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Schistosome infection aggravates HCV-related liver disease and induces changes in the regulatory T-cell phenotype

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SUMMARY

*Schistosome infections are renowned for their ability to induce regulatory networks such as regulatory T cells (Treg) that control immune responses against homologous and heterologous antigens such as allergies. However, in the case of co-infections with hepatitis C virus (HCV), schistosomes accentuate disease progression and we hypothesized that expanding schistosome-induced Treg populations change their phenotype and could thereby suppress beneficial anti-HCV responses. We therefore analysed effector T cells and n/iTreg subsets applying the markers Granzyme B (GrzB) and Helios in Egyptian cohorts of HCV mono-infected (HCV), schistosome-co-infected (*Sm*/HCV) and infection-free individuals. Interestingly, viral load and liver transaminases were significantly elevated in* Sm*/HCV individuals when compared to HCV patients. Moreover, overall Treg frequencies and Helios^{pos}Treg were not elevated in* Sm*/HCV individuals, but frequencies of GrzB$^+$Treg were significantly increased. Simultaneously, GrzB$^+$ CD8$^+$ T cells were not suppressed in co-infected individuals. This study demonstrates that in* Sm*/HCV co-infected cohorts, liver disease is aggravated with enhanced virus replication and Treg do not expand but rather change their phenotype with GrzB possibly being a more reliable marker than Helios for iTreg. Therefore, curing concurrent schistosome disease could be an important prerequisite for successful HCV treatment as co-infected individuals respond poorly to interferon therapy.*

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Abbreviations: GrzB, Granzyme B; HCV, hepatitis C virus; HD, healthy donors; *S. mansoni*, *Schistosoma mansoni*; SEA, soluble egg antigen; *Sm*/HCV, *Schistosoma mansoni*/HCV co-infection; Treg, Regulatory T cells

INTRODUCTION

Hepatitis C virus (HCV) is the second most common viral infection worldwide and considered the most important agent of liver disease and liver carcinoma. In general, approximately 70% of acute HCV cases develop chronic hepatitis of which 15–20% will eventually evolve into cirrhosis with a 10% risk of hepatocellular carcinoma (1). Currently, it is estimated that 180 million people are infected with HCV, and, interestingly, Egypt has the highest prevalence worldwide (31%) with 90% of those infected individuals being positive for the genotype 4 variant (2–5). HCV infections induce both CD4$^+$ and CD8$^+$ T-cell responses, and during acute infection, several studies have demonstrated that strong and sustained viral-specific CD4$^+$ and CD8$^+$ T-cell responses are required for spontaneous and successful viral clearance. Individuals who fail to mount or sustain such viral-specific responses usually develop persistent viraemia and chronic infection (6). Although the underlying mechanisms of dysfunctional virus-specific immune responses remain poorly understood, research has shown that chronic HCV-infected individuals present higher levels of peripheral CD4$^+$CD25$^+$ Foxp3$^+$ regulatory T cells (Treg) which are able to suppress virus-specific CD8$^+$ T-cell responses (7, 8). The high prevalence of HCV infection in Egypt stems from an

iatrogenic epidemic that was introduced in the 1950s due to mass treatment with antimony against schistosomiasis (9). Since then, concomitant human schistosomiasis and HCV infection are extremely common, especially in rural areas of Egypt (10, 11) and co-infected individuals present an accelerated onset of liver cirrhosis and hepatocellular carcinoma (12). Indeed, whereas 30% of HCV mono-infected individuals recovered from acute infections, all those with an additional *Schistosoma mansoni*-infection progressed to a chronic state (13). Moreover, compared to HCV mono-infected individuals, these patients exhibited higher HCV-positive RNA titres, higher necro-inflammatory and fibrotic scores in the liver and poor responses to interferon therapy (10, 14).

