HCV Infection Amplified Th2 Bias and Th17 Responses In *Schistosoma*-Infected Patients

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Schistosomiasis and HCV are the most prevalent infections in Egypt. *Schistosoma* infection is associated with bias towards Th2 responses. Plasma from 17 (*Schistosoma*-infected), 39 (*Schistosoma*/HCV co-infected) and 23 controls were collected. Cytokine multiplex array was used to measure 6 plasma cytokine levels representing the surrogate markers of three T helper lymphocytes subpopulations; IL-12 (Th1), IL-4 and IL-10 (Th2), IL-17, IL-22 and IL-23 (Th17). There was a significant increase in plasma levels of IL-17 and IL-23 in co-infected patients compared to *Schistosoma*-infected patients. As well, there was a significant increase in the Th2 regulatory cytokines IL-10 and IL-4 in co-infected group compared to *Schistosoma*-infected patients. However, no significant changes were observed in the Th1 cytokine (IL-12) among the groups. In conclusion, presence of HCV infection shifted the response towards Th2/Th17 pathway which may play a role in the progressive pathogenesis of HCV in *Schistosoma*-co-infected patients.

Schistosomiasis is a chronic helminthic disease infecting more than 200 million people worldwide (Chitsulo *et al.,* 2000). Infection with *Schistosoma mansoni* is endemic in Egypt, with a prevalence of 17.5%–42.9% (El-Khoby *et al.,* 2000; Hammam *et al.,* 2000). Morbidity in humans infected with *S. mansoni* results primarily from deposition of parasite ova in the portal areas with granuloma formation, which progresses to irreversible fibrosis and severe portal hypertension in more than 60% of cases. The severity of disease appears to be regulated by the balance between Th1- and Th2-type cytokines. Additionally, it is reported that *S. mansoni* infection is characterized by a strong Th2 immunologic bias (Estaquier *et al.,* 1997; Kamal *et al.,* 2001b; Sabin & Pearce, 1995).

The appropriate induction and balance between Th1 and Th2 subsets in response to an infection affects the immunopathogenesis of the disease (Koziel, 1999). Th17 cells have been recently described as a third independent effector cell subset differentiated from $CD4^+$ T cells upon antigenic stimulation (Chitsulo *et al.,* 2000; Schirren *et al.,* 2000; Takaki *et al.,* 2000; Wen *et al.,* 2011). Although the functions of Th17subset are not completely understood, several studies suggested the important role of Th17 cells in host defenses against extracellular pathogens and in the immunopathogenesis of some infectious diseases by producing their crucial regulatory cytokine IL-17 (Grzych *et al.,* 1991; Rutitzky *et al.,* 2008; Rutitzky *et al.,* 2009; Rutitzky & Stadecker, 2011; Shainheit *et al.,* 2008; Smith *et al.,* 2009).

During *Schistosoma* infection, the immune response progresses through at least three phases. The first phase occurs during the first three weeks of the infection, when the host is exposed to migrating immature and mature parasites. In this phase, the dominant response is Th1- like. The response is induced by non-egg antigens, such as the schistosomula and soluble worm antigen (SWA) (Dunne &

Cooke, 2005; Rutitzky *et al.,* 2008). In the second phase (beginning 4–5 weeks post infection), the parasites begin to produce eggs, and the immune response is characterized by a stronger Th2 response which is primarily induced by egg antigens (Gobert *et al.,* 2007). The granulomas that form around the eggs in the liver, develop to their maximum size around 8–9 weeks post-infection and are positively regulated by Th17 cells and its secreted IL17 cytokine (Grzych *et al.,* 1991). In the third phase, (beginning 11–13 weeks post-infection), the Th2 response is predominant and modulated the egg-induced granulomas which become smaller in size. At this stage, $CD4^+$ $CD25^+$ $Foxp3^+T_{reg}$ cells are induced mainly by egg antigens and down-regulated the egg-induced pathological immune responses (Burke *et al.,* 2009).

Th17 cells are directly associated with the severity of hepatic egg-induced granulomatous inflammation (Wen *et al.,* 2011). The level of Th17 cells in the host is determined by multiple factors including exposure to complex parasitic antigens that either induce or suppress the irgeneration. Lowering IL-17 levels may also favor the host's protective responses against *Schistosoma* infection (Shainheit *et al.,* 2011; Wen *et al.,* 2011). An increase in the frequency of Th17 cells located in the liver granulomas and spleens of *Schistosoma*-infected mice was reported which could be reduced by neutralization of IL-17 *in vivo* (Mbow *et al.,* 2013; Rutitzky *et al.,* 2005; Rutitzky *et al.,* 2008).

