

STAT6 Expression and IL-13 Production in Association with Goblet Cell Hyperplasia and Worm Expulsion of *Gymnophalloides seoi* from C57BL/6 Mice

Jin-Joo Lee^{1,†}, Donghee Kim^{2,†}, Kyoung-Ho Pyo¹, Min-Ki Kim¹, Hyo-Jin Kim¹, Jong-Yil Chai¹ and Eun-Hee Shin^{1,3,*}

¹Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, and Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul 110-799, Korea; ²Seoul National University Gang-Nam Hospital, Seoul 135-984, Korea; ³Seoul National University Bundang Hospital, Seongnam 463-707, Korea

Abstract: In intestinal helminth infections, Th2 immune responses are generally associated with mucin secretion for worm expulsion from the host intestine. In particular, IL-4 and IL-13 are the important cytokines related with intestinal mucus production via STAT6 signalling in nematode infections. However, this perspective has never been studied in *Gymnophalloides seoi* infection. The present study aimed to observe the STAT6 signalling and cytokine responses in C57BL/6 mice, a mouse strain resistant to infection with this trematode. The results showed that worm expulsion occurred actively during days 1-2 post-infection (PI), when goblet cells began to proliferate in the small intestine. The STAT6 gene expression in the mouse spleen became remarkable from day 2 PI. Moreover, *G. seoi* infection induced a significant increase of IL-13 from day 4 PI in the spleen of infected mice. Our results suggested that goblet cell hyperplasia and worm expulsion in *G. seoi*-infected mice should be induced by STAT6 signalling, in which IL-13 may be involved as a dominant triggering cytokine.

Key words: *Gymnophalloides seoi*, intestinal trematode, goblet cell hyperplasia, worm expulsion, IL-13, STAT6

In intestinal helminth infections, increased expressions of IL-4 and IL-13 can directly affect cell populations that express IL-4R, and IL-4R signalling is largely dependent on the actions of the signal transducer and activator of transcription 6 (STAT6) [1]. In mouse models, intestinal nematodes induce a strong Th2-biased response, represented by an increase of IL-4 and IL-13 expressions and causing hyperplasia of mucin-secreting goblet cells [1-3]. IL-4R α -deficient mice showed a delayed ability to resolve *Nippostrongylus brasiliensis* infection, with significantly prolonged fecal egg recovery and delayed worm expulsion [4]. The delayed worm expulsion was related to delayed intestinal goblet cell hyperplasia [4]. This result emphasized that the IL-13/IL-4R α /STAT6 signalling pathway was essential in control of intestinal nematode infections.

During the infection period, STAT6 is also critical in the de-

velopment of intestinal muscle hypercontractility, and both IL-4 and IL-13 influence the second message system of muscle cells by acting on the calcium channel or on the muscuranic receptor, either of which subsequently alters intestinal muscle contractility [1]. As increased contractility is associated with parasite expulsion [3], Th2-type immune response is an important mechanism behind worm expulsion from the intestine. A previous study in *T. spiralis*-infected mice has shown that both IL-4 and IL-13 induce hypercontractility of muscle cells via the STAT6 pathway, and this is a cellular mechanism for hypercontractility of the intestine [5].

The mechanisms of worm expulsion in intestinal trematode infections have been extensively studied in *Metagonimus yokogawai*, *Echinostoma hortense*, *Neodiplostomum seoulense*, and *Gymnophalloides seoi* [6]. In these models, several immune effector cells, including intestinal intraepithelial lymphocytes, mast cells, and goblet cells, were found in greater numbers along the villous epithelial layer of the small intestine in the respective definitive hosts [6]. Intestinal mastocytosis, goblet cell hyperplasia, eosinophilia, increase of mucosal IgA, and infiltration of macrophages into the small intestine have all been

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*Corresponding author (ehshin@snu.ac.kr)

†These authors contributed equally to this work.

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known to play important roles in the expulsion of intestinal trematodes [6,7]. However, trematode infections have generally not been the subject of study as much as nematode infections.