Schistosomiasis, caused by the helminth *Schistosoma*, remains one of the most important parasitic diseases worldwide, and current monitoring indicates that over 250 million individuals are infected (15, 16). In endemic areas, these infections have a detrimental impact on both financial and social sectors (17). *S. mansoni* and *S. haematobium* are endemic in many rural areas of Egypt, and community prevalence often ranges between 15 and 45% (18). Immunologically, one of the most interesting aspects of schistosome infection is the modulation of the immune system including a dynamic switch from an initial pro-inflammatory Th1 to a dominant helminth-beneficial Th2 immune response once female worms become fecund. As infection enters the chronic phase, a more regulated equilibrium ensues with high levels of anti-inflammatory cytokines (IL-10 and TGF-β) and elevated frequencies of regulatory T-cells (Treg) (19–22). In association, disease severity is partially controlled by the balance of Th1 versus Th2 type cytokines and/or the presence of Treg (19, 20, 23). We and others have shown the essential role of Treg using the murine model of *S. mansoni* and although Treg cannot prevent helminth infection per se, their necessity in controlling exaggerated immunopathology and effector T-cell responses has been well documented (23–25). Indeed, those studies demonstrated that isolated Treg, from infected but not naive mice, could specifically suppress schistosome-specific $CD4^+$ T-cell responses in an IL-10 independent manner and moreover changed their phenotype during infection (22, 24, 25). Specifically, schistosome-induced Treg acquired cytotoxic T-cell activity as Granzyme B (GrzB) was strongly upregulated (25).

Currently, no data are available whether there are characteristic changes from nTreg (naturally occurring, thymus derived) to iTreg (peripherally induced) during human schistosomiasis. Therefore, we investigated changes in the phenotype of $Foxp3^+$ Treg, during human schistosomiasis, which could be responsible for the reported failure to raise effective antiviral $CD4^+$ and $CD8^+$ T-cell responses (7, 8, 26). In turn, this would lead to loss of viral replication control and eventually to more pronounced liver disease. Thus, we analysed liver function and viral load as well as Treg T effector cell (Teff) frequencies and specifically the nTreg/iTreg distribution using the novel markers Helios and GrzB within patients with HCV mono-infection and those with concomitant *Schistosoma* infection.

MATERIALS AND METHODS

Study subjects and collection of blood samples

In collaboration with the Hepatology Outpatient Clinic of Cairo University Hospital in Egypt in 2009, chronic hepatitis C patients were recruited and further classified into HCV mono-infected (n = 15–HCV) or co-infected with *S. mansoni* (n = 16–*Sm*/HCV). Schistosome infection was diagnosed on the presence of *S. mansoni* eggs within the stool with the Kato-Katz method and/or the presence of high titres of schistosomal antibodies (>1/640) against adult worms (Fumouze Diagnostics, Levallois-Perret, France). The study protocol was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki, and ethical clearance was given by the Scientific Research Ethics Committee at the Faculty of Medicine, Cairo University in Egypt and by the Ethics Commission of the Faculty of Medicine at the Technischen Universität München (TUM) in Germany. Patients exhibiting other viral hepatic infections, hepatic cirrhosis, prolonged partial prothrombin time (PPT) and hepatocellular carcinoma were excluded, as well as other intestinal parasitic infections by sodium-acetate-formalin (SAF) enrichment method and microscopy. As the areas of patient recruitment in Egypt are considered malaria free, individuals were not tested for malaria. Peripheral blood mononuclear cells (PBMC) were isolated immediately after the collection of fresh heparinized blood using Ficoll-Hypaque density gradient centrifugation. Cell samples were subsequently cryopreserved in 10% DMSO (dimethyl sulphoxide) and 90% foetal calf serum (Sigma Chemicals, Hamburg, Germany; PAA Laboratories Inc., Cölbe, Germany) at −80°C and shipped to Germany on dry ice. HCV viral loads in plasma were quantified using real-time PCR (27). Further blood analysis of all study participants included routine laboratory tests such as liver enzymes AST, ALT and AFP (Reflovet Plus Reader, Roche, Penzberg, Germany), kidney function and coagulation tests, and a blood count. For comparison, further samples were collected from age-matched, infection-free volunteers, which were mainly relatives of the patient cohort and resided therefore in the same endemic area (n = 13). They are denoted in this study as ‘healthy donors’ (HD).

