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Research Article
Role of Toll Like Receptors 4, 5 and 9 Ligands in Pathogenesis and Outcome of Intestinal and Hepatic Schistosomiaisis Caused by *Schistosoma mansoni*

[1,2]Salwa A. Shams El-Din

[1]Department of Medical Parasitology, Faculty of Medicine, Menoufia University, Shebin El-Koom, Menoufia, Egypt
[2]Department of Microbiology and Parasitology, College of Medicine, King Abdulaziz University, Jedda, Saudi Arabia

Abstract
Human schistosomiasis is an endemic disease with approximately 207 million infected people worldwide and 280,000 deaths annually. Schistosomiasis is endemic in 76 countries more than 90% of people live in the African region. It is known that innate immunity plays an important role in disease pathogenesis. The Th2 response is critical for host survival. Innate immunity is initiated through Toll Like Receptors (TLRs). Macrophages also are stimulated by TLRs. The current study was aimed to assess the role of TLR ligands 4, 5 and 9 (LPS, flagellin and CpG, respectively) on parasite load, granuloma size and mice survival. Also it was aimed to assess the expression of macrophage immunohistochemically in liver of infected mice. It was found that decreased worm load, egg load and granuloma size were accompanied with TLR4 and TLR9 stimulation with upregulated expression of macrophages. These previous parameters were accompanied with increased survival of infected mice. Also there was a negative correlation between increased macrophage expression and granuloma size. So it can be concluded that LPS and CpG can alter pathogenesis of schistosomiasis with significant advancing effect on disease outcome and survival.

Key words: LPS, flagellin, CpG, TLRs, macrophage, schistosomiasis

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Corresponding Author: Salwa A. Shams El-Din, Department of Medical Parasitology, Faculty of Medicine, Menoufia University, Shebin El-Koom, Menoufia, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Schistosoma spp. are endemic parasitic trematodes infecting human. Schistosoma was found to infect about 207 all over the world with annual death rate reaching 280,000 deaths (WHO., 2010). People under infection risk are 779 million. The infection with Schistosoma is more endemic in76 countries in Africa (90%) (Amarir *et al*., 2011). This disease is an important major neglected disease of tropical nature. People in South America, Africa and the Middle East are infected with *S. mansoni,* while in China and the Philippines by *S. japonicum* and Middle East and Africa by *S. haematobium* (Ortega *et al*., 2010).

The host immune protective Th2 response to parasite eggs causes granuloma formation in affected tissues followed by collagen deposition and fibrosis (Ortega *et al*., 2010). Granuloma also protects host from Schistosome eggs and their antigens. The Th2 response is critical for host survival after egg deposition stage (Fallon *et al*., 2000). So Th2 response has two antagonizing roles in schistosomiasis; protect body from egg antigens as well as granuloma pathological consequences (Riner *et al*., 2013).

Innate immunity is initiated through many receptors as Toll-like receptors (TLRs) on macrophages and dendritic cells. TLR detect pathogen-associated molecules and stimulated by their specific ligands resulting in series of signaling ending in IFNs and other cytokines up regulation ending with the inflammatory response (Colonna *et al*., 2006). Most TLRs mainly promote Th1cells and inflammatory responses and initiation of Tumor Necrosis Factor-a (TNF-a) and interferon c (IFN-c). On the other hand, signaling of TLRs together with C-type lectins can result in anti-inflammatory responses (Geijtenbeek and Gringhuis, 2009).

More surprising, chronic TLR4 stimulation in immunodeficient mice without CD+4 cells can help parasite development (Lamb *et al*., 2010). Also, macrophage stimulation depends on TLR4 and TLR9 stimulation and induction of NF-κβ and interferon regulatory (IRF) family members host survival may be affected by increased liver and intestinal pathology (Gabhann *et al*., 2012).

Schistosomiasis japonicum in TLR deficient mice increased egg load and activated T cells and upregulated expression of some cytotoxic genes as assayed by Th1/Th2 cytokine secretion and DNA microarray analysis (Zhang *et al*., 2011).

The innate immunity in schistosomiasis should be studied as well as the relationship between its antigens and Antigen Presenting Cells (APC) From this point of view, the present study aimed at studying the role of stimulation of some pattern recognition receptors as TLR4 ligand (LPS), TLR5 ligand (flagellin) and TLR9 ligand (CpG) on *S. mansoni* disease outcome and immune response regarding parasite load, tissue egg burden, host survival, granuloma size, total leucocytic count and macrophage density in the affected liver tissue.