Egypt has one of the highest prevalence of hepatitis C virus (HCV) infection in the world with approximately10–25% of the population are infected with HCV, most of them living in the rural areas where schistosomiasis is endemic (Farid *et al.,* 2005; Kamel *et al.,* 1994). Patients co-infected with HCV and schistosomiasis exhibit unique clinical, virological, and histological patterns manifested by viral persistence with high HCV RNA titers, as well as higher necro-inflammation and fibrosis in the liver (Angelico *et al.,* 1997; Kamal *et al.,* 2000). Th1 responses have been associated with protective host defense against HCV (Fathy *et al.,* 2011). However, recent evidence suggests that HCV can also induce Th17 cells, although their role in antiviral host defense still unclear (Balanescu *et al.,* 2012; van de Veerdonk *et al.,* 2009). *In vitro,* stimulation of PBMCs isolated from patients infected with HCV alone produced Th1 cytokine profiles, in contrast to the Th2 predominant cytokines induced in patients co-infected with HCV and *S. mansoni* (El-Kady *et al.,* 2005; Kamal *et al.,* 2001a). Recently, a controversial data was reported about the effect of mono infection (HCV or *Schistosoma*) or co-infection with HCV and *Schistosoma* (Allam *et al.,* 2014; Loffredo-Verde *et al.,* 2015).

In the present study, the effects of co-infection with HCV on the Th1/Th2/Th17 cytokine patterns in *Schistosoma*-infected patients were investigated through measuring 6 cytokines representing surrogate markers of T helper lymphocytes subpopulations. They were IL-12 (for Th1), IL-4 and IL-10 (for Th2), IL-17, IL-22 and IL-23 (for Th17).

Patients and Methods

Patients

A total of 79 participants were enrolled from Al-Qaser El-Ainy University Hospital, Cairo University from Oct 2012- to Oct 2014. Aliquots of 10-15 ml blood were withdrawn from all enrolled subjects. Plasma from 17 (*Schistosoma* infected), 39 (*Schistosoma*/HCV co-infected) and 23 controls were collected. All subjects participated in the study signed written informed consents. The study was approved by the research ethics committee of Cairo University, Egypt. Inclusion criteria of the HCV-infected participants were based on seropositivity for HCV antibodies, HCV RNA genotype 4 as assessed by PCR, elevated aminotransferase levels for 6 months, liver biopsy sample showing evidence of chronic hepatitis, and no current or previous therapy with interferon (IFN) or

ribavirin. Inclusion criteria for *Schistosoma*-infected participants included history of schistosomiasis, detection of *S. mansoni* ova in stool or rectal biopsy sample, and/or seropositivity for schistosomal antibodies (indirect heamagglutination; Femouz laboratories). Exclusion criteria for all the participants were; positive serological markers for hepatitis A, hepatitis B, human immunodeficiency virus (HIV), or other hepatic or intestinal parasites. Autoimmune alcohol- or drug-induced liver disease as well as hepatocellular carcinoma were ruled out. All patients were subjected to a physical examination, and a clinical history was obtained. Plasma samples were stored at -80˚C before analysis. Age and gender data of the enrolled subjects is listed in table (1).

Table 1. Age and gender data of the enrolled subjects.

Schistosoma-infected patients (n= 17)		Co-infected patients (n= 39)		Controls (n= 23)	
Sex	Age ± SD	Sex	Age ± SD	Sex	Age ± SD
Male (n= 14)	52.4±11.9	Male (n= 27)	45.6±7.3	Male (n= 6)	48.8±16
Female (n= 3)	44.6±4.7	Female (n= 12)	44.6±8.9	Female (n= 17)	39.9±13

Methods

- Measuring cytokines levels in the plasma using multiplex assay

Cytokines concentrations in the plasma were determined by enzyme-linked immunosorbent assay (ELISA) using Milliplex^{TM} Multiplex kits (Millipore, Billerica, MA) according to manufacturer's instructions. Briefly, in a 96 well multiscreen filter plate, 25µL sample in duplicate was incubated with 25µL antibody coated beads overnight at 4^{o}C on a plate shaker. Plates were then washed 2 times on a vacuum apparatus and 25µL of secondary antibody was added and incubated at room temperature. After1 hour 25µL of strept-avidin-RPE was added directly to the secondary antibody and incubated for 30 minutes at room temperature with shaking. Plates were then washed 2 more times and 100µL of sheath fluid was added. Plates were shaken for 5 minutes and then read using Bio-Plex (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/ml. The cytokine analysis was conducted in the Cincinnati Children's Research Flow Cytometry Core facility.