G. seoi is an intestinal trematode reported in patients with acute pancreatitis or diabetes mellitus [8], and was subsequently found to be transmitted by consumption of raw oysters, *Crassostrea gigas* [9]. In animal studies, mice experimentally infected with *G. seoi* exhibited villous atrophy, crypt hyperplasia, and goblet cell hyperplasia with inflammatory reactions in the villous stroma and crypt [6,10]. It was shown that CD4⁺ T-cells play an important role as a mediator of goblet cell hyperplasia but did not cause functional activation of goblet cells [10]. Because goblet cell hyperplasia is induced in the early phase of infection, there is little information regarding cellular events, such as STAT6 signalling. Thus, *G. seoi* provides a decent trematode model for a study.

In this study, we observed STAT6 expression in the spleen of C57BL/6 mice (a resistant mouse strain to infection with *G. seoi*), together with worm recoveries and goblet cell responses during days 0-7 post-infection (PI) with *G. seoi*. In addition, we also observed IL-4 and IL-13 levels in the spleen cell cultures during goblet cell hyperplasia and STAT6 expression in these mice.

Specific pathogen-free (SPF) female C57BL/6 mice were purchased from Koatech Laboratory Animal Center (Pyeongtaek-shi, Gyeonggi-do, Korea). After an acclimation period of 1 week, mice were housed in an animal facility equipped with SPF conditions at the Seoul National University College of Medicine [11]. Seven-week-old C57BL/6 mice were infected with *G. seoi* metacercariae. Animal experiments were carried out in accordance with the guidelines of Institutional Animal Care and User Committee, Seoul National University College of Medicine, Seoul, Korea.

For preparation of *G. seoi* metacercariae, oysters (*Crassostrea gigas*) were collected from Aphaedo, Shinan-gun, a known endemic area [9,10]. Each oyster shell was detached, and the oyster body was treated with artificial digestive solution containing 0.6% pepsin (1:10,000; Sigma-Aldrich, St. Louis, Missouri, USA) and 0.8% HCl at 37°C for 10 min. The resulting product containing free metacercariae was washed several times with normal physiological saline. The solution was processed through Baermann's apparatus, and the presence of metacercariae was confirmed under a stereomicroscope. Each mouse was orally infected with 200 metacercariae in 0.2 ml of saline using a gavage needle (Cadence Science, Lake Success, New York, USA).

Mice were euthanized under ether anesthesia to recover the worms and to collect the spleen for immunoassays. After incubation for 1 hr in Baermann's apparatus, the intestinal segments were returned to Petri dishes and examined under a stereomicroscope for residual flukes. The flukes were collected from the bottom of the tube equipped in the apparatus.

For the observation of goblet cells, 2-cm intestinal segments from the middle portion of the jejunum were washed with normal saline (0.9% sodium chloride) and fixed with 10% formalin. The tissues were embedded in paraffin and sectioned to a 5 µm thickness. The slides were deparaffinized serially with xylene and alcohol before staining, then oxidized by 1% periodic acid (Sigma Aldrich, Poole, UK) for 5 min, and reacted with Schiff's reagent to produce a colored end product. They were counterstained with hematoxylin (Sigma). Goblet cell (GC) numbers were counted per 10 villus-crypts units.

The mouse spleen was isolated aseptically, and single cell suspensions of splenocytes was prepared in cold Hank's balanced salt solution (HBSS) (Sigma) containing 2% FBS (Gibco BRL, Rockville, Maryland, USA). After splenocytes were washed with 2% FBS-HBSS and centrifuged at 1,500 rpm for 10 min, RBC in the cell pellet was lysed with the lysis buffer (NH₄Cl 150 mM, KHCO₃ 1 mM, EDTA 0.01 mM) for 1 min. After further washing, the supernatant was decanted and splenocytes were resuspended in complete RPMI media containing 1% HEPES buffer, 10% FBS, 50 mM 2-ME, 2 mM L-glutamate, 1% glucose, 2% sodium bicarbonate, 1% sodium pyruvate, and 1% antibiotics. The resulting collection of splenocytes were divided into and incubated in 96-well plates (5 × 10⁵ cells per well) (Nunc, Roskilde, Denmark) under 37°C and 5% CO₂ atmosphere. Culture supernatant was collected after 72 hr of incubation and stored at -80°C.

