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Differential effect of hesperidin on Th1, Th2, Th17, and proinflammatory cytokines production from splenocyte of *Schistosoma mansoni*-infected mice

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Abstract

The present study was conducted to evaluate the potential effect of hesperidin on the regulating Th1, Th2, Th17, and the proinflammatory cytokines production from splenocyte of acute murine schistosomiasis. Each mouse was infected with 100 cercariae of Schistosoma (S.) mansoni *and at 8 weeks post-infection, mice were sacrificed, splenocytes were prepared and cultured in the presence of either soluble adult worm antigen (SWAP) or soluble egg antigen (SEA) of S. mansoni with either 50 or 100 μM hesperidin. Hesperidin, in both tested concentrations, significantly augmented interleukin (IL) 4 ($p < 0.05$), IL-10 ($p < 0.01$), and interferon γ (IFN-γ) ($p < 0.05$) production in response to SAWP and SEA stimulation. However, IL-13, IL-17, IL-1, and tumor necrosis factor α (TNF-α) production were significantly ($p < 0.01$) reduced. Interleukin 12 production was not significantly ($p > 0.05$) changed with hesperidin treatment at 50 μM, while 100 μM concentration of hesperidin significantly reduced IL-12 production in response to SWAP ($p < 0.05$) and SEA ($p < 0.01$) stimulation. The results suggested that hesperidin preferentially modulated in vitro production of Th1, Th2, Th17, and proinflammatory cytokines and this could reduce immunopathology of schistosomiasis.*

Key words: *hesperidin, schistosomiasis, Th1, Th2, Th17, proinflammatory cytokines production.*

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Introduction

In *Schistosoma* (*S.*) *mansoni* infection, the outcome of the immune response determines the balance between protective immunity and immunopathology [1]. The morbidity in *S. mansoni* infection arises from the granulomatous response to eggs that become trapped in the host tissues with subsequent fibrosis. Granuloma formation is dependent on $CD4^+$ T cell responses [2, 3], while schistosomiasis is associated with an imbalance in T helper 1 (Th1)/Th2 cytokines [4]. Differentiation of $CD4^+$ T cells into Th1 or Th2 lymphocyte population depends to a great extent upon the relative abundance of various cytokines during the priming of the antigen-specific lymphocyte population by antigen-presenting cells [5, 6]. Meanwhile, interleukin (IL) 12 and IL-4, the key promoters of Th1 and Th2 cell populations respectively, both are mutually antagonistic. Interleukin 4 is capable of inhibiting the expression of the β2 subunit of the IL-12 receptor [7] while IL-12 is responsible for the suppression of IL-4 production in a interferon γ (IFN-γ)-dependent manner [8]. Therefore, modulation of both Th1 and Th2 responses could down regulate the granulomatous inflammation and consequently reduce morbidity from schistosomiasis.

In the course of an infection, Th1 response is dominant in the first 3-5 weeks of infection during the migration and the maturation of parasites while Th2 cytokine expression becomes dominant shortly after egg laying begins; with IL-4, IL-5, IL-10, and IL-13 are the principal cytokines secreted by lymphoid cells after stimulation with schistosome egg antigens [9]. The secretion of Th1 cytokines, IFN-γ and IL-2, is concurrently down-regulated at the time

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the Th2 response reaches their peak [10]. There is a current clear consensus that Th2 responses contribute substantially in mediating the formation of the egg induced granulomas, as an abrogation of the type 2 responses by ablation of STAT6 [11] or IL-4 α receptor [12] expression greatly reduces the granuloma formation. Th2 activation appears to be necessary in protecting the host from the lethal hepatic and intestinal damage during acute infection [13] and keeping Th1 inflammatory immunopathology in check [14]. In contrary, immunization studies suggested that these Th2 responses might not provide protective immunity; instead, the Th1 immune response appeared to be important in the induction of resistance against *S. mansoni* in the murine model [15, 16]. Several studies have reported that the protection in radiation-attenuated vaccine model of murine schistosomiasis is dependent on the Th1-associated humoral and cell-mediated immune responses [17-19]. Nevertheless, in the absence of Th1-type responses a reduction in worm burdens was observed, suggesting that the Th2-type responses might also possess a role in protection [20, 21]. Accordingly, a balance between Th1 and Th2 response is an important factor to maintain protective immunity and to reduce the pathological changes induced by schistosomiasis.