Flowcytometry cell staining

Phenotypic and functional characteristics of regulatory and effector T-cell subsets were investigated using multicolour surface and intracellular stainings based on flow cytometry technology. The following staining panel was designed in order to characterize Treg and T effector cells: CD8 AmCyAn, CD127 PE, Granzyme B FITC (Becton Dickinson, Heidelberg, Germany), CD3 APC-A750, CD25 PE-Cy7 (Beckmann Coulter, Krefeld, Germany), CD103 PE-Cy5, Foxp3 PB, Helios FITC (BioLegend, San Diego, CA, USA) and CD4 A700 (Affymetrix, Vienna, Austria). Viability was assessed by addition of ethidium monoazide ([EMA], Molecular Probes/Invitrogen, Karlsruhe, Germany) and did not differ between patient groups (average mean of viable cells in % ± SD: HD 76·6% ± 11·7%; *Sm*/HCV: 77·3% ± 14·1%; HCV: 74·0% ± 16·2%). In short, following surface staining, PBMCs were fixed and permeabilized for 20 min at 4°C in 100 μL fixation/permeabilization working solution using the Foxp3 staining buffer set from eBioscience (Affymetrix). After washing, cells were resuspended in permeabilization buffer containing anti-human antibodies specific to intracellular and intranuclear proteins for 30 min at 4°C. Cell acquisition was performed using the LSRII flow cytometer (Becton Dickinson) equipped with a high-throughput system. Sample analysis was performed using FlowJo version 9.5.3 (Tree Star, Ashland, OR, USA).

Serum cytokine detection

Levels of IL-6 and IL-8 were measured in individual plasma samples using the human Th1/Th2/Th8/Th17/Th22 13plex Kit FlowCytomix according to manufacturer's instructions (Affymetrix). Acquisition of the multiplex immunoassay was performed using the LSRII flow cytometer. Sample analysis was performed with Flowcytomix™ Pro 3.0 Software (Affymetrix).

Statistical analysis

All statistical tests were performed with PRISM® 5.01 (GraphPad Software Inc., San Diego, CA, USA). D'Agostino and Pearson omnibus normality tests were performed, and parametrically distributed data were analysed with unpaired *t*-test (2 groups) and Mann–Whitney U-test was used for nonparametric data. For more than two groups, 1-way ANOVA tests was conducted and if data were nonparametric, a Kruskal–Wallis test with a confidence interval of 95% was employed. Results with a P value of $<0{\cdot}05$ were considered as significant and P values $<0{\cdot}01$ as highly significant.

RESULTS

Co-infected patients present higher viral load and stronger liver pathology

Recruitment of patients was based on the following parameters: viral titres, liver transaminases levels, ultrasonography and schistosoma infection. Within the three different groups, the base-line characteristics such as age, gender and body mass index (BMI) were similar (Table 1). Basic haematological parameters such as complete blood count, coagulation rates and partial thromboplastin time (PTT) were all comparable (data not shown). *S. mansoni* eggs and specific antibodies were only detected in the *Sm*/HCV groups (Table 1). The percentage of lymphocytes was also comparable between the groups (Table 1). Liver inflammation was assessed by measurement of total bilirubin, alpha fetoprotein (AFP) and liver transaminases (AST/ALT) in plasma. Whereas levels of the total bilirubin and AFP did not significantly differ between the groups (Table 1), both ALT (Figure 1a) and AST (Figure 1b) levels were significantly higher in the *Sm*/HCV group. Furthermore, HCV-RNA titres were also significantly higher in the co-infected patient group (Figure 1c) indicating that this cohort had an aggravated disease state.