Culture supernatants of splenocytes were assayed for IL-4 and IL-13 using ELISA kits (BD Pharmingen, San Diego, California, USA). *G. seoi* antigen for in vitro stimulation of splenocytes was prepared from metacercariae (GMAg) and adult worms (GAAg). The worms were homogenized using a glass-teflon homogenizer and were spun down at 13,200 rpm at 4°C for 20 min. Each resulting supernatant was filtered through a 0.45 µm disposable syringe filter (MFS Inc., Pleasanton, California, USA) and stored at -80°C until required. Protein concentrations were determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). GMAg and GAAg were added 100 µg/ml per well for cell stimulation. To examine STAT6 expression, the splenocytes were

lysed using radioimmunoprecipitation assay (RIPA) buffer ($1 \times$ PBS, 1% NP-40, 0.05% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with 10% protease and phosphatase inhibitor cocktail (Pierce, Rockford, Illinois, USA), and kept for 30 min on ice. The lysate was obtained by centrifugation at 13,200 rpm for 30 min at 4°C. The protein concentration was assessed by BCA protein assay kit (Pierce) using bovine plasma globulin as the standard and was read at 562 nm of spectrophotometer. The determined protein samples were boiled with sample buffer for 3 min at 99°C. Equal amounts of protein were loaded on each lane of 10% SDS-PAGE gel, and were separated in the running buffer at 100 V. After the gel was transferred onto a PVDF membrane (BioRad, Richmond, California, USA), the PVDF membrane was blocked with $1 \times$ TBS-T containing 5% skim milk for 2 hr at room temperature. Subsequently, the membrane was incubated with anti-STAT6 (Santa Cruz, California, USA) and anti- β -actin antibodies (Santa Cruz) for overnight at 4°C. The membrane was washed 3 times for 10 min with TBS-T ($1 \times$ TBS, 0.01% Tween-20), and then incubated with HRP-conjugated secondary antibody

(1:2,000) in blocking buffer with 5% skim milk for 1 hr at room temperature. After washing, the protein band on the membrane was visualized by LAS-4000 system (Fujifilm, Tokyo, Japan). Data are presented as mean \pm SEM. Statistical significance of data was analyzed using the Student's t-test. A P -value of <0.05 was considered statistically significant.

C57BL/6 mice were infected with 200 metacercariae of *G. seoi*, and the worm burden in the small intestine was examined everyday for 6 days PI (Fig. 1). *G. seoi* worms were rapidly expelled from the small intestine, and were found with rare frequency on day 6 PI. The worm recovery was 51.8%, 5.7%, and 4.0% on days 1, 3, and 5 PI, respectively (Fig. 1A). The proliferation of goblet cells in *G. seoi*-infected mice was remarkable (Fig. 2), when compared to uninfected mice. The goblet cell count was highest on day 2 PI. The counts per 10 villus-crypt units (VCU) were 5.1 ± 2.7 (day 0 PI), 10.1 ± 3.2 (day 1 PI), 19.2 ± 3.7 (day 2 PI), 13.3 ± 3.7 (day 3 PI), 13.1 ± 5.8 (day 4 PI), 9.0 ± 2.2 (day 5 PI), and 8.7 ± 3.3 (day 6 PI) (Fig. 1B). Goblet cell hyperplasia continued during those 6 days, until the worms were expelled.