Recently, Th17 cell was involved in the research of adjustment net of cytokines in schistosomiasis in an animal model [22]. Interleukin 17 producing cells were identified as a distinct lineage of CD4+ T cells with a potent proinflammatory properties [23, 24]. Interleukin 17-producing cells were first shown to mediate the immunopathology in several autoimmune diseases such as allergic encephalomyelitis, collagen induced arthritis, and inflammatory bowel disease [25-28]. In severe schistosomiasis, pronounced immunopathology correlated with an increase in IL-17 production by granuloma cells in the lesions of the infected animals was observed [29, 30]; such pathology was reduced by *in vivo* neutralization of IL-17 [29]. In these circumstances, activated dendritic cells (DC) could initiate an innate proinflammatory cascade leading to the differentiation of pathogenic Th17 cells in response to schistosome egg antigens [31]. Such interactions were shown to be conducive to the differentiation of Th2 [32] or T regulatory cell responses [33, 34] however, the data bout IL-17 as an adjusted factor in schistosomiasis, was still insufficient. Therefore, the balance of Th1-, Th2-, and Th17-type lymphocyte populations in the host after exposure to infection is crucial to the development of protective immunity or immunopathology.

Hesperidin (HES, 5,7,3´-trihydroxy-4´-methoxy-flavanone 7-rhamnoglucoside, CAS number: 520-26-3) a flavanone-type flavonoid, is abundant in citrus fruits and has been reported to possess many biological activities: antifungal and antiviral activities [35, 36], inhibition of cell cycle progression in human pancreatic cells [37], reduction of reactive oxygen species [38] and triggering an *in vitro* caspase dependent apoptosis in human polymorphonuclear neutrophils [39], cytotoxic effects on human colon cancer cells accompanied with DNA fragmentation and caspase-3 activation [40], an antioxidant effect [41, 42], anti-inflammatory effects on rodent cell lines and human cell lines [43, 44], suppression of mRNA and protein expression of cyclooxygenase-2 (COX-2) in LPS-induced macrophages [45, 46], reduction of tumor necrosis factor α (TNF-α) production and inhibition of infection-induced lethal shock [47], downregulation of mRNA and protein expression of TNF-α, IL-1β, IL-6, IL-8, IL-12, and iNOS [46, 48-52], suppression of eosinophil infiltration, allergic airway inflammation, IL-13, IL-5, and IL-17 production in mouse model of allergic asthma [53, 54], enhancement the production of IL-4, and IL-10 in bronchoalveolar lavage fluid of mouse with acute lung inflammation [48], and increased IFN-γ release from pollen-stimulated PBMC of patients with seasonal allergic rhinitis to pollen [55]. However, there no studies was reported on the effect of HES on cytokine profile and Th1/Th2 bias of *S. mansoni*-infected mice. The aim of the current study was to evaluate the *in vitro* ability of HES to regulate proinflammatory, Th1-, Th2-, and Th17-type cytokines production using murine model of *S. mansoni*.

Material and methods

Mice and parasite

Eight-weeks-old MF1 male mice were purchased from the Biological Supply Centre, King Fahd Centre for Medical Researches, College of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia. Mice were housed in stainless-steel cages under strict hygienic conditions at 25-28ºC, 8-12 h light and free access to feed and water. *S. mansoni* (John Bruce Egyptian strain) cercariae were collected by illumination of the infected *Biomphalaria alexandrina* snails under a 60-watt lamp for 2 h as previously described [56].

Infection

Mice were infected by tail immersion method according to Bruce and Radke [57] with some modifications. Briefly, mice were individually placed in mouse retraining chambers, allowing the tail outside the chamber. After cleaning the tail with dechlorinated tap water, it was inserted into a vial containing 100 cercariae in 2 ml dechlorinated tap water and located into an exposure board. Mice were left in contact with the infective cercariae for 2 h, and then removed from the chamber and tails were allowed to dry. The remaining cercariae were counted and mice receiving less than 95% of the cercariae were excluded from the experiment.

Reagents

Hesperidin (HES, purity ≥ 97.0%) was dissolved in dimethylsulfoxide (DMSO) and then diluted with Roswell

Park Memorial Institute (RPMI) 1640 medium (Invitrogen) at a final DMSO concentration ≤ 0.05% (v/v). All reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Antigens

Soluble adult worm antigen (SWAP) and soluble egg antigen (SEA) of *S. mansoni* were purchased from Schistosome Biological Supply Center, Theodore Bilharz Research Institute (TBRI, Imbaba, Giza, Egypt). Antigen preparations were suspended in sterile 0.01 M phosphate buffered saline (PBS), pH 7.2, protein content was measured by Lowry's method [58]. Antigens were diluted with sterile RPMI-1640 medium and stored at –80°C as aliquots until used.