Table 1 Baseline characteristics of healthy donors, *Schistosoma mansoni*/HCV co-infected and HCV mono-infected patients

	Healthy donors (HD)	*S. mansoni*-HCV co-infected patients (*Sm*/HCV)	HCV mono-infected patients (HCV)
Number (Male: Female)	13 (8:5)	16 (10:6)	15 (8:7)
Age, years	37·0 ± 8·9	40·5 ± 7·9	40·5 ± 9·7
Body mass index	25·5 ± 3·9	26·7 ± 2·9	26·6 ± 3·6
Total Bilirubin	0·7 ± 0·2	0·8 ± 0·4	0·7 ± 0·2
AFP (U/mL)	n.d.	5·2 ± 5·2	6·7 ± 3·8
Lymphocytes (%)	40·7 ± 8·7	44·7 ± 10·1	51·4 ± 14·6
Stool (Kato-Katz)	0/13	8/16	0/15
Schistosome mAb	0/13	16/16	0/15

Gender, age and body mass index (BMI) of healthy donors (HD, $n = 13$), *S. mansoni* and HCV co-infected (*Sm*/HCV, $n = 16$) and HCV mono-infected (HCV, $n = 15$) are shown. Liver damage was evaluated by measuring levels of total bilirubin and alpha fetoprotein (AFP). Schistosome infection was diagnosed by counting *S. mansoni* eggs/g stool and the detection of antischistosomal antibodies (schistosome mAb) within plasma. Percentages of total leucocytes are indicated for each patient as lymphocytes %. Data are shown as mean ± SD; n.d., not determined.

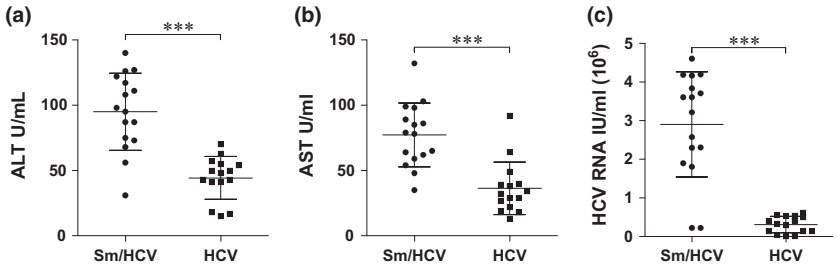

Figure 1 Elevated HCV-RNA and liver transaminases (ALT/AST) in the plasma of *Schistosoma*/HCV-co-infected patients. To determine the extent of liver damage between HCV mono-infected and *S. mansoni*/HCV (*Sm*/HCV) co-infected individuals, liver transaminases (a) ALT and (b) AST were measured in plasma. (c) HCV-RNA levels were ascertained by real-time PCR. Symbols show individual measurements within the patient groups and graphs show mean ± SD. Asterisks show statistical differences (Student's *t*-test (c) or Mann–Whitney U-test (a, b)) between the groups indicated by the brackets (***$P < 0{\cdot}001$).

Co-infected patients present higher levels of circulating IL-8 when compared to mono-infected individuals

The cytokine milieu within plasma was analysed using a multiplex cytokine assay. (Figure 2a) shows that in comparison to HD, *Sm*/HCV co-infected individuals had significantly higher levels of circulating IL-8. No differences between the two HCV-infected groups could be determined. Some HCV-infected individuals did, however, present high amounts of IL-6 (Figure 2b). No differences were found in IL-12p70, IFN-γ, IL-17A, IL-2, IL-10, IL-22, IL-13, IL-4, IL-1β, TNFα and IL-5 (data not shown).

Frequency of effector CD4$^+$ and CD8$^+$ T cells is increased in infected patients, whereas the distribution of GrzB-producing CD4$^+$ and CD8$^+$ effector T cells is not altered

