Th17 Cytokines Modulates Neutrophil Response to Co-Infection with *Schistosoma* SEA and HCV Core Protein

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**Abstract**—This study aimed to investigate the role of neutrophils in *Schistosoma/HCV* co-infection in the presence of Th17 cytokines (IL-17 and IL-22). In *in vitro* studies on the effects of SEA antigens of *Schistosoma* and HCV core protein on healthy neutrophil functions and *in vivo* studies on effects of co-infection on plasma IL-17 and IL-22 levels as well as neutrophil mediators; TNF-α and H2O2 levels (using ELISA) were included. A significant decrease in TNF-α production and increase in H2O2 production by (SEA+core) stimulated neutrophils comparing to SEA or core protein was observed. IL-17 significantly increased TNF-α but not hydrogen peroxide. IL-22 significantly increased TNF-α and decreased H2O2. Significant increases in TNF-α and H2O2 levels in coinfected plasma. IL-17 levels were significantly higher and IL-22 level was significantly lower in coinfected plasma comparing to *Schistosoma* infected plasma. Conclusively, IL-17 and IL-22 have differential effects on Neutrophil response to co-infection with *Schistosoma* and HCV.

**Keywords**—Co-infection, Hepatitis C virus, *Schistosoma*, Th17 cytokines.

**LIST OF ABBREVIATIONS IN THE ORDER OF APPEARANCE**

- Soluble egg antigen (SEA)
- Hepatitis C virus (HCV)

**I. INTRODUCTION**

**SCHISTOSOMIASIS** affects almost 240 million people worldwide. The infection is prevalent in tropical and subtropical areas, mostly in poor communities without potable water and adequate sanitation. Egypt is one of the highest endemic areas of schistosomiasis. Additionally, among *Schistosoma* infected populations, hepatitis C virus (HCV) co-infected 20-50% of *Schistosoma*-infected patients in Egypt [1]-[6]. The association between HCV and schistosomiasis in Egypt is due to the mass parenteral anti-Schistosoma treatment [7]. Immunological state and HCV co-infections are important factors for the diverse clinical patterns observed for schistosomiasis [8]-[10].

Patients coinfected with HCV and schistosomiasis exhibit a unique clinical, virological and histological pattern manifested by high HCV-RNA titres, as well as higher levels of inflammation in the liver [11], [12]. Dual infections are associated with significant changes on the host immune responses including cytokine shift pattern alteration, cytotoxic T-lymphocyte response and other impaired immunologic functions with diminished capacity to clear the virus [13], [14]. The interactions between the two infections are complicated but several studies reported that Th17 cells and their products like IL-17, and IL-22 play an important role in the immunopathogenesis of schistosomiasis [11], [12], [15]-[18]. Additionally, recent studies reported that in animal models, lack of IL-17A signaling lead to an ineffective neutrophil response to infections with bacteria, fungi or parasites with ultimate decrease in animal survival [19]-[21]. The aim of this study was to investigate the role of neutrophils in case of *Schistosoma/HCV* co-infection focusing on the HCV core protein and SEA antigens. The relationship between cytokines patterns in the plasma and effect of the HCV core and SEA proteins on neutrophils functions were examined.

This study included two main parts; *in vitro* studies on the effects of SEA antigens of *Schistosoma* and HCV core antigens on neutrophil functions in the presence of Th17 cytokines (IL-17 and IL-22), and *in vivo* studies on the effects of co-infection on the Th17 plasma cytokines levels as well as TNF-α and H2O2 levels as functional mediators of neutrophil activation.

**II. MATERIALS AND METHODS**

**A. In Vivo Study**

Three groups of patients were enrolled (Table 1): Group 1 included 18 patients infected with schistosoma alone, Group 2 included 50 patients coinfected with chronic HCV and schistosomiasis, and Group 3 included 17 control healthy individuals. Plasma were collected and kept frozen at -20°C till performing quantitative ELISA for different cytokines (IL-17, IL-22 and TNF-A). The study was approved by the research ethics committee of Cairo University. All participants gave consent.
inform informed consent before participation in the study and their demographic data were recorded.