Splenocyte preparation

Spleens were removed from infected mice aseptically after 8 weeks of infection. Single cell suspensions were prepared as described previously [59] with some modifications. Briefly, cells suspension were obtained by forcing tissues through sterile 70-mm nylon mesh (Becton Dickinson) and washed one time with ice-cold RPMI-1640 medium. Erythrocytes were lysed from the dispersed spleen cell suspensions by hypotonic shock followed by extensive washing with RPMI-1640 medium. After cells counting and viability percentages calculation by using trypan blue exclusion method, cells were resuspended in appropriate volume of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1mM Na pyruvate.

Cytokine production

The effect of HES on different cytokines (IL-1, IL-4, IL-10, IL-12, IL-13, IL-17, TNF-α and IFN-γ) production in response to SAWA and SEA stimulation was tested. Splenocytes from infected mice were suspended in complete tissue culture medium (CM) at a final concentration of 1.5×10^6 cells/ml. Splenocytes from each mouse were cultured in triplicates, 1.5×10^5 cells/well, in a total volume of 200 µl/well into flat-bottom 96-well plates (Falcon) containing either sterile SAWA (2 µg/well) or SEA (1 µg/well) with either 50 or 100 µM HES, and HES free cultures containing ≤ 0.05% (v/v) DMSO (CM) used as control. Then plates were incubated either at 24 h (for IL-4 production) or 72 h (for assessment of other cytokines) at 37°C in humidified 5% CO_2 atmosphere. At the end of the incubation periods, cell-free supernatants were harvested and stored at –80°C until used for cytokine determination assays.

Cytokine determination

The amount of IL-1β, IL-4, IL-10, IL-12, IL-13, IL-17A, TNF-α, and IFN-γ were quantified in culture supernatants by sandwich enzyme linked immunosorbent assays (ELISA). Briefly, high protein binding ELISA plates (Nunc, Denmark) were coated with 2 µg/ml of capture monoclonal antibody (eBioscience, Birmingham, USA) against the target cytokine for at least 18 h at 4°C in 0.05 M carbonate/bicarbonate buffer, pH 9.6. After blocking the free spots of the wells with 5% non-fat milk, culture supernatants were dispensed and incubated for 2 h at room temperature (RT). Plates were washed, then 1 µg/ml of the detecting biotin labeled antibodies (eBioscience) was added and incubation for 1 h at RT. Unbound biotine was removed by 5 times washing, 100 µl/well of 1 : 4000 diluted peroxidase-labeled streptavidin (eBioscience) was added and plates were incubated for 1 h at RT. Ortho-phenylenediamine (OPD) was then added (100 µl/well) and the plates were placed in the dark at RT for 30 min. Finally, the 2N H_2SO_4 (100 µl/well) was added and the absorbance was measured at 492 nm using microplate reader (BioTech, CA, USA). Cytokine concentrations were determined by reference to standard curves construction with known amounts of mouse recombinant IL-1β, IL-4, IL-10, IL-12p70, IL-13, IL-17A, TNF-α, or IFN-γ (eBioscience).

Statistical analysis

The statistical tests were performed with the SPSS (version 16) software. Significant differences were determined by one-way analysis of variance (ANOVA) and applying Tukey's test for multiple comparisons with a level of significance set at $p < 0.05$.

Results and discussion

Cytokines play an important role in the immunological pathogenesis of schistosomiasis. The disease is associated with an imbalance in Th1/Th2 cytokines [4]. Therefore, modulation of Th1, Th2, and Th17 responses as well as proinflammatroy cytokines could downregulate granulomatous inflammation and consequently controls the outcome of immunopathology in schistosomiasis. To investigate the regulatory role of HES on cytokine secretion during acute schistosomiasis, splenocytes isolated from 8 weeks *S. mansoni* infected mice and cultured with either 50 or 100 µM HES in response to SWAP and SEA of *S. mansoni*. Cytokine production was quantified by using sandwich ELISA. The tested HES concentrations were chosen according to Yeh *et al.* [48], who showed that 50 µM HES suppressed the *in vitro* expression of IL-8, TNF-α, IL-1β, IL-6, IL-12, ICAM-1, and VCAM-1 on epithelial cell line (A549) and myelomonocytic cell line (THP-1).

