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Therapeutic effect of soluble worm protein acting as immune regulatory on colitis

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ABSTRACT

Objective: To investigate the anti-inflammatory effect of the protein derived from the soluble factor of *Heligmosomoides polygyrus* (*H. polygyrus*) excretory-secretory in a colitis model.

Methods: Colitis was induced by providing drinking water containing 3% dextran sodium sulfate (DSS) for a week. DSS was administrated in a cycle protocol, each cycle consisted of 7 days of 3% DSS in the drinking water and followed by 7 days of regular water. This study consisted of five treatment groups, including Groups A (control) received untreated water, B (DSS only, without excretory-secretory), and C–E injected (*i.p.*) with excretory-secretory protein (*H. polygyrus* excretory-secretory total, excretory-secretory 28 kDa and excretory-secretory 55 kDa, respectively). Mice received injection every week. The injection of excretory-secretory was started from the 6th weeks and continued until 11 weeks. At the end of 11 weeks of the experiment, mice were sacrificed, colon tissue was removed and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, flow cytometry, real-time PCR and histology examination.

Results: Mice received *H. polygyrus* excretory-secretory 55 kDa reduced mono-nuclear cell infiltrations. *H. polygyrus* excretory-secretory 55 kDa induced the down-regulation of mRNA interferon-γ expression. There were significant differences in the expression of mRNA interferon in the colon of mice after the administration of the excretory-secretory 55 kDa protein fraction compared with other groups ($P < 0.001$), whereas mRNA transforming growth factor-β expression up regulated in the colon of mice after the administration of the excretory-secretory 55 kDa protein fraction compared with total excretory-secretory group ($P < 0.05$). The treatment of colitis in mice with excretory-secretory 55 kDa protein fractions modulated interleukin-10 (IL-10) expression, whereas excretory-secretory total and excretory-secretory 28 kDa protein fractions insufficient promoted IL-10 expression. Excretory-secretory 55 kDa proteins fraction promoted IL-10 expression via Foxp3-independent pathways.

Conclusions: Excretory-secretory 55 kDa protein could reduce inflammation and have potential therapy. *H. polygyrus* excretory-secretory 55 kDa was the soluble factor that may help in the development of novel treatments to cure colitis.

1. Introduction

Ulcerative colitis is a form of inflammatory bowel disease, or chronic inflammation of the colon. Colitis is characterized by spontaneous recurrences and the social conditions of the patient. Ulcerative colitis patients have a high risk of complications, including colon carcinoma and rectal carcinoma [1].

The prevalence and incidence of ulcerative colitis have increased in developing countries in recent years [1,2]. The highest prevalence of worm infections in developing countries

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is likely the cause of the low incidence of certain diseases, such as inflammatory bowel disease.

The prevalence and incidence of colitis in Asia have been increasing. An experimental model of ulcerative colitis in rodent was induced by administration of drinking water containing dextran sodium sulfate (DSS) [3]. Colitis is induced by DSS causing rodents to show many symptoms of human ulcerative colitis, such as bloody feces, diarrhea, loss of body weight, shortening of the large intestine and mucosal ulceration as well as lesions of human ulcerative colitis [3,4].

The number of $GR1^+$ and $CD11b^+$ cells of DSS-induced mice increased compared to controls [5]. In patients with colitis, macrophages are activated and show increased production of cytokines, such as interferon-γ (IFN-γ), interleukin-12 (IL-12) and IL-23 [6,7]. Th1 cells activate $CD4^+$ T cells, resulting in them secreting IL-2 and IFN-γ. The secretion of IFN-γ plays a role in macrophage activation. Activated macrophages secrete IFN-γ, which is a pro-inflammatory cytokine [8].

Worm therapy has recently been developed. This therapy is widely used in China, Russia and Japan. *Schistosoma mansoni* infection has a protective response to trinitro-benzene-sulfonic acid-induced colitis in mice [9,10]. *Hymenolepis diminuta* infection is able to cure colitis in mice [11], whereas *Trichuris suis* is a therapy for Crohn disease [12] and *Trichinella spiralis* protects against colitis induced by dinitrobenzene sulfonic acid [13]. The mechanisms of human disease modification by these various worm species can be studied using a mouse model of *Heligmosomoides polygyrus* (*H. polygyrus*) infection [14,15].

Many studies have shown that living nematodes have therapeutic uses. The use of worms has become an alternative therapy, but this method has disadvantages. Patients must be infected by the parasite into the body, thus, a large number of living nematodes can implement these therapies. It has been studied about the inflammatory response caused by a parasite and the consequences resulting from the nematode that lives in the body during therapy.