To analyse possible alterations within effector T-cell populations, PBMCs from individuals in all three groups were analysed using a 9-colour ICS assay Teff/Treg panel. As effector T cells loose CD127 expression upon activation and do not express the Treg-specific transcription factor Foxp3, frequencies of total CD4$^+$ and CD8$^+$ T-cell populations were defined using the marker combination CD4$^+$CD127^{neg}Foxp3$^-$ and CD8$^+$CD127^{neg}Foxp3$^-$ (28–30). As shown in (Figure 3(a and b), the frequency of CD4$^+$CD127^{neg}Foxp3$^-$ and CD8$^+$CD127^{neg}Foxp3$^-$ T effector cells was significantly higher in both HCV-infected cohorts when compared to HD. Viral infections are known to induce both CD4$^+$ and CD8$^+$ immune responses, and as the majority of CD8$^+$ effector T cells produce the serine protease Granzyme B, the prevalence of these effector T-cell populations was further assessed by staining GrzB intracellularly (Figure 3c,d). Frequencies of GrzB-producing CD8$^+$CD127^{neg}Foxp3$^-$ and CD4$^+$CD127^{neg}Foxp3$^-$ effector T cells were not altered within the different groups (Figure 3c,d, respectively).

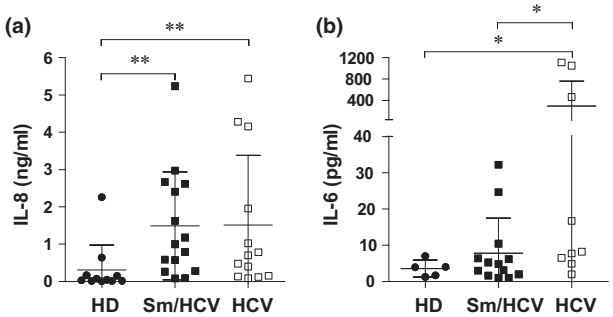

Figure 2 Co-infected patients produce more IL-8 than HCV mono-infected individuals. (a) IL-8 and (b) IL-6 levels in individual infection-free volunteers (HD), HCV and *Sm*/HCV were analysed in the plasma with a multiplex cytokine assay. Symbols show individual data and graphs depict mean ± SEM. Statistical significances between the indicated groups were obtained after Kruskal–Wallis test and Mann–Whitney U-test (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$).

nTreg/iTreg ratio is altered in HCV-infected patients but only Sm/HCV co-infected individuals display increased frequencies of GrzB-producing CD4$^+$CD127^{lo}Foxp3$^+$ Treg

As we have shown previously that Foxp3$^+$ Treg are elevated during murine *S. mansoni* infection (24, 31) and moreover that GrzB is upregulated in Treg from schistosome-infected mice, we investigated whether this is the case in human schistosomiasis as well. To characterize Treg in PBMCs from all three groups of individuals, we defined this cell subset using the marker combination: CD4$^+$ CD127^{lo} Foxp3$^+$. The Treg phenotype was then expanded upon by additionally analysing intracellular expression of GrzB or Helios which allows the discrimination between nTreg and infection-induced iTreg (32). As shown in (Figure 4a), the frequency of CD4$^+$CD127^{lo}Foxp3$^+$ Treg was comparable between the three groups. However, upon further characterization of the Treg phenotype using the novel marker