Effect of hesperidin on Th2 cytokine production

Data of the present study showed that both tested concentrations (50 and 100 µM) of HES significantly ($p < 0.05$) increased the IL-4 production (Fig. 1A). Interleukin 4 operates as the key cytokine driving the Th2 response [60]. In the absence of IL-4, some strains of mice overproduce sev-

eral pro-inflammatory mediators, including TNF-α and nitric oxide (NO), leading to an acutely fatal disease following infection [61]. Moreover, IL-4 has been shown to contribute in the induction of the protective immunity in the radiation attenuated vaccine model [20, 21]. Therefore, IL-4 is an important cytokine for the regulation of immunopathology in schistosomiasis and may have a role in the protective immunity.

Similarly, data of the current study clearly showed that HES at 50 and 100 μM concentrations significantly ($p < 0.01$) enhanced IL-10 production in response to both SWAP and ESA stimulation (Fig. 1B). Interleukin-10 is an important cytokine produced by a variety of cell types, including CD4+ and CD8+ T cells, monocytes/macrophages, mast cells, keratinocytes, eosinophils, and various tumor cells [62]. It was originally characterized by its ability to down-regulate Th1 response development, but is now known to be a major immunoregulatory cytokine influencing Th cell development as well as the production of numerous proinflammatory cytokines [10]. It has been reported that IL-10 have a protective function during schistosomiasis, where up to 30% mortality was observed in IL-10-deficient mice at the acute stage post-infection [63]. As in IL-4-deficient animals, liver damage caused by excess production of TNF-α, nitric oxide and reactive oxygen intermediates appears to be the primary cause of mortality in IL-10 deficient mice [64]. Therefore, upregulation of IL-10 could be downmodulate excessive production of inflammatory mediators and protect host tissues from further damage.

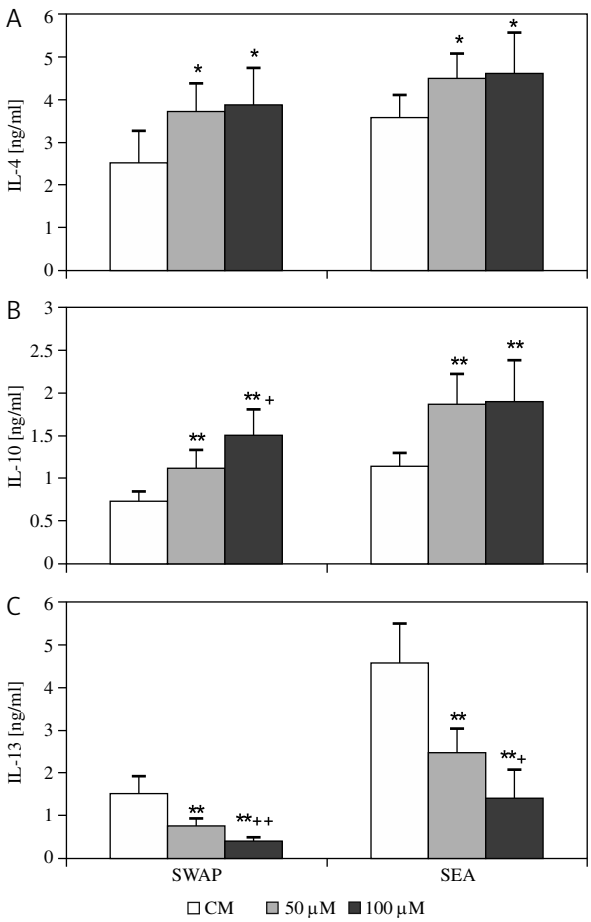

Fig. 1. The effect of hesperidin on Th2 cytokines production in response to SWAP and SEA stimulation. Columns represent mean of 5 individual mice, and the bars denote SD about the mean. Significant differences between culture medium (CM) and HES are denoted by asterisks (*$p < 0.05$, **$p < 0.01$) and between HES concentrations by plus ($^{+}p < 0.05$, $^{++}p < 0.01$) above the columns

In contrast, IL-13 secretion significantly ($p < 0.01$) decreased with HES treatment in a dose dependent manner (Fig. 1C). Several studies have been pointed out the important role of IL-13 in the development of liver fibrosis in mice and humans [13, 65-67]. Mice deficient in IL-13 survived longer than similarly infected wild-type animals, and IL-13-blocking studies have shown that this cytokine was the key mediator of fibrosis in schistosomiasis [68]. Although the average size of egg induced granulomas was unaltered to IL-13-deficient mice, the marked fibrotic changes were almost completely ablated in the absence of IL-13 even after several months of infection [66]. Fibrogenic effect of IL-13 was completely TGF-β independent [69]. Consequently, down regulation of IL-13 might decrease tissues fibrosis accompanied with schistosomiasis.