The inflammatory responses to a parasite and the consequences resulting from the presence of living nematodes in the body during therapy have been studied. During worm infection, macrophages are activated to produce Th2 cells, which inhibit Th1 response. However, allowing nematodes to infect and remain alive inside the human body is still difficult for patients to accept [9,16]. Many studies have reported that treatment with living worm infection or worm extracts could reduce inflammation associated with autoimmune diseases, such as rheumatoid arthritis. However, living parasites have the disadvantage of potential side effects due to invasion of the parasite to other tissues in the human host [17–20].

Based on the above reason, the worm infection causes pathological disorders. Therefore, the therapy using living parasites is necessary to be replaced with a soluble protein produced by parasites [12,21–23]. Treatment with excretory-secretory proteins could overcome the disadvantages of treatment using living parasites. We examined potential mechanisms that contribute to DSS infection by nematode *H. polygyrus*. Therefore, therapies using excretory-secretory proteins are more favorable than therapies that allow living nematodes to be maintained in the host. We also examined the effect of *H. polygyrus* excretory-secretory on mice with DSS-induced colitis.

2. Materials and methods

2.1. Animals

Female BALB/c mice, aged 8–10 weeks and weighing 20–25 g were maintained in a relative humidity of 50%–55% with a preset light–dark cycle (12:12 h). Mice were given normal drinking water *ad libitum* during the experimental periods. The mice were housed under specific pathogen-free conditions. All experimental procedures involving animals were conducted in accordance to institutional guidelines and the current regulations and approved by the Ethical Committee Brawijaya University, Malang, Indonesia (No. 288/EC/KEPK-JK/11/2012).

2.2. Worm establishment

BALB/c mice were infected with 300 third-stage larvae (L3) *H. polygyrus*. L3 *H. polygyrus* were provided by Jikei University of Tokyo and maintained in Laboratory of Parasitology, Faculty of Medicine, Brawijaya University, Indonesia. Adult worms were collected 14–20 days post-infection and were used for *in vitro* culture to measure secreted proteins.

2.3. Excretory-secretory antigens of H. polygyrus

Excretory-secretory was collected from adult worms. Briefly, the worm was washed several times with sterile culture medium (RPMI-1640, 100 IU/mL penicillin, 100 IU/mL streptomycin) (Sigma–Aldrich, St. Louis, MO, USA) and incubated in 10 mL of culture medium at 37 °C in a 5% CO_2 atmosphere. Culture supernatants were removed every 24 h for 3 days and were stored at −20 °C, and the conditioned medium containing the excretory-secretory products was collected, centrifuged at 4000 r/min for 30 min, fractionated into low and high-molecular-weight fractions using 50 kDa MWCO Amicon Ultra Centrifugal (Amicon, Danvers, MA) and stored at −20 °C until use.

2.4. Induced colitis and injected excretory-secretory in mice

BALB/c mice were given drinking water containing 3% DSS (ICN Biomedical Inc, CA, USA) for 7 days. DSS was administered in a cycle protocol, with each cycle consisting of 7 days of 3% DSS and followed by 7 days of regular water. Colitis was induced by cyclical DSS treatments, which consisted of 7 days of 3% DSS followed by 7 days of untreated water. The injection of excretory-secretory (40 μg/mL) was started from 6th weeks and then, continued until 11 weeks. The body weights of the mice were measured every week. Induction of colitis was determined by the observations of weight loss, fecal blood. Blood in the feces was detected using an occult blood detection kit (Hemoccult). Female BALB/c mice were adaptively fed for 1 week and then randomly divided into five groups. Group A was regarded as normal control group with free access to drinking water. Group B was DSS model group, freely drinking 3% DSS solution for 7 days followed water for 7 days. Group C was *H. polygyrus* excretory-secretory total treatment group, with the same drinking solution as in Group B, with intraperitoneal

injection of *H. polygyrus* excretory-secretory total. Group D was *H. polygyrus* excretory-secretory 28 kDa treatment group, with the same drinking solution as in Group B, with intraperitoneal injection of *H. polygyrus* excretory-secretory 28 kDa. Group E was *H. polygyrus* excretory-secretory 55 kDa treatment group, with the same drinking solution as in Group B, with intraperitoneal injection of *H. polygyrus* excretory-secretory 55 kDa. At the end of 11 weeks, mice in all groups were sacrificed and colonic tissues were collected for analysis.