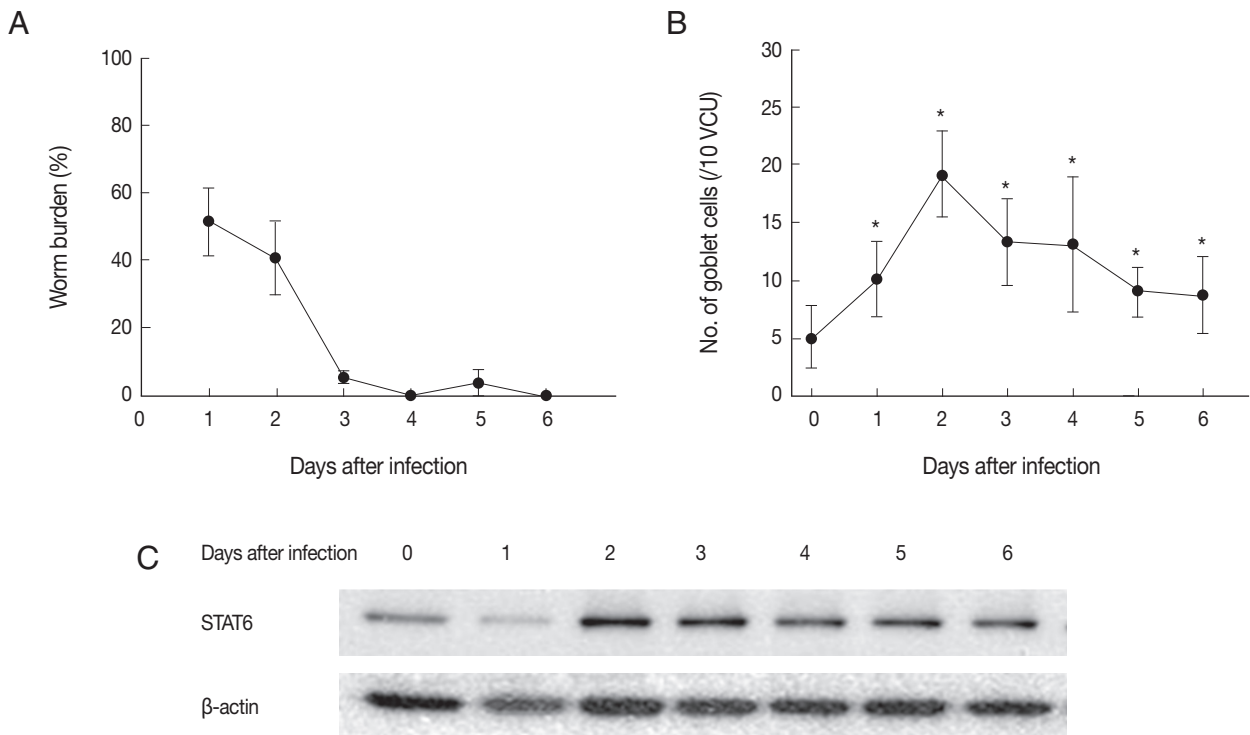


Fig. 1. Worm recovery, goblet cell responses, and STAT6 expression in C57BL/6 mice infected with 200 metacercariae of *G. seoi* and sacrificed every day after infection. (A) Chronological worm recovery (%) in mice infected with *G. seoi*. Each value represents mean \pm SD from 3 mice. (B) Goblet cell numbers per 10 villus-crypt unit (VCU) in the small intestine of mice infected with *G. seoi*. * $P < 0.05$. (C) STAT6 expression in the spleen of mice infected with *G. seoi*. Splenocytes were harvested from mice during experimental period and lysed using RIPA buffer. STAT6 concentration in the cell lysate was determined by Western blot analysis. β -actin was used as the house-keeping protein.