MATERIALS AND METHODS

Assessment of effect of used TLRs ligands on survival of mice: One hundred and twenty Swiss albino male, pathogen-free, 4-6 weeks old and weighing 20-25 g were used. The animals were obtained from the Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt and housed throughout the study at the SBSC, TBRI, Giza, Egypt. Mice were divided randomly into 4 groups (30 mice each). Mice were held in specific pathogen free facilities with free access to food and water and allowed to acclimatize for seven days prior to use.

Group 1 : Thirty infected untreated mice were used as control group

Group 2 : Thirty infected mice were injected individually with lipopolysaccharides (*Escherichia coli* 0111B4, Sigma-Aldrich, Sigma Chemical Co., St. Louis, USA). Intraperitoneal (ip) as 1 mg kg^{-1} LPS dissolved in sterile saline solution once on the first day after infection (Toldo *et al*., 2015)

Group 3 : Thirty infected mice were injected individually by100 μg kg^{-1} flagellin (InvivoGen, San Diego, CA, USA) intraperitoneally for 10 days starting from first day after infection (Xiao *et al*., 2015)

Group 4 : Thirty infected mice were injected individually with CpG oligodeoxynucleotide (InvivoGen, San Diego, CA, USA), subcutaneously in tail at a dose of 50 μg in 10 μL Phosphate Buffered Saline (PBS) once on the first day after infection (Israely *et al*., 2014)

Mortality was calculated in all groups of mice at 6, 8 and 10 weeks post infection.

Preparation of parasites and mice infection: *Schistosoma mansoni* cercariae were obtained from laboratory bred infected *Biomphalaria alexandrina* snails in SBSC at TBRI. Each mouse was experimentally infected with 60±10 *S. mansoni* cercariae suspended in 0.3-0.5 mL saline by subcutaneous injection through the loose skin over the upper region of the back (Abaza *et al*., 2013).

Assessment of used TLRs ligands on pathogenesis of the disease: Another 120 Swiss albino male, pathogen-free, were used, divided, grouped, infected and injected with drugs as described before.

The assessment of drug effects was done at 6, 8 and 10 weeks post infection through.

Assessment of worm load by porto-mesenteric worm burden assay: Through fine needle connected to a perfusion pump used to suck the worms from hepatic and intestinal tissue. The recovered worms were then counted (Smithers and Terry, 1965) at the end of 6, 8 and10th week Post Infection (PI).

Assessment of tissue egg count per gram intestine: At the end of perfusion, weighed fragments of small intestinal tissues (after being opened and cleaned from fecal content) were removed for the estimation of tissue egg loads. The fragments were incubated separately in 5 mL 5% KOH at 37°C for 24 h for hydrolysis. The number of ova/0.1 mL was counted in 3 slides and the mean was calculated and multiplied by 50 to calculate mean number of ova/5 mL, representing the number of ova in the weight of the intestine previously digested (Cheever, 1968).

$$\text{Number of ova/g tissue} \times 1 = \frac{\text{Number of ova/5 mL}}{\text{Intestine weights (g) recorded before digestion}}$$

Assessment of hepatic granulomas diameter: From each mouse, a small piece of liver was removed and preserved in 10% formalin. Hepatic sections were prepared and stained with Hematoxylin and Eosin (H and E) (Drury and Wallington, 1980). The slides were microscopically examined to determine the pathological changes and the granuloma diameter according to the criteria of Jacobs *et al.* (1997) summarized as follows: (1) Single egg granulomas were selected for diameter measurements, (2) Greatest diameter and its perpendicular diameter were measured and the mean of both diameters was considered granuloma diameter, (3) Mean diameter of 10 granulomas was estimated for each slide and (4) Mean of them in different treatment groups was calculated.

Total leukocytic count: Blood samples were collected from each mice in different groups for total leucocytic count (Becton Dickinson and Company, 1998).

Immunehistochemical staining of IHC macrophages ($CD68^+$): Briefly, paraffin blocks were used. Slides were dewaxed and rehydrated in distilled water. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. Sections were incubated with 10% normal goat serum (Dako Cytomation, Carpinteria, CA) for 20 min and incubated with primary antibody at room temperature. Primary antibodies used for $CD68^+$ (clone PG-M1, Dako, Glostrup, Denmark). Subsequently, sections were incubated with peroxidase-labelled secondary antibody (Dako Cytomation) for 30 min at room temperature. For visualization of the antigen, sections were immersed in 3-amino-9-ethylcarbazole plus substrate-chromogen (DakoCytomation) for 30 min and counterstained with Gill's haematoxylin (Droeser *et al.*, 2013). Immunoreactivity for $CD68^+$ protein marker was scored semiquantitatively by evaluating the mean number of positive cells over the whole microscopic field for 10 fields (Zlobec *et al.*, 2008).