Statistical Analysis

Data for clinical and demographic characteristics are presented as mean ± SD. Continuous variables (such as cytokines levels) were compared between the enrolled patients using Student's *t* test, or Wilcoxon Rank Sum Test, as appropriate with a significance value at $P \leq 0.05$. ANOVA test was used to examine the difference between groups of the enrolled subjects. All statistical analyses were completed with the help of Graph Pad Prism 6 Software (San Diego, California, USA).

Results

Table (2) shows the statistical analysis of cytokine levels in *Schistosoma* versus co-infected patients.

Table 2. Statistical analysis of the plasma cytokine levels in *Schistosoma-infected*, co-infected patients, and controls.

	IL - 10	IL - 4	IL - 12	IL - 23	IL - 17	IL - 22
ANOVA	*P*<0.001	*P* =0.035	Ns	Ns	*P*<0.001	Ns
Schisto & controls	*P* =0.008	Ns	Ns	Ns	*P*=0.005	Ns
Schisto & coinf	*P* <0.001	*P* =0.002	Ns	*P* =0.049	*P* <0.001	*P* =0.001
Coinf & controls	*P* =0.007	Ns	Ns	Ns	*P* =0.003	Ns

*Significantly different as compared to controls at $P < 0.05$. NS= not significant

Comparison of the plasma levels of Th1 cytokine (IL-12) in *Schistosoma*-infected and co-infected patients

No significant changes were observed in the plasma levels of Th1 cytokine IL-12 in the plasma from *Schistosoma* infected patients (77.44 ± 35.88 pg/ml, *P*= 0.47) or HCV co-infected patients (136.3± 15.03 pg/ml, *P*= 0.65) compared to the control group (118.7 ± 40.38 pg/ml). In addition, no significant change was observed between that of HCV co-infected patients (*P*=0.08) and *Schistosoma* infected ones (Table 2 and Fig. 1).

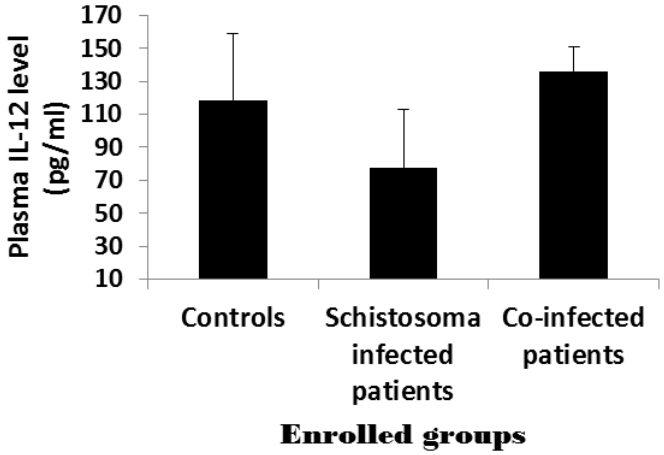

Figure 1. Comparisons of the plasma levels of Th1 cytokine (IL-12) in *Schistosoma*, co-infected patients, and controls. Plasma IL-12 levels were determined by enzyme-linked immunosorbent assay (ELISA) using Milliplex™ Multiplex kits (Millipore, Billerica, MA) as described in material and methods. Concentrations were calculated from standard curves using recombinant IL-12 proteins and expressed in pg/ml. No significant differences were seen between groups.

Comparison of the plasma levels of Th2 cytokines (IL-4 and IL-10) in *Schistosoma*-infected and co-infected patients

As shown in Fig. (2), and Table (2), there was a significant decrease in the plasma levels of Th2 regulatory cytokines IL-10 in *Schistosoma*-infected patients (10.70 ± 2.883 pg/ml, p= 0.009) compared to the control, while a significant increase was observed in that of HCV co-infected patients (38.66 ± 4.663 pg/ml, *P*= 0.007) compared to the control (21.89 ± 2.677 pg/ml).

However, there was no significant change observed in the plasma levels of IL-4 (193.5 ± 55 pg/ml, p= 0.18) of *Schistosoma*-infected patients or HCV co-infected patients (581 ± 74 pg/ml, *P*= 0.28) compared to the control (425.8 ± 133 pg/ml).