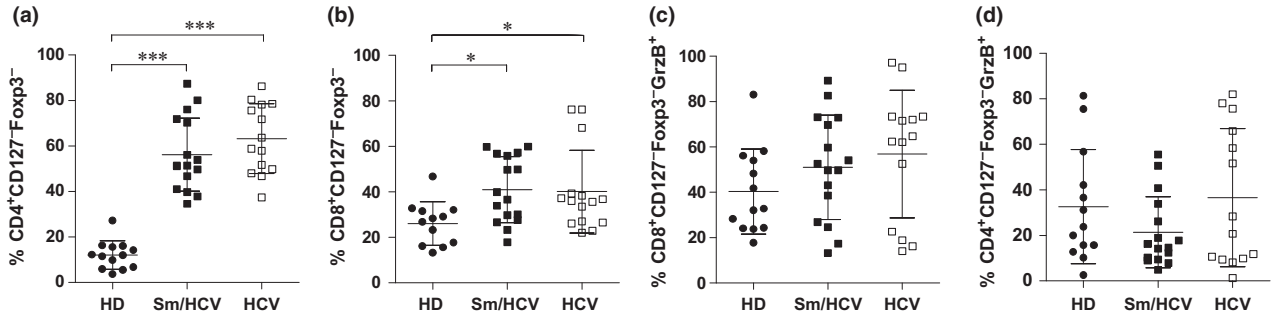

Figure 3 Frequency of effector $CD4^+$ T and $CD8^+$ T cells is increased within HCV-infected patients. Frequencies of T-cell subsets were analysed within PBMCs using a 9-colour ICS assay 'Teff' panel. Frequency of (a) $CD4^+CD127^-Foxp3^-$ T cells and (b) $CD8^+CD127^-Foxp3^-$ T cells in all three groups. The function of effector T-cell populations was further assessed by determining the frequencies of (c) $CD8^+CD127^-Foxp3^-GrzB^+$ and (d) $CD4^+CD127^-Foxp3^-GrzB^+$ T cells. Symbols show individual T-cell frequencies, and graphs show data as mean ± SD. Statistical significances between the indicated groups were calculated using ANOVA (a, b, c) and Kruskal–Wallis test (d) (*$P < 0{\cdot}05$, ***$P < 0{\cdot}001$).

Helios, we detected significantly higher frequencies of $CD4^+CD127^{lo}Foxp3^+Helios^{pos}$ Treg within the HCV mono-infected group when compared to HD (Figure 4b). This phenotype was further reflected in the frequency of $CD4^+CD127^{lo}Foxp3^+Helios^{neg}$ cells as this population was significantly reduced in the HCV mono-infected group compared to HD individuals (Figure 4c). However, levels of $CD4^+CD127^{lo}Foxp3^+GrzB$-producing Treg within the *Sm*/HCV group were significantly increased, when compared to both the HCV mono-infected individuals and HD (Figure 4d).

DISCUSSION

Helminths have co-evolutionally adopted several mechanisms to evade host immune responses as they rely on their host's survival for a successful life cycle. In schistosomiasis or Bilharzia, it has been shown that for the parasite, a balanced immune response by the host is more acceptable than no immune response at all. This balance or even 'tolerance' is maintained by strong immune regulatory processes such as regulatory T cells induced during the chronic infection. We have previously shown that during schistosome infection, Tregs expand and change their phenotype acquiring stronger suppressive properties and expressing molecules like cytotoxic GrzB (24, 31, 33). However, little remains known about the impact of schistosome infection on other chronic diseases in humans. The central aim in this study was therefore to determine whether schistosome-induced immunomodulation could have an impact on the severity and course of another chronic liver disease, hepatitis C virus infection and whether this is associated with functional effector T cell and nTreg/iTreg changes.

Within our study, we found that HCV patients with schistosoma co-infection had significantly higher liver

