China. *Acta Trop*. 2020; 202:105246. Epub 2019/11/02. <https://doi.org/10.1016/j.actatropica.2019.105246> PMID: 31672488.
39. Klein SL, Flanagan KL. Sex differences in immune responses. *Nature reviews Immunology*. 2016; 16(10):626–38. Epub 2016/08/23. <https://doi.org/10.1038/nri.2016.90> PMID: 27546235.
40. vom Steeg LG, Klein SL. Sex Matters in Infectious Disease Pathogenesis. *PLoS pathogens*. 2016; 12(2):e1005374. Epub 2016/02/20. <https://doi.org/10.1371/journal.ppat.1005374> PMID: 26891052.
41. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. *Nature reviews Immunology*. 2010; 10(8):594–604. Epub 2010/07/24. <https://doi.org/10.1038/nri2815> PMID: 20651746.
42. Spolarics Z. The X-files of inflammation: cellular mosaicism of X-linked polymorphic genes and the female advantage in the host response to injury and infection. *Shock*. 2007; 27(6):597–604. Epub 2007/05/17. <https://doi.org/10.1097/SHK.0b013e31802e40bd> PMID: 17505297.

43. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod.* 2008; 78(3):432–7. Epub 2007/11/16. <https://doi.org/10.1095/biolreprod.107.063545> PMID: 18003947.
44. Aomatsu M, Kato T, Kasahara E, Kitagawa S. Gender difference in tumor necrosis factor-alpha production in human neutrophils stimulated by lipopolysaccharide and interferon-gamma. *Biochemical and biophysical research communications.* 2013; 441(1):220–5. Epub 2013/10/22. <https://doi.org/10.1016/j.bbrc.2013.10.042> PMID: 24140406.