2.5. RNA preparation and RT-qPCR

Colons were removed from euthanized mice. The colons were homogenized for total RNA extraction, and oligo nucleotide primers purchased from Integrated DNA Technologies (Coralville, USA) were used for the relative quantification of total RNA (ABI-7500 system, software version 1.3) using a SYBR Green protocol. In brief, total RNA was isolated using Tri Reagent (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. RNA was quantified by UV spectro-photometry. The Applied Biosystems kit (Carlsbad, CA, USA) was used to generate cDNA from RNA according to the standard protocol. cDNA was subjected to real-time quantitative PCR. The PCR reactions were performed using the SYBR Green PCR Master Mix (Roche Diagnostic, USA), and the reaction volumes included 24 μL of cDNA obtained from the colons of the mice and 1 μL of test cDNA per reaction. The qPCR primer sequences were as follows: IFN-γ (forward, 5′-TGAACGCTACACACTGCATCTTGG-3′; reverse, 5′-TGAACGCTACACACTGCATCTTGG-3′); transforming growth factor-β (TGF-β) (forward, 5′-CCTCCCCCATGCCGCCCTCG-3′; reverse, 5′-CCAGGAATTGTTGCTATATTTCTG-3′); Foxp3 (forward, 5′-ACTGCTGGCAAATGGAGTCT-3′; reverse, 5′-AAGTAGGCGAACATGCGAGT-3′); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-TTCACCACCATGGAGAAGGC-3′; reverse, 5′-GGCATGGACTGTGGTCATGA-3′).

After an initial denaturation for 10 min at 95 °C, 35 amplification cycles were performed with the following steps: 30 s of 94 °C denaturation, 30 s of 45 °C annealing, and 1 min of 72 °C extension. The mRNA levels were measured using the $2^{-\Delta\Delta ct}$ method and were normalized to the level of GAPDH mRNA.

2.6. Analysis of cytokine expression by a FACS Calibur flow cytometer

Single cell suspensions derived from mesenteric lymph nodes were plated in 96-well plates (1×10^5 cells per well), stimulated with anti-CD3 mAb (145-2C11; BD Bioscience), and then were cultured for 48 h, followed by PMA-Ionomycin and Golgi Plug™ stimulation. The stimulated cells were stained with fluorescein isothiocyanate-conjugated anti-mouse CD4 and phycoerythrin-conjugated anti-mouse IL-10 (BD Biosciences) and then, subjected to intracellular cytokine using the BD Cytofix/Cytoperm kit (BD Bioscience). Cytokine expression was measured by a FACS Calibur flow cytometry and analyzed by using Cell Quest software.

2.7. Histological examination

For microscopic histological evaluation, formalin-fixed tissues were embedded in paraffin, and 5 mm sections were stained with hematoxylin and eosin. The sections were prepared for light microscopy and then were evaluated by 2 pathologists blinded. The total histology damage score was calculated using the following sub scores: loss of architecture, 0–4; inflammatory infiltrate, 0–4; goblet cell depletion, 0 or 1; ulceration, 0 or 1; muscle thickening, 0–2; and presence of crypt abscesses, 0 or 1.

2.8. Statistical analysis

All data were presented as the means ± SD values of five mice in each group. An ANOVA test (analysis of variance) was conducted to determine the statistical significance of difference between groups, with $P < 0.05$ being considered significant.

3. Results

3.1. Protein profiles of H. polygyrus excretory-secretory

Proteins profile of soluble factors was identified from *H. polygyrus* excretory-secretory. The soluble factors were fractionated into low and high-molecular-weight fractions using a 50 kDa MWCO Amicon. *H. polygyrus* excretory-secretory proteins were separated into two groups by molecular weight, using 50 kDa as the cut-off. Coomassie blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed the presence of proteins with two molecular weights 28 kDa and 55 kDa (Figure 1).

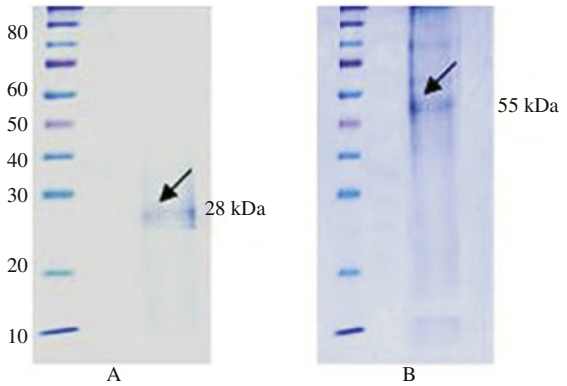

Figure 1. Identification of *H. polygyrus* excretory-secretory soluble protein (A) 28 kDa (< 50 kDa MW cut off columns) and (B) 55 kDa (> 50 kDa MW cut off columns).
The soluble protein fractions were run on one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Representative results from four independent experiments are shown.