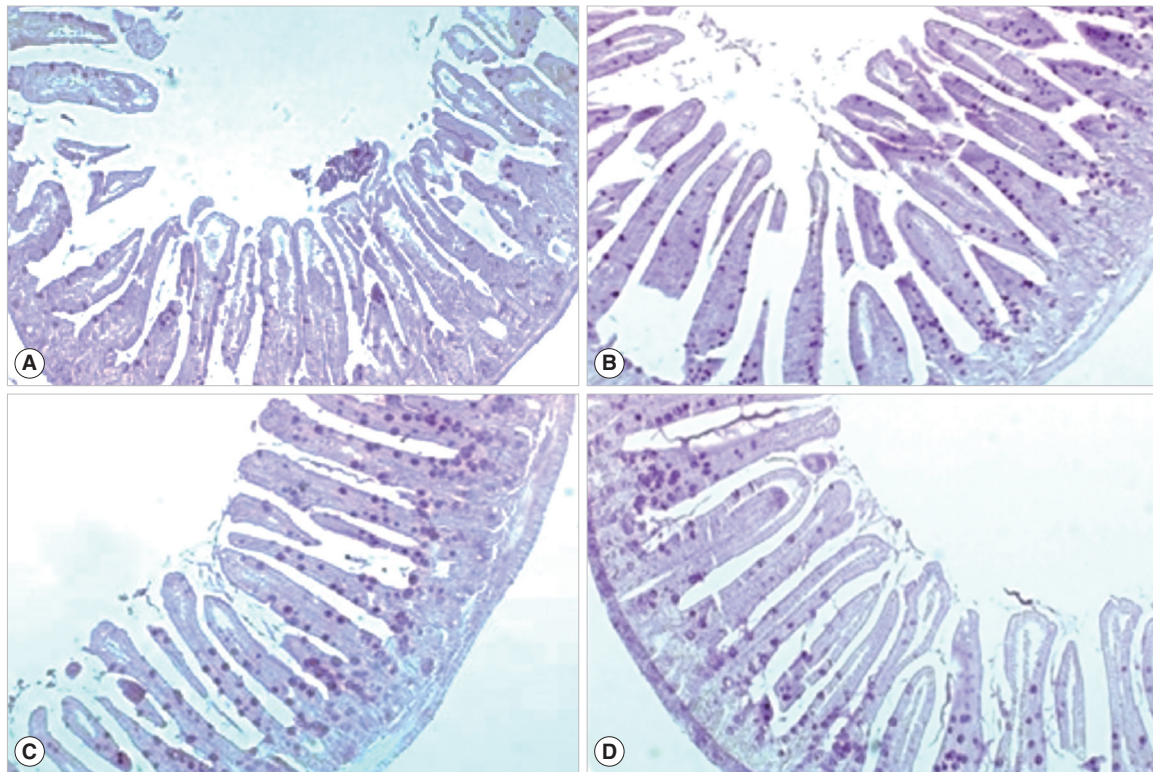


Fig. 2. Intestinal sections showing goblet cell hyperplasia in the jejunum of a normal (A) and *G. seoi*-infected C57BL/6 mice (B, day 2 PI; C, day 3 PI; D, day 4 PI). Periodic acid Schiff (PAS) stain. $\times 100$.

From day 2 PI, STAT6 was highly expressed (Fig. 1C). To determine cytokines associated with STAT6 expression, the in vitro-cultured splenocytes were assayed for IL-4 and IL-13. When splenocytes were cultured with GMaG, these cells produced significantly high levels of IL-13 from day 4 PI ($P < 0.05$), suggesting that IL-13 might be triggering IL-4R α in *G. seoi* infection (Fig. 3A). IL-4 was not significantly increased during *G. seoi* infection (Fig. 3B).

In our previous study which investigated the role of CD4⁺ T-helper cells in C57BL/6 (resistant) and ICR (susceptible) mice, C57BL/6 showed high numbers of mucosal goblet cells and increased mRNA expressions of IL-4 and IL-5 in mesenteric lymph node (MLN) cells during the early stage of infection [10]. In addition, when CD4⁺ T-cells from *G. seoi*-infected mice were adoptively transferred to other infected mice, these mice showed increased worm expulsion and goblet cell hyperplasia [10]. However, alteration of the terminal sugar of mucins, which is indicative of functional activation of goblet cells, was independent from CD4⁺ T-cells in *G. seoi* infection [10]. This implied that goblet cell hyperplasia should be regulated by CD4⁺ T cells but alteration of the terminal sugar chain of mucins

may be probably induced by a non-CD4⁺ T-cell-dependent process [10].

In general, Th2 immune responses in intestinal helminth infection can be represented by goblet cell hyperplasia, increased mucin secretion, mast cell hyperplasia, and increased intestinal muscle contractility [2,5,12-16]. These immune responses have been well studied in nematode infections [1-5,13-16]. IL-4 and IL-13 play an important role in generation of intestinal muscle contractility in mice infected with *Heligmosomoides polygyrus* and *N. brasiliensis*, partly dependent on STAT6 expression [17]. Also in *T. spiralis* infection, the enteric muscle function was important in worm expulsion, and STAT6 activated by IL-4 and IL-13 was critical in the development of intestinal muscle hypercontractility [18]. Thus, IL-4 and IL-13 have multiple effects on induction of mastocytosis, mucin secretion, and smooth muscle contractility [19]. Since IL-4 and IL-13 share the receptor IL-4R α for mediating STAT6 signalling pathway, a dominant expression of either IL-4 or IL-13 can contribute to the host defense [16,17].

However, the role of IL-4 and IL-13 is quite different from each other as reported in intestinal nematode and cestode in-