Statistical methods: Data collected were managed and statistically analyzed using SPSS version 11 software. Kruskal Wallis' test, Wilcoxon signed rank test, K-test and F-test were used to compare means and their SD from three or more quantitative variables and also chi square test. Pearson correlation measured the association between two quantitative variables. The p-values<0.05 were considered statistically significant (Addelman, 1970).

Ethical considerations: The study was conducted during the period from June, 2013 to March, 2014 at the animal house of the SBSC, TBRI, Giza and Department of Parasitology Department, Faculty of Medicine, Menoufia University, Egypt. The study was approved by Menoufia University ethics committees on July, 2013. The animal experimental study was carried out according to the internationally valid guidelines and ethical conditions for using animals in research (NRC., 2006).

RESULTS

Evaluation of survival of mice after treatment with TLRs ligands (drugs): Early in infection, at 6th and 8th weeks, mortalities were more recorded in G1 and G3 and the differences in survival were significant ($p<0.001$) (Table 1). However, late with disease chronicity, mortalities increased in all groups and significantly in G1 and G4 ($p<0.001$) with highest survival rate in G2 (Table 1). *Schistosoma mansoni* eggs surrounded by tissue granuloma were found in intestine and liver of all groups by histopathological examination of haematoxylene and eosin stain. The granuloma is formed of chronic mononuclear inflammatory cells mainly lymphocytes and plasma cells (Fig. 1 and 2).

Table 1: Survival of mice among the studied groups

	Studied groups									
	G1		G2		G3		G4			
Duration PI	No.	%	No.	%	No.	%	No.	%	X^2-test	p-value
6 wpi										
Alive	21	70.0	30	100.0	15	50.0	27	90.0	25.38	<0.001*
Dead	9	30.0	0	0.0	15	50.0	3	10.0		
8 wpi										
Alive	21	70.0	30.0	100.0	12	40.0	27	90.0	17.23	<0.001*
Dead	9	30.0	0.0	0.0	18	60.0	3	10.0		
10 wpi										
Alive	15	50.0	18	60.0	12	40.0	15	50.0	2.40	0.49
Dead	15	50.0	12	40.0	18	60.0	15	50.0		
X^2 test	20.0	7.2	0.61	21.5						
	20.0	7.2	0.61	21.5						
p-value	<0.001[1]*		0.007[1]*		0.44[1]		<0.001[1]			
	<0.001[2]*		0.007[2]*		0.44[2]		<0.001[2]			

Test: Chi square (X^2), 1: Relation of 10 weeks with 6 weeks, 2: Relation of 10 weeks with 8 weeks, *Significant

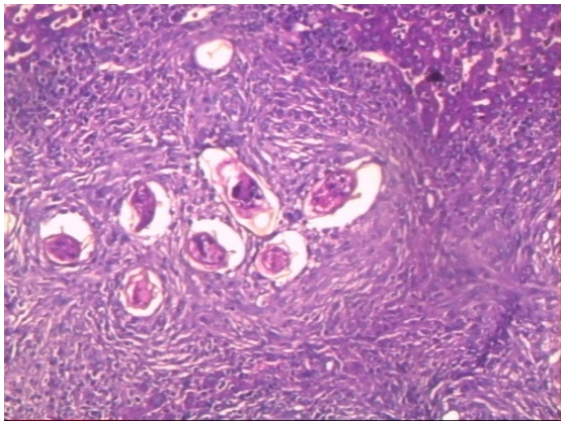

Fig. 1: *Schistosoma mansoni* granuloma around eggs in liver tissue (H and E stain×200)

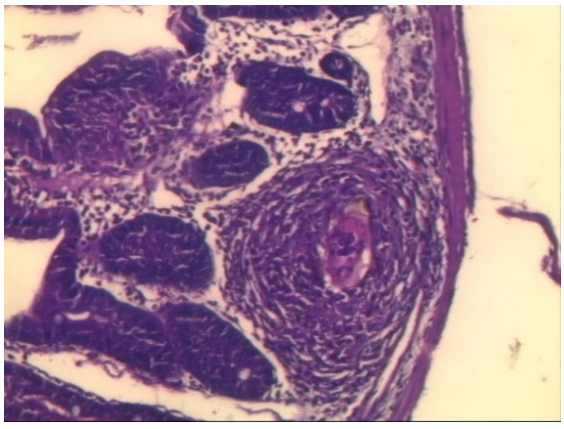

Fig. 2: *Schistosoma mansoni* granuloma around egg in intestinal tissue (H and E stain×100)