B. In Vitro Study

Isolation of neutrophils from whole blood: Fifteen ml of fresh blood was withdrawn from healthy consenting adults. Neutrophils were isolated from the EDTA anti-coagulated venous blood as described previously [22] with some modifications. Briefly, the blood was centrifuged with Ficol Hypaque (Amersham Pharmacia, Piscataway, NJ) density gradient at 400 x g for 25 min to remove the mononuclear cells. In the cell sediments RBCs were lysed using ACK lysis buffer (Sigma, St. Louis, MO). Resulting neutrophils preparations were >98% pure as assessed by flow cytometry and more than 95% of the neutrophils were viable as measured by trypan blue. Purified neutrophils were washed and re-suspended in DMEM media (Sigma, St. Louis, MO) at the proper concentration.

In vitro activation of neutrophils: Schistosoma SEA (Theodore Bilharz Research institute, Giza, Egypt) and LPS-free HCV-related core protein (ViroGen Corp, USA) were used at the optimum concentrations which were 3 µg/ml, and 100 µg/ml for the HCV core protein and Schistosoma SEA, respectively. Additionally, the concentrations of IL-17 and IL-22 (R & D Systems, Inc.) were optimized and used at 10 ng/ml and 100 ng/ml respectively.

Measurement of TNF-α production by activated neutrophils using ELISPOT assay: An enzyme-linked immunospot (ELISPOT) assay was performed with TNF-α ELISPOT kit (catalog no. 3510-2H; Mabtech, Sweden) according to the manufacturer’s instructions. Briefly, 96-well nitrocellulose-bottomed plates (Millipore, Bedford, MA) were coated with murine anti-human TNF-α MAb at a concentration of 10 µg/ml in PBS and incubated at 4°C. After 24 h, the plates were washed and blocked with RPMI with 10% fetal bovine serum (FBS). Then, neutrophils were added at a concentration of 10⁵ cells/well in a 100 µl volume of complete medium (RPMI 1640 containing 10% FBS). For stimulation, Schistosoma SEA and LPS-free HCV-related core protein were added according to the experiment protocol. After an 18-h incubation at 37°C and 5% CO₂, the plates were washed 5 times with washing buffer (PBS containing 0.5% Tween 20 (Sigma, St. Louis, MO) using an automatic plate washer (Bio Tec Instruments, Inc.). Biotinylated anti-human TNF-α MAb (clone TNF5-biotin) was added at a concentration of 1 µg/ml in dilution buffer (PBS containing 0.05% FBS), and the plates were incubated at room temperature for 2 h. Plates were then washed again five times, and streptavidin-horseradish peroxidase (1:100) in dilution buffer was added. The plates were incubated at room temperature for 1 h. After, five washes with washing buffer, tetramethylbenzidine (TMB) substrate (MabTech) was added. After the spots were developed in 10 to 15 min, the plates were washed with distilled water and air dried. The number of spots was enumerated using an automated ELISPOT 3B analyzer (CTL, Cleveland, OH) [23].

Assessment of H₂O₂ generation by activated neutrophils using flow cytometry: H₂O₂ generation by stimulated neutrophils was measured using dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich) as previously described [24]. Briefly, isolated Neutrophils (1x 10⁶/well) were placed in a 96-well microplate and incubated with the stimuli or controls in a humidified atmosphere of 5% CO₂ at 37°C. After 24 h incubation, 20 µM/ml of DCF-DA was added as intracellular reactive oxygen species marker for 20-30 min. Then, cells were washed by PBS and fixed with 1% paraformaldehyde. Before analysis, cells were washed with PBS and resuspended in PBS. The number of positive cells was measured using flow cytometry (BD) as described previously [24].

Quantitative measurement of plasma cytokines levels and H₂O₂: TNF-α, IL-17 and IL-22 were measured using Human ELISA kits (Booster Immunoleader, USA) according to the manufacturer’s instructions. H₂O₂ was measured using colorimetric method (Bio-diagnostic, Egypt) as described [25].

Statistical Analysis: Statistical analysis was performed using paired Student’s t test and Graph Pad Prism software. Results with a p value < 0.05 were considered significant.