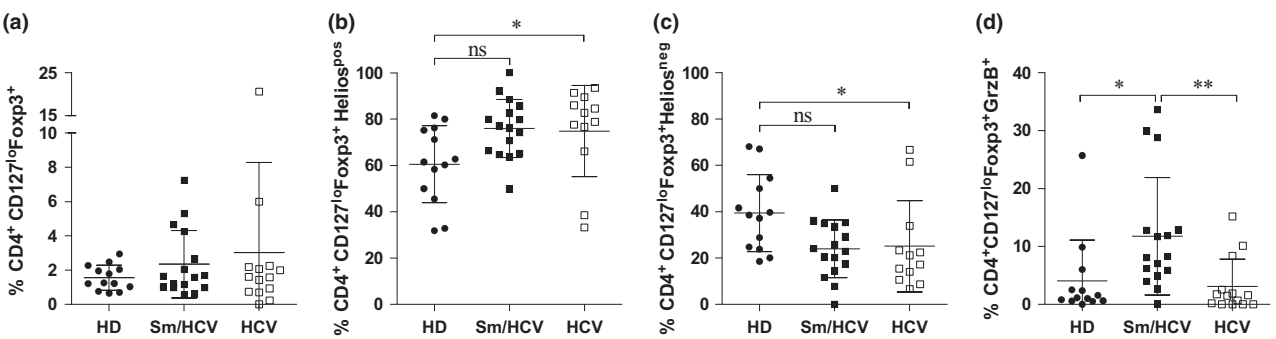

Figure 4 Pronounced increase of $CD4^+CD127^{lo}Foxp3^+GrzB^+$ Treg in *Sm*/HCV-infected individuals. (a) The frequency of $CD4^+CD127^{lo}Foxp3^+$ Treg was determined in all three groups. Treg subsets were then further differentiated into $CD4^+CD127^{lo}Foxp3^+Helios^{pos}$ (b) and $CD4^+CD127^{lo}Foxp3^+Helios^{neg}$ (c). The Treg phenotype was further analysed using the marker combination $CD4^+CD127^{lo}Foxp3^+GrzB^+$ within PBMCs of individual donors (d). Symbols show T-cell frequencies of each individual, and data are displayed as mean ± SD. Statistical significances between the indicated groups were obtained after Kruskal–Wallis test and Mann–Whitney U-test (*$P < 0{\cdot}05$ and **$P < 0{\cdot}01$).

transaminases levels as well as elevated HCV-RNA titres, indicating that concomitant schistosome infection indeed aggravates liver disease and inhibits virus elimination. As previous studies revealed that ALT and AST levels in endemic normals and *S. mansoni*-mono-infected individuals were comparable (El-Kady *et al.*, 2005), our current findings substantiate other studies performed in Egypt demonstrating aggravated liver disease and less controlled viral replication in schistosome-co-infected HCV patients (10, 12, 34, 35). Viral clearance is known to correlate with strong and sustained virus-specific $CD4^+$ and $CD8^+$ T-cell responses during acute HBV infection (36), and chronicity develops in such individuals who fail to mount or sustain such responses (6). Mechanisms responsible for such dysfunctional HCV-specific $CD8^+$ T cells are still not understood, and several explanations have been proposed, including the lack of different $CD4^+$ helper T-cell functions or the suppressive activity of Treg (37). Indeed, chronic HCV infection *per se* can lead to the expansion of $CD4^+CD25^+Foxp3^+$ T cells that reportedly suppress $CD8^+$ T-cell responses to different viral antigens (7, 8, 36, 38). As liver biopsies from chronic HCV-infected individuals showed an influx of Treg, it has been suggested that such cells aid in controlling hepatic immune responses (39). In association, Bolacchi *et al.* detected peripheral $CD4^+CD25^{hi}$ Treg that secreted TGF-β in response to HCV peptides, and in turn, this was inversely correlated to liver inflammation (26). Concerning the expansion or even the change of phenotype of Treg during human schistosomiasis, controversial data have been put forward in contrast to the consistent findings in mice (24, 33, 40, 41). When compared to both HCV mono-infected and HD, the frequency of $CD4^+CD127^{lo}Foxp3^+$ Tregs in this study was not elevated in the schistosome co-infected group. However, we did find a higher frequency of GrzB-positive Treg within the *Sm*/HCV group, which confirmed our previous findings in *S. mansoni*-infected mice where GrzB was upregulated in schistosome-induced Treg which simultaneously had an enhanced suppressive capacity on antigen-specific effector T cells (24, 25). Therefore, a potential regulatory scenario is that schistosome-induced Treg have an influence on the effector T-cell populations by acting directly on virus-specific effector T cells via GrzB. As this study revealed no correlations between GrzB-producing Treg and effector T-cell populations, future studies should concentrate on elucidating possible interactions between $GrzB^+$Treg and distinct effector T-cell subsets that have phenotypes unique in their expression of cytokines (IFN-γ, IL-2) or activation markers (CD44, CD69).