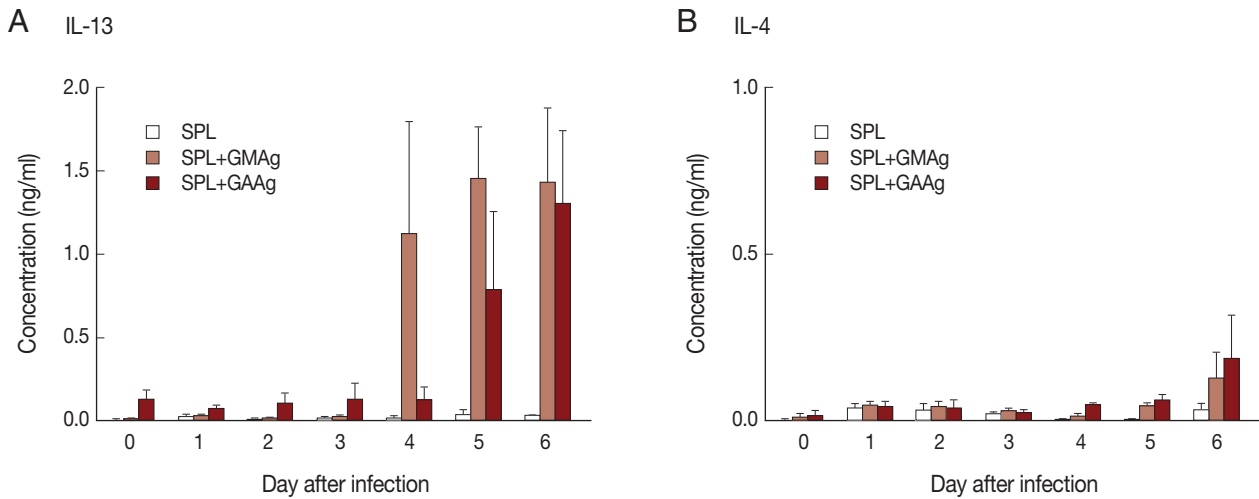


Fig. 3. IL-13 and IL-4 expressions in the spleen of mice infected with *G. seoi*. (A) IL-13 concentrations in the spleen after infection, as determined from the supernatant of cultured cells stimulated with *G. seoi* antigen, using an IL-13 ELISA kit. (B) IL-4 concentrations in the spleen after infection, as determined from the supernatant of cultured cells stimulated with *G. seoi* antigen, using an IL-4 ELISA kit. SPL, spleen cells; GMAg, *G. seoi* metacercaria antigen; GAAg, *G. seoi* adult antigen.

fections [14-17,19]. It has been shown that mast cell hyperplasia is strongly influenced by IL-4, whereas goblet cell hyperplasia is apparently much more dependent on IL-13 [15]. However, the roles of IL-4 and IL-13 are not well-known in trematode infections. In particular, very little is known about the STAT6 signalling mechanism during an intestinal trematode infection.

In a trematode infection, it was reported that *G. seoi* induced intestinal inflammation characterized by marked mucin hypersecretion and goblet cell hyperplasia in rodent models, which emphasized the role of goblet cell hyperplasia on parasite expulsion [6,10]. In our study, the number of goblet cells was the highest on day 2 PI, and subsequently, the worm expulsion occurred maximum by day 3 PI. Meanwhile, the expression of STAT6 was increased from day 2 PI in the spleen. This suggests that *G. seoi* infection may be controlled by STAT6-based Th2 immune responses, as was previously reported in intestinal nematode infections [1-6,16-18].

Our results showed that, in the presence of *G. seoi* infection, the cytokine which was directly associated with STAT6-related immune response was IL-13 but not IL-4. This suggests that IL-13 may be associated with goblet cell hyperplasia and worm expulsion. However, IL-13 production was significantly increased on days 4-6 PI rather than in an earlier course of infection. In comparison, IL-4 production in splenocytes stimulated with GMAg or GAAg never increased during the infection period. In our previous study, C57BL/6 mice infected with *G. seoi* induced

cytokines related with Th2- as well as Th1-type immune responses in MLN [10]. Both Th1 cytokines (IL-1 α , IL-1 β , IL-2, IFN- γ , and TNF- α) and Th2 cytokines (IL-4, IL-5, and IL-13) increased on day 2 PI [10]. Therefore, it is suggested that IL-4 may be induced in the early stage of infection within day 3 PI in the regional MLN and but not in the spleen. In the case of IL-13, it may become induced in the early stage in MLN and reach the peak in later stages in the spleen.

IL-4 and IL-13 have been found to mediate specific functions during Th2 immune responses, with IL-4 facilitating humoral IgG1 and IgE production and IL-13 contributing to epithelial hyperplasia and eosinophil recruitment in peripheral tissues [12,20]. In *G. seoi* infection in C57BL/6 mice, IL-13 production, together with STAT6 expression, may contribute to goblet cell hyperplasia and worm expulsion.

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Conflict of interest

We have no conflict of interest related to this work.

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