Effect of hesperidin on Th1 and Th17 cytokine production

As depicted in Fig. 2A, HES at 50 and 100 μM concentrations significantly ($p < 0.05$) enhanced IFN-γ production in response to both SWAP and SEA stimulation. From the aforementioned data, HES stimulated IL-4 and IFN-γ production from splenocytes of *S. mansoni* infected mice. Such increase in IFN-γ with IL-4 have also been reported in another model in which splenocytes treated with aqueous leaf extract of *Morinda lucida*, showed increased in both IFN-γ and IL-4 production [70]. Interferon γ plays a crucial role in the protective immunity against *S. mansoni* infection in radiation attenuated vaccine model [71, 72]. Previous reports suggested that IFN-γ downregulated granuloma formation both *in vitro* and *in vivo* models [73, 74]. In addition, IFN-γ has a potent antifibrogenic effect [75]. Therefore, modulation of IFN-γ production has a beneficial effect and may relieve pathology of schistosomiasis and maintained protective immunity.

On the other hand, IL-17 highly significantly ($p < 0.01$) reduced with HES treatment in response to both SWAP and SEA antigens (Fig. 2B). It has been reported that in the natural and induced models of severe disease, pronounced immunopathology correlated with an increase in IL-17 production [29, 30]. Hence, the reduction of IL-17 leaded to amelioration of schistosomiasis-induced pathology.

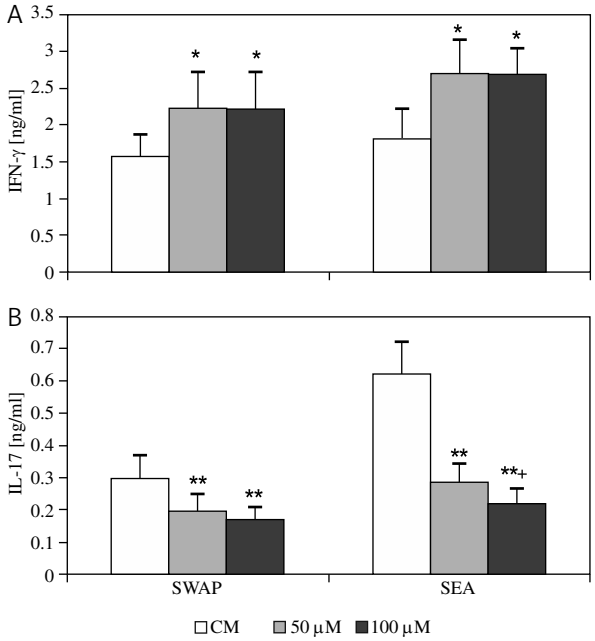

Fig. 2. The effect of hesperidin on Th1 (IFN-γ) and Th17 (IL-17) cytokines production in response to SWAP and SEA stimulation. Columns represent mean of 5 individual mice, and the bars denote SD about the mean. Significant differences between culture medium (CM) and HES are denoted by asterisks (*$p < 0.05$, **$p < 0.01$) and between HES concentrations by plus ($^{+}p < 0.05$) above the columns