3.2. Effect of H. polygyrus excretory-secretory protein on mice with colitis

To analyze the protein response of mice, colitis was induced by the *ad libitum* 3% DSS (w/v) in the drinking water for 7 days. Mice were injected (*i.p.*) with 40 μg of excretory-secretory protein (*H. polygyrus* excretory-secretory total, excretory-secretory 28 kDa and excretory-secretory 55 kDa) every week. Body weight changes were monitored and showed as the percentage of the original body weight. Body weight and stool consistency were monitored every week. Loose stools and occult blood in feces were detected in mice at the end of the experiment (Week 11). Treatment with the two fractions of *H. polygyrus* excretory-

secretory were performed using the 50 kDa MW cut-off value which revealed that the 28 kDa fraction did not affect DSS-treated mice, whereas the > 50 kDa fraction (excretory-secretory 55 kDa) maintained normal body weight of DSS-treated mice (Figure 2).

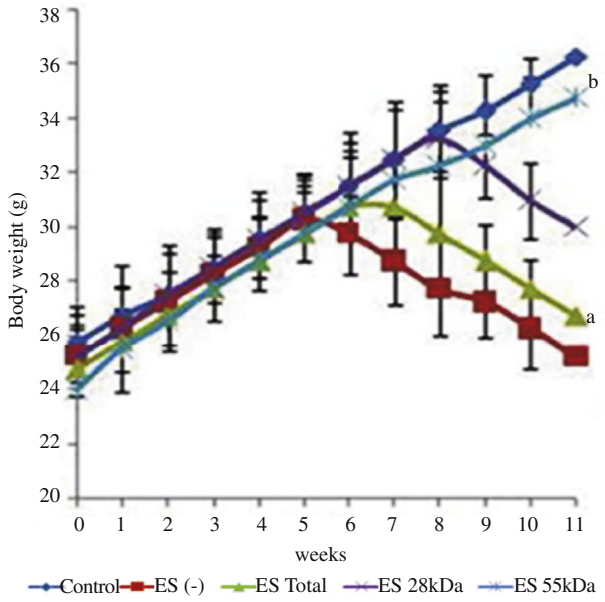

Figure 2. Excretory-secretory protein injection effects on mice with DSS-induced colitis.
ES: Excretory-secretory. Data were obtained from four independent experiments (n = 4), each of which contained five mice (total n = 20, error bar = SD). Body weight loss was observed in mice without excretory-secretory treatment (Group B) and C–D treated with excretory-secretory total and excretory-secretory 28 kD, respectively. Mice of Group B (DSS-H_2O only) showed significantly body weight loss compared to excretory-secretory 55 kDa treated mice ([a]: $P < 0.05$) whereas excretory-secretory 55 kDa injected mice were not significantly decreased compared to control. [b]: $P < 0.001$. Representative results obtained from five independent experiments are shown.

In our study, DSS administration resulted in a significant loss of body weight after 5 weeks ($P < 0.05$), and injection with total excretory-secretory or the excretory-secretory 28 kDa protein fractions resulted in a significant body weight loss after 7 and 8 weeks ($P < 0.01$) (Figure 2). In contrast, the excretory-secretory 55 kDa protein fraction significantly ameliorated DSS-induced colitis ($P < 0.05$).

3.3. Treatment with the excretory-secretory 55 kDa protein in DSS-induced colitis

The DSS only group showed extensive ulceration, with severe inflammatory cell infiltration. Treatment with total excretory-secretory resulted in severe inflammation, with the most severe change being the development of deep ulcers. The normal structure of the mucosa completely disappeared (Figure 3A,B). In mice that received the excretory-secretory 28 kDa protein fraction, inflammation was insufficiently suppressed (Figure 3C), and the mice showed diffuse mono-nuclear cell infiltration. On the other hand, in mice that received the excretory-secretory 55 kDa protein fraction, the structure of the epithelium was maintained. Further analysis showed that administration of the excretory-secretory 55 kDa protein fraction reduced inflammatory cell infiltration (Figure 3D) and the histological damage scores of DSS-induced colitis in mice. *H. polygyrus* proteins also protected against DSS-induced damage to the colon. When colon pathology was quantified, mice that received excretory-secretory 55 kDa protein fraction had significantly lower histological scores ($P < 0.001$) than untreated excretory-secretory mice (Figure 3E).