III. RESULTS

A. In Vitro Results on The Effect of Co-Infection:

As shown in Fig (1A), SEA or core protein induced a significant decrease in the production of TNF-α compared to that produced by non-activated neutrophils. Similarly, there was a marked decrease in TNF-α production by neutrophils stimulated with both SEA and core protein compared to that produced in response to only SEA or core.

Moreover, SEA alone or core protein alone induced a significant decrease in hydrogen peroxide generation by neutrophils as compared to control (Fig 1B). However, both SEA and core protein induced a significant increase in hydrogen peroxide produced by neutrophils as compared to that produced in response to only SEA or core.

In the absence of IL-17, SEA and core significantly decreased TNF-α production by neutrophils (Fig 2A). Additionally, there was a marked increase in TNF-α production in the presence of IL-17, and IL-22 as shown in Fig 2A. In contrast, as shown in Fig 2B, H₂O₂ generation by neutrophils in the presence of SEA and core didn’t show any significant changes in the presence of IL-17, but it is significantly decreased in the presence of IL-22.

B. In vivo results on the effect of co-infection

68 patients and 17 control healthy individuals were enrolled in the in vivo study. The study was approved by the research ethics committee of Cairo University. The patients were recruited from Al-Qaser El-ainy University Hospital. All participants gave informed consent before participation in the study. The demographic data of the enrolled subjects are represented in Table (1). There is no significant difference in the age of males and females within each group (Schistosoma http://dx.doi.org/10.15242/IICBE.C0215058
group, co-infected group and control group) or between the different groups as compared to control subjects.

The in vivo results were confirmatory to these results. There was a significant increase in TNF-α and hydrogen peroxide levels in coinfected plasma comparing to those in Schistosoma infected plasma (Figure 3). In parallel, IL-17 level was markedly higher and IL-22 level was markedly lower in coinfected plasma than those in Schistosoma infected plasma (Figure 4).

IV. DISCUSSION

Schistosomiasis and HCV are endemic diseases in Egypt [26], [27]. Moreover, the prevalence of coinfection with both diseases is high [28], [29]. Neutrophils may play important roles in responses to Schistosoma-induced inflammation and HCV infections. Previous studies investigated the cellular immune response to HCV core protein however the response of neutrophils to HCV core has not been studied. Additionally, recruitment of neutrophils by IL-17 has been studied in different inflammatory conditions. However, there is limited studies investigated the role of Th17 cytokines on neutrophil recruitment during schistosomiasis or in case of co-infection. The aim of this study was to investigate the role of neutrophils in Schistosoma/HCV co-infection focusing on the HCV core protein and Schistosoma soluble egg antigen (SEA) as the main immune modulators in both infections.

The results of the in vitro study revealed that neutrophils activated with only SEA or core protein produced a significant decrease in the production of TNF-α (82.6 % and 59.5 %, respectively) compared to that produced by non-activated neutrophils. Similarly, there was a marked decrease in the production of TNF-α by neutrophils stimulated with both SEA and core protein compared to that produced in response to only SEA or core (75% and 89%, respectively). This finding may suggest that SEA and HCV core protein have inhibitory effects on TNF-α production by neutrophils and their effects are agonistic Therefore, coinfection may be associated with suppression of TNF-α production by neutrophils which dampened the immune responses to HCV and leading to high viral load.

Hydrogen peroxide is well known to induce tissue damage when its level increased and not sequestered by antioxidants [33]-[38]. In the current study it was found that SEA alone or core protein alone induced a significant decrease in hydrogen peroxide generation by neutrophils as compared to control. However, there was a significant increase in hydrogen peroxide produced by neutrophils activated with both SEA and core protein by 98 % and 100 %, respectively, compared to that produced in response to only SEA or core protein which further supporting that both SEA and HCV core are agonistic to neutrophils. So, it seems that combined infections will lead increasing H2O2 production by neutrophils and expansion of tissue damages produced by Schistosoma eggs and HCV infection. However, the mechanism of agonist is not known and is the focus of our current studies.

The decrease of TNF-α with its immune suppressive effect, and increase in the hydrogen peroxide levels with their tissue damage effects may explain the mechanisms of the increase of liver fibrosis and rapid progress to hepatocellular carcinoma in coinfected patients.