In our study, we further analysed the phenotype of effector $CD4^+$ and $CD8^+$ T cells using GrzB as a functional marker. Even though there were significantly increased frequencies of $CD4^+$ and $CD8^+$ effector T cells within *Sm*/HCV and HCV mono-infected patients, frequencies of GrzB-secreting $CD8^+$ and $CD4^+$ effector T cells were not altered. This could be due to the fact that all HCV patients were chronically infected and therefore lacked appropriate cytotoxic effector responses as described before (6, 36). As GrzB expression is not the only hallmark of $CD8^+$ and $CD4^+$ T-cell activation, future studies should for example aim at deciphering whether viral-specific $CD8^+$ and $CD4^+$ T-cell responses are modulated in co-infected individuals by using an HCV genotype 4a peptide library. Such findings would expand on previous studies that have demonstrated that co-infected patients exhibit either completely absent or transiently weak HCV-specific IFN-γ secretion by $CD4^+$ T cells (42). In addition, all HCV-infected patients had significantly elevated frequencies of total $CD4^+$ and $CD8^+$ effector T cells, whereas viral loads were only elevated in the *Sm*/HCV group. This suggests that the effector $CD4^+$ and $CD8^+$ T-cell populations might not act properly on viral clearance and might be somehow influenced by the schistosome co-infection.

Another major finding within this study was the characterization and distribution of nTreg and iTreg within the Treg populations of the different groups. To discriminate between these Treg subsets, we employed the novel marker Helios, a transcription factor of the Ikaros family of transcription factors. This marker is currently proposed to be selectively expressed on nTreg and can therefore be used to discriminate between nTreg and iTreg (32, 43). We found significantly higher frequencies of $Helios^{pos}$nTreg and decreased percentages of $Helios^{neg}$iTreg within the HCV mono-infected group, when compared to healthy individuals. Although a tendency towards higher frequencies of $Helios^{pos}$nTreg was observed within the *Sm*/HCV co-infected group compared to healthy controls, no significant differences could be determined, possibly due to the high variation within the group itself. These data suggest that nTreg might increase during HCV infection, which is somewhat counterintuitive, and indeed, recently the selectiveness of Helios is being controversially discussed as it was also shown to be a marker of T-cell activation and proliferation and was further detected in human and murine effector $CD4^+$ and $CD8^+$ T cells (32, 43, 44). More specifically, Akimova *et al.* (43) reported that Helios expression within human and murine effector T cells regresses under resting conditions, whereas constantly high expression of the transcription factor could be detected within nTreg. In addition, Gottschalk *et al.* generated iTreg from naïve murine $CD4^+$ T cells *in vitro* and *in vivo* using different stimulatory conditions and demonstrated

that these iTreg only expressed Helios after being activated by antigen-presenting cells (APCs) and downregulated Helios expression under resting conditions. In contrast, nTreg constantly expressed the transcription factor Helios. iTreg generated without APCs but in the presence of TGF-β and TCR stimulation showed no Helios expression (44). The study clearly demonstrates that Helios expression within iTreg is derived from an APC-dependent stimulus. In this regard, our findings of higher levels of Helios^{pos} Treg might not only pertain to the nTreg compartment as *in vivo* many iTreg will have been generated upon APC contact. We therefore believe that Helios might be a better marker to detect activated iTreg in humans rather than as a discrimination tool to dissect nTreg from iTreg. In this context, it will be interesting to investigate the phenotype of schistosome-induced 'Helios^{pos}' iTreg' and moreover whether such cells simultaneously upregulate GrzB as well.

Previous studies have shown that HCV/*S. mansoni*-infected individuals have enhanced pathology when compared to *S. mansoni*-infected patients. Moreover, these studies have associated this pathology with differences in immune profiles of those groups including Treg numbers (10, 12, 13, 34, 35). Expanding on those findings, we focused our study here on differences between HCV and HCV/*S. mansoni*-infected individuals and deciphered that Treg have a distinct immune profile: elevated frequencies of $CD4^+CD127^{lo}Foxp3^+GrzB^+$ Treg. In conclusion, this study provides additional knowledge on the broad influence that chronic helminthic infections have on immune responses to unrelated antigens such as HCV. As with their ability to suppress autoimmune diseases, this phenomenon probably stems from the nature of their general immunosuppressive features that arise due to the expansion and/or induction of regulatory populations such as Tregs (40, 45–48). As in this co-infection scenario there is a detrimental effect on the patient's response to antiviral regimes, it will be important to study these immune responses after helminth treatment as this might improve the outcome of HCV therapy.

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DISCLOSURES

None of the authors have a conflict of interest.

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