3.4. Effect of proteins derived from the soluble factor of H. polygyrus on the cytokine profile in mice colitis model

RT-PCR analysis was performed to determine the gene expression pattern of cytokines in mice treated with *H. polygyrus*

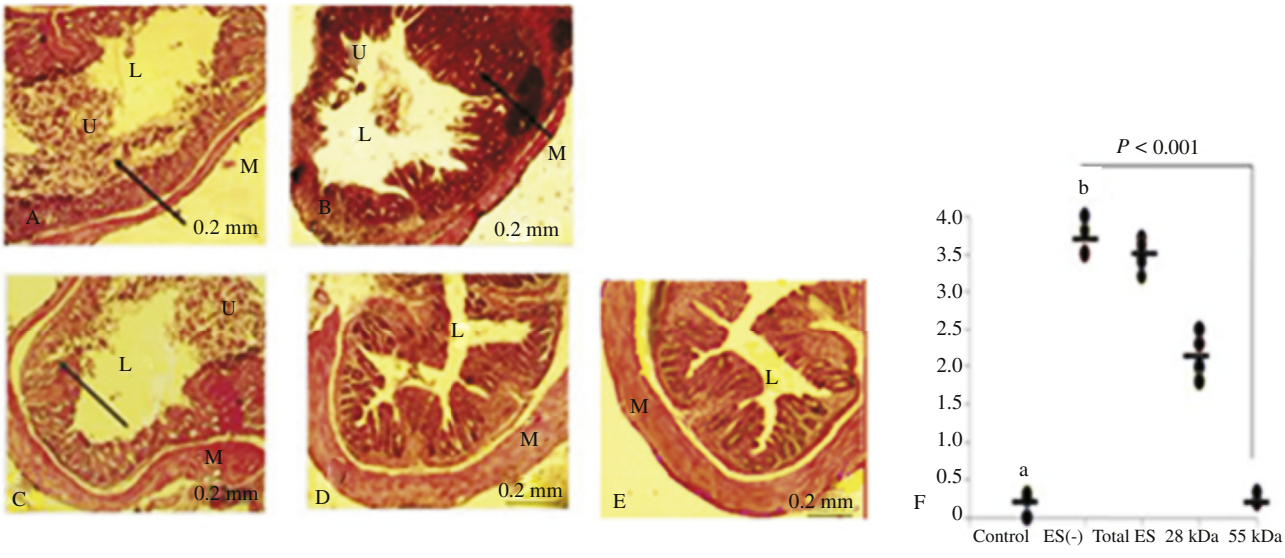

Figure 3. Histological changes during chronic DSS colitis mice after 11 weeks of treatment with excretory-secretory protein.
A: Mice were given untreated water as the control; B: Mice were given DSS only; C–E: Mice were injected with *H. polygyrus* excretory-secretory total, excretory-secretory 28 kDa and excretory-secretory 55 kDa, respectively; F: Histological colitis scores of individual mice are shown as circles, and the averages of each group are shown as horizontal bars. Each symbol represents an individual animal. Representative pictures of each group are shown. Groups B and C had significantly higher colitis scores than Group E ([a]: $P < 0.05$ and [b]: $P < 0.01$, respectively). L: Gut lumen; M: Muscle; U: Ulcer; Arrow: Inflammatory infiltrate; ES: Excretory-secretory. Magnification 200×.

excretory-secretory. The expression of IFN-γ, TGF-β, and Foxp3 were measured in mice that received DSS with or without excretory-secretory injection. Expression levels of mRNA were analyzed by PCR. The expression level of IFN-γ in the group treated with DSS only (Group B) was greatly higher than Groups D and E. The IFN-γ expression levels in mice treated with total excretory-secretory and the excretory-secretory 28 kDa protein fraction were slightly different (Figure 4A). Surprisingly, the excretory-secretory 55 kDa protein fraction induced the down-regulation of IFN-γ mRNA expression. The expression of IFN-γ was the lowest in the colon of mice after the administration of the excretory-secretory 55 kDa protein. The results showed that mice received the excretory-secretory 55 kDa protein fraction showed the highest IFN-γ expression. This study suggested that excretory-secretory 55 kDa injection was able to increase production of Th2 cytokines.

groups ($P < 0.001$) (Figure 5A). Furthermore, IL-10 expression of mice injected with excretory-secretory total or the excretory-secretory 28 kDa protein fractions was significantly lower than excretory-secretory 55 kDa group ($P < 0.001$). The data indicated that injection of excretory-secretory total or excretory-secretory 28 kDa were insufficient promoted IL-10 expression (Figure 5B,C). Whereas, mice treated with the excretory-secretory 55 kDa protein fraction (Group E) showed the highest IL-10 expression (Figure 5D).