A significant increase in the Th2 regulatory cytokines IL-10 (38.7 ± 4.66 pg/ml, p = 0.001) and IL-4 (580 ± 74 pg/ml, p = 0.002) were observed in the plasma from HCV co-infected patients compared to that from *Schistosoma*-mono infected patients (10.7 ± 2.88pg/ml and 190 ± 55pg/ml, respectively), (Table 2).

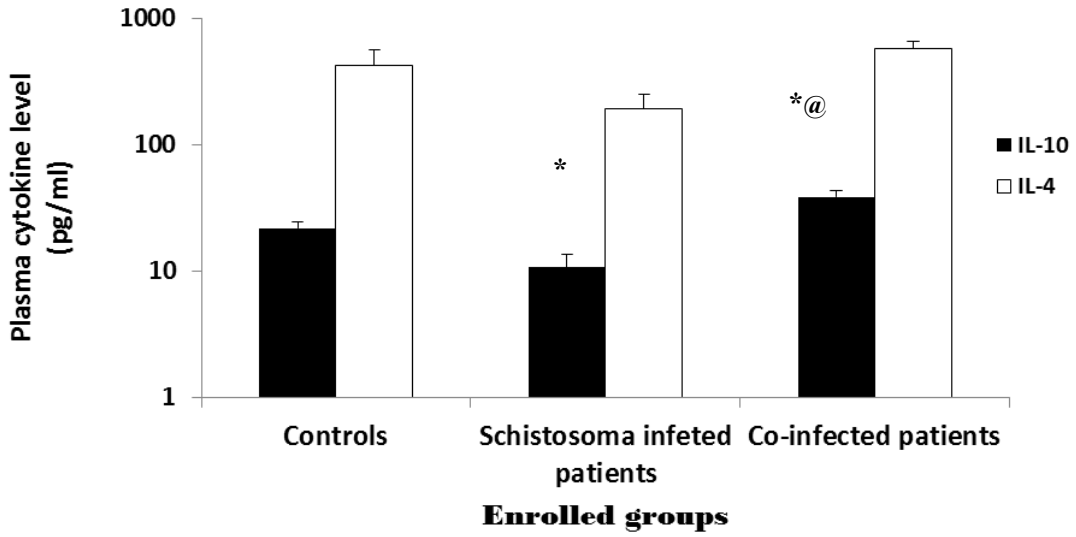

Figure 2. Comparisons of the plasma levels of Th2 cytokines (IL-4 and IL-10) in *Schistosoma*, co-infected patients, and controls. Plasma IL-4, and IL-12 levels were determined by enzyme-linked immunosorbent assay (ELISA) using Milliplex™ Multiplex kits (Millipore, Billerica, MA) as described in material and methods. Concentrations were calculated from standard curves using recombinant IL-4 and IL-10 proteins and expressed in pg/ml. There was a significant increase in the Th2 regulatory cytokines IL-10 (38.7 ± 4.66 pg/ml, *P* <0.001) and IL-4 (580 ± 74 pg/ml, *P* <0.005) in the co-infected group compared to the *Schistosoma*-infected patients (10.7 ± 2.88 and 190 ± 55). *Significantly different as compared to controls at *P* < 0.001; @, Significantly different as compared to *Schistosoma* infected patients at *P* < 0.001

Comparison of the plasma levels of Th17 cytokines (IL-17, IL-22 and IL-23) in *Schistosoma*-infectedand co-infected patients

The level of IL-17 cytokine secreted from Th17 cells showed a significant decrease (24.18±6.384 pg/ml, *P*=0.003) in plasma from *Schistosoma*-infected patients while a significant increase was observed in that of co-infected patients (96.24±11.89 pg/ml, *P*=0.005) in comparison with the control group (52.38±5.889 pg/ml) (Fig 3). No significant change observed in plasma levels of IL-22 (582±133 pg/ml, *P*=0.30) or IL-23 (397±208.8 pg/ml, *P*=0.39) in *Schistosoma*-infected patients compared to that of the control (210.4±117.2 pg/ml, 1932±142.5 pg/ml, respectively). Additionally, there was no significant change in the plasma levels of IL-22 (1445±161 pg/ml, *P*=0.51) and IL-23 (9285±1515 pg/ml, *P*=0.41) in co-infected patients compared to that of the control (2104±172.2 pg/ml, 1932±1425 pg/ml, respectively).