Although SEA and core significantly decreased TNF-α production by neutrophils, there were 90% and 80% increases of TNF-α in the presence of IL-17, and IL-22, respectively. In contrast, H2O2 generation by neutrophils in the presence of SEA and core didn’t show significant changes in the presence of IL-17, but hydrogen peroxide significantly decreased (73 %) in the presence of IL-22. Our results clearly showed that Th17 cytokines, IL-17 and IL-22, not only helps in the recruitment of neutrophils as reported by several studies [11],[39], but differentially modulated the response of neutrophils to both Schistosoma SEA and HCV core protein.

To confirm the in vitro results, 68 patients were enrolled (18 Schistosoma infected and 50 with chronic HCV and schistosomiasis coinfection) as well as 17 control healthy individuals. The in vivo data confirmed the in vitro data. There was a significant increase in the plasma levels of TNF-α and H2O2 in coinfected compared to those in Schistosoma infected patients (29.6% and 21.4%, respectively). Plasma TNF-α levels were not significantly (p= 0.0953) different between Schistosoma infected patients and controls (873.6 ± 269 pg/ml, and 536.5 ± 85.24 pg/ml, respectively). However, a significant decrease in the level of plasma H2O2 was observed in Schistosoma infected patients compared to that in control subjects. It has been reported that H2O2 is involved in toxicity against S. mansoni [40].

Human blood plasma is continuously generating H2O2 [37], [38]. The increase in co-infected plasma H2O2 level in this study may be due to the increase in the level of xanthine oxidase which increases due to tissue injury and is usually involved in peroxide generation. This explanation is supported by previous studies [41]-[43].

In parallel to neutrophil mediators, the level of coinfected plasma IL-17 was markedly higher than that of Schistosoma infected patients or control subjects (80.6% and 42.6%, respectively). However, there was a non-significant decrease in plasma IL-17 levels from Schistosoma infected plasma compared to controls. Regarding, plasma IL-22 level in coinfected patients, it was markedly lower (51.3 %) compared to that from Schistosoma infected patients but not significantly different compared to that in control subjects. However, this IL-22 plasma level was significantly increased (43.6%) in Schistosoma infected plasma compared to that in control subjects.
The marked increase in IL-17 level in coinfected plasma is supported by several studies. Th17 cells have been shown to play an important pathogenic role in an increasing number of inflammatory [44]-[47] and infectious conditions [48], [49]. They regulate the immune responses by secreting IL-17 and thereby stimulating the production of additional proinflammatory and chemotactic molecules [50]. However, IL-22 which is also secreted by Th17 cells is reported to be a protective cytokine and so the decrease of its plasma level in coinfected patients comparing to Schistosoma infected ones is supported by this fact [11].

In conclusion, Th17 cytokines modulates neutrophil responses during co-infection with Schistosoma and HCV. Moreover, there are differential effects of IL-17 and IL-22 on neutrophil responses to co-infection with Schistosoma and HCV.

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**TABLE I**

**DEMOGRAPHIC DATA OF THE ENROLLED SUBJECTS**

<table>
<thead>
<tr>
<th>Schistosoma patients (n=17)</th>
<th>Co-infected patients (n=50)</th>
<th>Controls (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Sex</td>
<td>Sex</td>
</tr>
<tr>
<td>Male (n=14)</td>
<td>Male (n=20)</td>
<td>Male (n=7)</td>
</tr>
<tr>
<td>52.8 ± 11.5</td>
<td>43.75 ± 7.4</td>
<td>35 ± 16</td>
</tr>
<tr>
<td>Female (n=3)</td>
<td>Female (n=30)</td>
<td>Female (n=10)</td>
</tr>
<tr>
<td>44.7 ± 4.7</td>
<td>44.5 ± 8.4</td>
<td>40 ± 13</td>
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</tbody>
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**FIGURE LEGENDS**

**Fig. 1:** TNF-α production (A) and peroxide generation (B) by activated neutrophils in response to Schistosoma SEA/core-HCV.