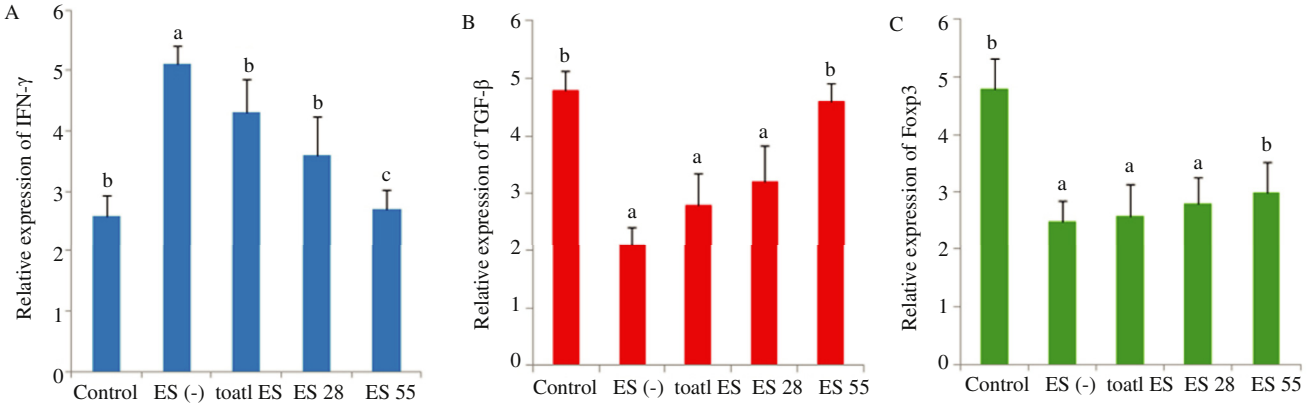

Figure 4. The effect of excretory-secretory protein treated on cytokine expression.
A: The expression of IFN-γ gene of excretory-secretory 55 kDa was decreased significantly ($P < 0.05$) with groups which received total excretory-secretory, excretory-secretory 28 kDa or excretory-secretory (–); B: Groups that received excretory-secretory 55 kDa showed elevated levels of TGF-β significantly ($P < 0.05$); C: No significant changes were observed in Foxp3 gene expression among ES protein treatment, but there is significant difference between control and treatment groups. ES: Excretory-secretory. Different letters denote significant differences among control and treatment ($P < 0.05$).

We found significant up-regulation of TGF-β mRNA expression in T cells isolated from colonic tissues in Group E compared to Groups C and D ($P < 0.05$) (Figure 4B). This up-regulation was only observed in mice that were treated with the excretory-secretory 55 kDa protein fraction of *H. polygyrus*. The injection of mice with excretory-secretory proteins caused strong TGF-β mRNA expression. The expression of TGF-β mRNA was detected in a significantly greater proportion of Group E compared to Group B (DSS only group) ($P < 0.01$) (Figure 4B). This indicated the essential role of TGF-β in the suppression of colitis by the excretory-secretory 55 kDa protein fraction. Meanwhile, mice that received the excretory-secretory 55 kDa protein fraction were clearly unable to induce Foxp3 expression (Figure 4C). This study indicated that excretory-secretory *H. polygyrus* production clearly did not drive the expression of Foxp3.

3.5. Effect of soluble factor excretory-secretory 55 kDa protein on IL-10 expression

Soluble factor excretory-secretory 55 kDa protein might promote IL-10 expression, and then it must have generated iTreg (induced Treg) cells. In mice received DSS only, it showed that IL-10 expression was lower than excretory-secretory treatment