A significant increase in the level of IL-17 (96.24±11.89 pg/ml, *P*<0.001), IL-22 (1440±160pg/ml, *P*=0.001) and IL-23 (9300±1500 pg/ml, *P*=0.05) were observed in plasma from co-infected patients compared to that from *Schistosoma* mono-infected patients (24.2±6.38 pg/ml, 580±133 pg/ml, 400± 200pg/ml, respectively), (Fig 3, and table 2).

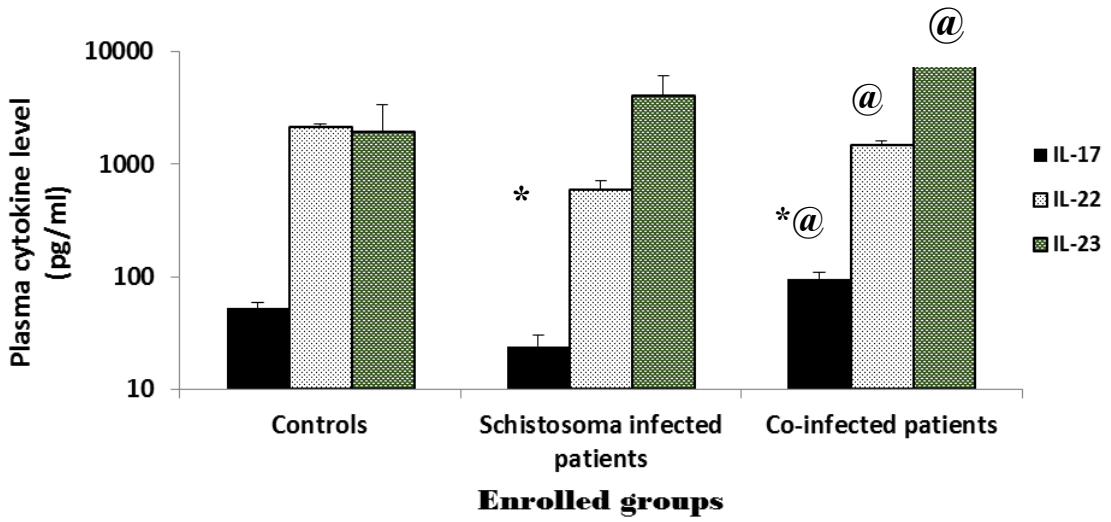

Figure 3. Comparisons of the plasma levels of Th17 cytokines (IL-17, IL-22 and IL-23) in *Schistosoma,* co-infected patients, and controls. Plasma IL-17, IL-22 and IL-23 levels were determined by enzyme-linked immunosorbent assay (ELISA) using Milliplex™ Multiplex kits (Millipore, Billerica, MA) as described in material and methods. Concentrations were calculated from standard curves using recombinant IL-17, IL-22 and IL-23 proteins and expressed in pg/ml. There was a significant increase in the plasma levels of IL-17 (96.24 ± 11.89 pg/ml, p<0.001) and its related Th17 cytokines, IL-22 (1440 ± 160 pg/ml, p <0.005) and IL-23 (9300 ± 1500 pg/ml, *P* = 0.049) in co-infected patients as compared to *Schistosoma*-infected patients (24±6.4 pg/ml, 580 ± 133 pg/ml, 400 ± 200 pg/ml, respectively). *, Significantly different as compared to controls at *P* < 0.001; @, Significantly different as compared to *Schistosoma* infected patients at *P* < 0.05

Discussion

Schistosomiasis is a chronic parasitic infectious disease which is widely endemic in different parts of the world (Chitsulo *et al.,* 2000). *Schistosoma* infection is highly controlled by the immune system through the induction of Th1, Th2 and Treg cells, as well as Th17 cells at different stages of the disease (Estaquier *et al.,* 1997; Malaquias *et al.,* 1997; Mbow *et al.,* 2013; Wen *et al.,* 2011). Patients co-infected with HCV and *Schistosoma* have a worse pathogenesis, with higher incidence of cirrhosis, hepatocellular carcinoma, poor response to IFN based-therapy, and higher mortality compared to patients infected with *Schistosoma* alone (Kamal *et al.,* 2001a). Previous studies investigated the potential mechanisms that explain differences in the clinical outcome during the course of co-infection, as well as the role of HCV-specific $CD4^{+}$T cell response and cytokines induced during HCV infection focusing on Th1/Th2 cytokine patterns (Kamal *et al.,* 2001a; Kamal *et al.,* 2001b). In the current study, the effect of HCV infection on the plasma levels of Th1/Th2/Th17cytokines in *Schistosoma* mono-infected and *Schistosoma/*HCV co-infected patients was compared. Six plasma cytokines representing surrogate markers of T helper lymphocytes subpopulations were measured; including IL-12 (for Th1), IL-4 and IL-10 (for Th2), IL-17, IL-22 and IL-23 (for Th17).