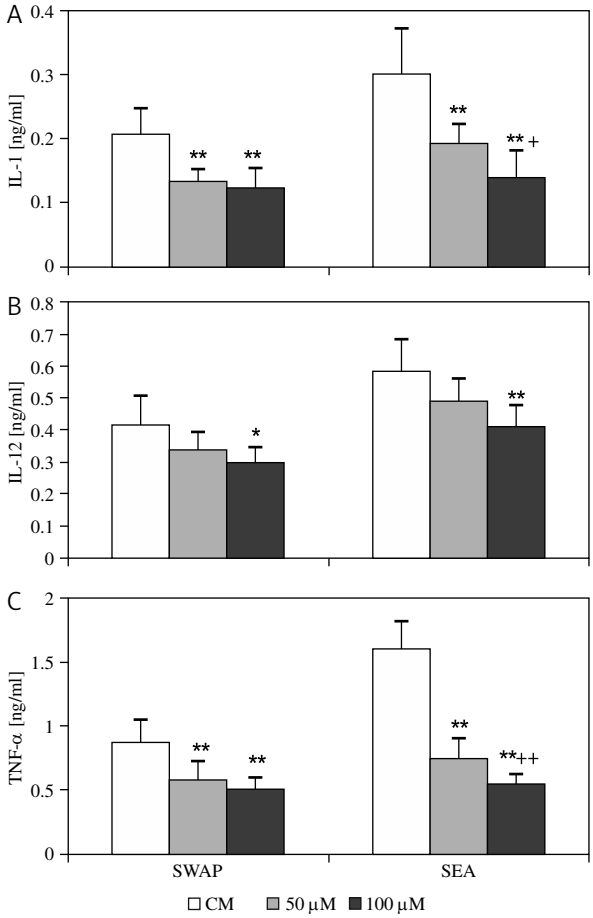

Fig. 3. The effect of hesperidin on IL-1β (A), IL-12 (B), and TNF-α (C) production in response to SWAP and SEA stimulation. Columns represent mean of 5 individual mice, and the bars denote SD about the mean. Significant differences between culture medium (CM) and HES are denoted by asterisks (*$p < 0.05$, **$p < 0.01$) and between HES concentrations by plus ($^{+}p < 0.05$, $^{++}p < 0.01$) above the columns

Effect of hesperidin on proinflammatory cytokine production

To evaluate the potential antiinflammatory effect of HES, IL-1β was determined in culture soap of HES treated splenocytes in response to SWAP and SEA antigens. As depicted in Fig. 3A, HES at both tested concentrations significantly ($p < 0.01$) reduced IL-1β production in response to *S. mansoni* antigens stimulation in a dose dependent manner. The significance of this key proinflammatory cytokine in immunopathology of schistosomiasis was unclear and needs further investigations. However, in some strains of mice, IL-1 is known through its capacity to increase the synthesis of other cytokines and promote B cell maturation, differentiation, and antibody production [76, 77], elicited down-regulation of IFN-γ in *S. mansoni* infection, and failed to enhance production of specific antibodies. Administration of IL-1 was associated with aggravation of the worm burden and/or egg load [78] therefore, the reduction of IL-1 is in favor of the host.

Data of the current study showed that HES significantly decreased IL-12 secretion at 100 μM concentration in response to SWAP ($p < 0.05$) and SEA stimulation ($p < 0.01$), however lower concentration of HES (50 μM) not significantly ($p > 0.05$) altered IL-12 production in response to either SWAP or SEA stimulation (Fig. 3B). Indeed, IL-12 involved in differentiation of naive $CD4^{+}$ lymphocyte to Th1 subset [79]. Several studies suggested that IL-12 possessed a role in the protective immunity against *S. mansoni* [16, 18, 80], however, IL-12 had no effect on immunity when administered alone during a normal infection [17]. Moreover, IL-12 deficient mice showed a substantial reduction in the level of protection induced by attenuated larvae [20]. On the other hand, IL-12 might have a role in regulation of granuloma formation, as mice vaccinated with SEA plus IL-12 developed smaller granulomas and less-severe fibrosis in the subsequent infection [60].

Our data clearly showed that HES highly significantly ($p < 0.01$) decreased TNF-α production in response to SWAP and SEA in a dose dependent manner (Fig. 3C). The pathology associated with schistosomiasis was largely attrib-

uted to the intense granulomatous reactions and subsequent fibrosis induced by parasite eggs that become trapped in host organs such as the liver and intestine. Currently, it is well documented that TNF-α plays an important role in granulomatous inflammation. It restored the ability of T cell deficient mice to mount a granuloma around schistosome eggs [81]; and caused tissue damage by the exacerbation of the granuloma formation and overproduction of reactive oxygen species [82]. Therefore, downregulation of TNF-α could largely reduce pathology associated with schistosomiasis [83].

Conclusions

From aforementioned data, HES up regulated IL-4, IL-10, and IFN-γ secretion; and down regulated IL-1, IL-12, IL-13, IL-17, and TNF-α production from splenocytes of 8 weeks *S. mansoni* infected mice. This preferentially modulation of cytokine production could reduce immunopathology of schistosomiasis. The precise mechanism by which HES regulated production of these cytokines was unclear and needs further investigations, however, HES has been shown to reduce IL-5 production in experimental ovalbumin-induced asthma model via inhibition of GATA-3 transcription factor [53]. Therefore, HES might modulate cytokine production through regulation of transcription factors such as c-maf, GATA-3, NFAT, and STAT6.

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The authors declare no conflict of interest.

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