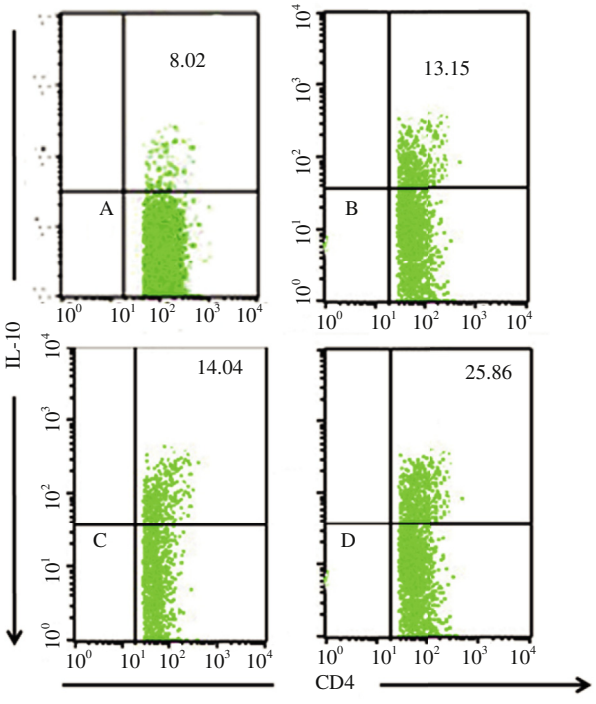

Figure 5. Effect of IL-10 on suppressing development of colitis.
A: IL-10 was observed in mice without excretory-secretory treatment (DSS only group); B: Mice were treated with total excretory-secretory protein; C: Mice were treated with excretory-secretory 28 kDa protein; D: Mice were immunized with excretory-secretory 55 kDa protein. Representative flow cytometry plots show cells gated as $CD4^{+}IL\text{-}10^{+}$.

4. Discussion

Worm therapy is widely used because it can reestablish immune system balance. The ability of worms to protect animals from various diseases, such as colitis, encephalitis, rheumatoid arthritis, asthma and diabetes mellitus has been studied experimentally. Exposure to worms suppresses Th2 allergic responses and the autoimmune response [24]. *H. polygyrus* excretory-secretory has been shown to reduce inflammation of the intestine [24,25].

In this study, we identify the protein profile of soluble factors derived from *H. polygyrus* excretory-secretory. We also demonstrated that *H. polygyrus* excretory-secretory 55 kDa has maintained the body weight of DSS-treated mice. Further analysis showed that administration of the excretory-secretory 55 kDa protein fraction reduced inflammatory cell infiltration. This protein also protected against DSS-induced damage to the colon. This study showed that the excretory-secretory 55 kDa protein has a capacity to suppress colitis induced by DSS. Although the exact mechanism(s) by which this protein increases the body weight of DSS-treated mice is still unknown, some studies have suggested that the 55 kDa protein band contains the protein *H. polygyrus* calreticulin, which is detected in all life stages and in the culture supernatant of adult *H. polygyrus* worms [26]. Calreticulin is a key protein in the regulation of cell function; it is a proven molecular adjuvant of the immune response [27]. Calreticulin can enhance the immune system [28]. The results of the current study indicated that the ability of mice to develop colitis was inhibited by the administration of the *H. polygyrus* excretory-secretory 55 kDa protein fraction.

The excretory-secretory 55 kDa protein fraction was effective as an ulcerative colitis therapy. Excitingly, we found down-regulation of IFN-γ expression of mice treated with the excretory-secretory 55 kDa protein fraction. We demonstrated that the excretory-secretory 55 kDa protein fraction decreased the expression levels of inflammatory cytokines. Therefore, our results indicated the possibility that the inhibition of inflammation by *H. polygyrus* excretory-secretory 55 kDa was a potentially effective strategy for reducing the risk of colitis.

IFN-γ has been implicated in the response to worm infection [29] and is considered to have an important role in murine models of colitis. Therefore, inhibition of inflammation by the excretory-secretory 55 kDa protein fraction of *H. polygyrus* indicates its potential for reducing the risk of colitis. These findings combined with our data suggest that IFN-γ also has an important pro-inflammatory role in DSS-induced colitis and in other models of ulcerative colitis, such as chronic intestinal inflammation. We found significant up-regulation of TGF-β mRNA expression of T cells isolated from colonic tissues. This up-regulation was only observed in mice that were treated with *H. polygyrus* protein excretory-secretory of 55 kDa. It suggests that TGF-β signaling to mucosal T cells is an important requirement for worm protection against colitis.

Based on our findings, we concluded that proteins obtained from *H. polygyrus* could serve as an alternative therapy to reduce inflammation in ulcerative colitis. Our findings also suggested that TGF-β signaling to mucosal T cells was an important aspect of the protection against colitis. Attenuation of inflammation after treatment with *H. polygyrus* proteins may also be linked to regulatory T cells, as we found significant up-regulation of TGF-β mRNA expression in T cells isolated from the mesenteric lymph nodes of mice treated with *H. polygyrus* excretory-secretory 55 kDa proteins. Our result demonstrated that mice received the excretory-secretory 55 kDa protein fraction were insufficient for their suppressive activity of Foxp3. These results indicated that the excretory-secretory 55 kDa protein fraction did not induce Foxp3 Treg and more likely suppresses colitis directly.