Multiple reports suggested that Th1 responses have protective role in host defense against HCV (Fan *et al.,* 1998; van de Veerdonk *et al.,* 2009). Our results revealed that there was no significant change observed in the plasma level of Th1 IL-12 cytokine in *Schistosoma*-infected patients or HCV co-infected patients compared to that of control subjects. Furthermore, no significant change was observed between plasma IL-12 levels in co-infected patients and *Schistosoma* mono-infected ones. These results are supported by previous reports that indicated that stimulation with HCV antigens produced a Th1 cytokine profile (Hoffmann *et al.,* 2000; Kamal *et al.,* 2001b; Pereira *et al.,* 1995). *Schistosoma* infection may down-regulate the stimulatory effect of HCVon Th1 cytokines and this may lead to chronicity of HCV infection in co-infected patients (El-Kady *et al.,* 2005; Farid *et al.,* 2005). Recent studies reported that schistosomiasis may not affect the outcome of HCV infection in genotype 4-infected patients (Allam *et al.,* 2014), however, *Schistosoma* infection might aggravate HCV-related liver disease through induction of changes in the regulatory T-cell phenotype (Loffredo-Verde *et al.,* 2015).

In the current study, there was a significant increase in the Th2 regulatory cytokines IL-10 and IL-4 in the plasma of HCV co-infected patients compared to that of *Schistosoma*-mono infected ones. The potential synergistic effect of HCV antigens with *Schistosoma* antigens in co-infected patients may be the reason for the increase in Th2/Th0 cytokinesas reported by other studies (Fan *et al.,* 1998; Kamal *et al.,* 2001a; Malaquias *et al.,* 1997). Thus, it is speculated that the increase in Th2 responses during HCV co-infection may be responsible for the increase in the pathogenicity in those *Schistosoma*-HCV co-infected patients.

Th17 responses are critical for mucosal and epithelial host defense against extracellular bacteria and fungi (van de Veerdonk *et al.,* 2009). Recent studies have reported that Th17 responses can also contribute to viral persistence and chronic inflammation associated with parasitic infection (Balanescu *et al.,* 2012; Mbow *et al.,* 2013; van de Veerdonk *et al.,* 2009). The type of microorganisms and the associated microenvironment in which they trigger the Th17 response determine the outcomes of the disease and the balance between Th17

induced protection and immunopathogenesis (van de Veerdonk *et al.,* 2009).

In the current study, the plasma levels of IL-17 cytokine in *Schistosoma* infected patients showed a significant decrease compared to the control group. A critical pathogenic role of IL-17 produced by Th17 cells in murine schistosomiasis had been reported (Rutitzky *et al.,* 2005; Rutitzky *et al.,* 2008). Th17 cells increased in the liver granulomas in murine schistosomiasis and neutralization of IL-17 *in vivo* resulted in significant reduction of hepatic inflammation (Mbow *et al.,* 2013). However, the potential role of Th17 cells in HCV/*Schistosoma* immunopathogenesis in humans is still controversial. In the current study, there was a significant increase in plasma IL-17 level and its related Th17 cytokines, IL-22 and IL-23 in co-infected patients compared to that in *Schistosoma* mono- infected patients. Our results are in accordance with previous studies which demonstrated anincrease in circulating Th17, intrahepatic IL-17 positive cells, as well as HCV-specific Th17 cells. The increase in IL-17 was correlated with severity of liver inflammation in chronic HCV infected patients (Balanescu *et al.,* 2012; Fathy *et al.,* 2011).

In conclusion, our data indicated that HCV infection amplified the Th2/Th17 bias during *Schistosoma* co-infection which may play a role in the progressive pathogenesis of HCV in *Schistosoma* co-infected patients. This study significantly contributes to our understanding of immunity to schistosomiasis and HCV co-infection and may aid in developing intervention tools to protect hosts from infection or restrain the immuno-pathogenesis of co-infection.

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