Activation of neutrophils with SEA alone or core protein alone led to a significant decrease (p<0.001) in the production of TNF-α by (17.39 ± 1.41 pg/ml) and (40.5 ± 17.45 pg/ml) respectively as compared to that of control non-activated neutrophils (100 ± 4.2 pg/ml). Additionally, there was a significant decrease in the production of TNF-α by neutrophils stimulated with both SEA and core protein (4.35 ± 1 pg/ml) as compared to SEA alone (17.39 ± 1.41 pg/ml) or core protein alone (40.5 ± 17.45 pg/ml) at p < 0.001 (Fig. 1A). However, hydrogen peroxide production was significantly increased by neutrophils stimulated with both SEA and core protein (142 ± 20 μMole/L) as compared to SEA alone (3 ± 0.15 μMole/L) or core protein alone (0 μMole/L) at p < 0.001 or controls as well (100 ± 2.25 μMole/L) at p < 0.05 (Fig. 1B). Although this production showed a significant decrease as compared to control in response to SEA alone or core protein alone.

**Fig. 2:** TNF-α production (A) and peroxide generation (B) by activated neutrophils in response to Schisto SEA/core-HCV in the presence of Th17 cytokines.

As shown in Fig. 2A&B, addition of IL-17 to SEA and core significantly increased TNF-α production (43.49 ± 2 pg/ml) but not hydrogen peroxide generation (120 ± 15 μMole/L) as compared to those without IL-17 by (4.35 ± 1 pg/ml) and (142 ± 20 μMole/L) respectively. However, addition of IL-22 significantly increased TNF-α (19.6 ± 7 pg/ml) and decreased hydrogen peroxide (38 ± 1.4μMole/L) as compared to those without IL-22 at p < 0.001 (Fig. 2A and B).

**Fig. 3:** Hydrogen peroxide and TNF-α levels in plasma of the enrolled subjects.

The in vivo results was confirmatory to the in vitro results were there was a significant increase in the level of TNF-α (1241.8 ± 232 pg/ml and hydrogen peroxide (302.7 ± 48.8μMole/L) in coinfected plasma as compared to those in Schistosoma infected plasma (873.6 ± 269 pg/ml) and (237.7 ± 24.7μMole/L) respectively at p< 0.05 (Fig. 3).

There was a non-significant (p= 0.0953) increase in the level of TNF-α when Comparing Schistosoma infected plasma (873.6 ± 269 pg/ml) to control plasma (536.5 ± 85.24 pg/ml). However, a significant decrease in the level of peroxide was observed in Schistosoma infected plasma (237.7 ± 24.7μMole/L) as compared to that (494.7 ± 12.6 μMole/L) in control plasma at p < 0.001 (Fig. 3).

**Fig. 4:** Th17 cytokine levels in plasma of the enrolled subjects

In parallel to neutrophil mediators, the level of coinfected plasma IL-17 was markedly higher (96.4 ± 12.5 pg/ml) than that in Schistosoma infected plasma (18.7 ± 4.8 pg/ml) or in control subjects (55.3 ± 27 pg/ml) at p < 0.001. However, there was a non-significant decrease in Schistosoma infected plasma as compared to controls (p= 0.0817) as shown in Fig. 4.

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Regarding, IL-22 level it was markedly lower (0.54 ± 0.3 ng/ml) than that in Schistosoma infected plasma (1.11 ± 0.43 ng/ml) but not significant as compared to that in control subjects (0.625 ± 0.29 pg/ml) at p < 0. However, this level showed a significant increase in Schistosoma infected plasma (1.11 ± 0.43 ng/ml) as compared to that in control subjects at P< 0.05 01 (Fig. 4).

Fig. 1: TNF-α production (A) and peroxide generation (B) by activated neutrophils in response to Schistosoma SEA/core-HCV.

Fig. 2: TNF-α production (A) and peroxide generation (B) by activated neutrophils in response to Schistosoma SEA/core-HCV in the presence of Th17 cytokines.

Fig. 3: Hydrogen peroxide and TNF-α levels in plasma of the enrolled subjects.

Fig. 4: Th17 cytokine levels in plasma of the enrolled subjects

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