Considering the potential of excretory-secretory 55 kDa proteins in protecting against colitis, this study aimed to evaluate some soluble proteins regarding their ability to induce the IL-10 production. Protein product excretory-secretory 55 kDa of worm could act as immunogens. Those immunogen might induce IL-10. Attenuation of inflammation after excretory-secretory 55 kDa treatment might also be linked to regulatory T cells as a results of IL-10 expression in $CD4^+$ T cells population from excretory-secretory 55 kDa protein-treated mice. These results suggest that either cells, which did not express Foxp3, have something to do with the production of IL-10 and TGF-β for the induction of Tregs other molecules and neighboring cells have to be present.

The excretory-secretory 55 kDa protein fraction increased IL-10 expression, indicating that *H. polygyrus* excretory-secretory was able to influence the recently discovered pro-inflammatory IFN-γ pathway. This result relates to the evidence that excretory-secretory 55 kDa protein prevented excessive cell inflammation. Excretory-secretory 55 kDa protein promoted the production of iTregs as indicated by the increased expression of IL-10, and they were functionally able to suppress the development of inflammation.

The data presented in this study showed that *H. polygyrus* excretory-secretory 55 kDa proteins could inhibit DSS-induced murine colitis and IL-10 helped mediate the immune response in colitis. First, these results showed that worm infection provokes a Th2-type response. Here, we have observed an increase in IL-10 expression. This finding is consistent with other reports of increased IL-10 expression with various anti colitis strategies [30,31]. Second, IL-10 is important in regulating effectors responses that emerge in response to infection [32–35]. *H. polygyrus* excretory-secretory 55 kDa proteins increased Th2 in this mouse model of colitis, which suppressed Th1 by reducing inflammation in colitis. Our data established that excretory-secretory 55 kDa proteins increased the expression of IL-10 *in vivo* via Foxp3-independent pathways. Our data are consistent with a previous report that the products secreted by *H. polygyrus* exert their anti-inflammatory effects by actively modulating host immune effector mechanisms via promoting IL-10 independent regulatory pathways [35,36].

Secretion of antigen in the range of 50–70 kDa is venom allergen-*Ancylostoma* secreted protein-like-1 (VAL-1), VAL-2 and VAL-5, which is suspected as excretory-secretory products [36]. These proteins indirectly activate dendritic cells to stimulate T cells that secrete IL-10. This suggests that the protein contains O-glycan VAL is able to increase the stimulation of dendritic cells to interact with T cells to produce IL-10 [22]. It is reasonable to assume that tolerogenic dendritic cell and T cell interactions are an important part of the process. Secretion of protein excretory-secretory 55 kDa was produced by *H. polygyrus* that altered dendritic cell function and this factor reached their cellular targets.

The recent advances in excretory-secretory had already identified the candidates and we have described here, provided exciting data onto the ability of proteins to modulate host immunity. The excretory-secretory 55 kDa protein could replace live parasite therapy with non-living parasite products. Our results suggest that the beneficial effect of *H. polygyrus* excretory-secretory proteins is linked to stimulation of regulatory T cells

and suppression of pro-inflammatory T cells. Therefore, we conclude that treatment of worms excretory-secretory proteins has therapeutic potential.

In recent studies, excretory-secretory had previously identified the candidate colitis treatments described here, providing exciting data regarding the ability of proteins to modulate host immunity. Our results demonstrated the beneficial effect of *H. polygyrus* excretory-secretory proteins and that it was linked to the stimulation of regulatory T cells and the suppression of pro-inflammatory T cells. This study indicates the potential of excretory-secretory proteins derived from worms for colitis therapy. Our results strongly suggested that worm excretory-secretory proteins are useful as living worms for the treatment of colitis. By identifying excretory-secretory proteins that modulate immune responses and prevent autoimmune diseases, it may be possible to design a model of novel therapeutic approaches that may not require infection with living worms. In this research, we established excretory-secretory proteins as an alternative therapy for colitis in place of living worms. Therefore, we concluded that treatment with worm excretory-secretory proteins has therapeutic potential.

Conflict of interest statement

We declare that we have no conflict of interest.

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