Evaluation of used TLRs ligands on pathogenesis of the disease: Regarding 6th and 8th week post infection, the mean numbers of adult *S. mansoni* detected inG1 (control group) mice were 33.75 and 20.25, respectively. The mean numbers of adult *S. mansoni* detected in G2 mice were 23.25 and 11.35, respectively. The mean numbers of adult *Schistosoma mansoni* found in G3 were 9 and 7.75, respectively. The mean numbers of adult *S. mansoni* detected in G4 mice were 11.5 and 10.5, respectively and the difference between groups was significant (p = 0.006 and 0.005 at 6th and 8th weeks, respectively) (Table 2 and Fig. 3). Late at 10th week post infection, the mean numbers of adult *S. mansoni* found in G1, G2, G3 and G4 were 18.0, 17.75, 14.5 and 16.0, respectively and the difference between groups was nonsignificant (p = 0.06) (Table 2 and Fig. 3).

At 6th week post infection, the mean number of *S. mansoni* eggs found in intestinal tissue of mice in G1 (control group), G2, G3 and G4 were18803.3, 14106.7, 1631.3 and 10422.3, respectively (Table 3 and Fig. 4) and the difference was statistically significant (p = 0.02). At 8th week post infection, the mean number of *S. mansoni* eggs found in intestinal tissue of mice in G1 (control group), G2, G3 and G4 were 21255.3, 17880.3, 2558.7 and 18997.3, respectively (Table 3 and Fig. 4) and the difference was statistically significant (p = 0.03). At 10th week post infection, the mean number of *S. mansoni* eggs found in intestinal tissue of mice in G1 (control group), G2, G3 and G4 were 23757.7, 12522.0, 5110.7 and 13294.0, respectively (Table 3 and Fig. 4) and the difference was statistically significant (p = 0.02).

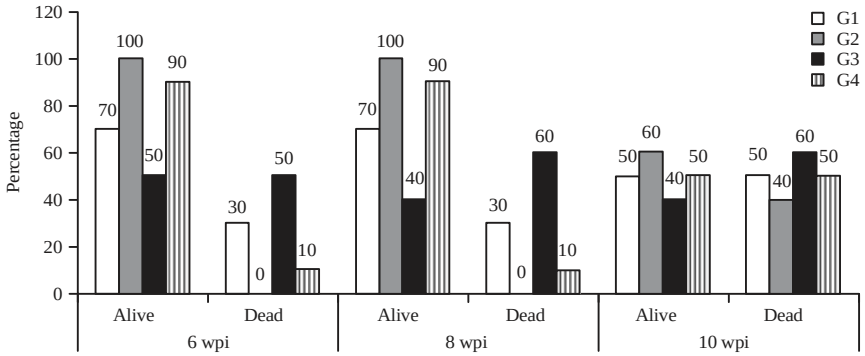

Fig. 3: Survival of mice among the studied groups

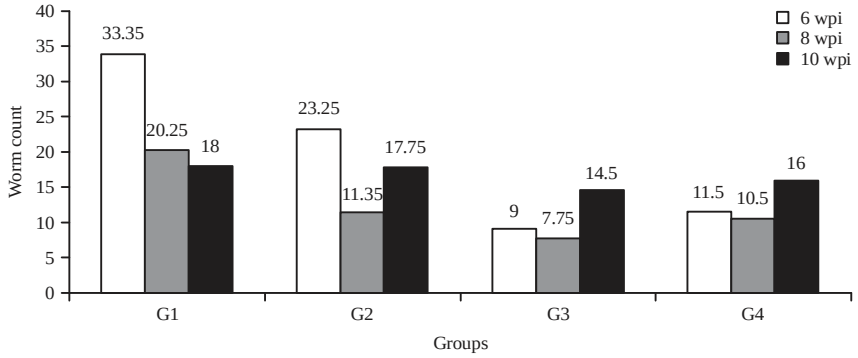

Fig. 4: Worm count among the studied groups

Table 2: Worm count among the studied groups

	Studied groups					
Duration PI	G1 (X±SD)	G2 (X±SD)	G3 (X±SD)	G4 (X±SD)	Kruskal wallis	p-value
6 wpi	33.75±4.19	23.25±4.57	9.00±1.15	11.5± 5.0	12.63	0.006*
8 wpi	20.25±1.26	11.35±1.91	7.75±0.50	10.5±1.29	12.94	0.005*
10 wpi	18.00±0.82	17.75±0.50	14.5±3.7	16.0±1.41	7.29	0.06
Wilcoxon signed rank	1.84	1.82	1.34	1.83		
	1.84	1.60	1.83	1.83		
p-value	0.07[1]	0.07[1]	0.18[1]	0.07[1]		
	0.07[2]	0.11[2]	0.07[2]	0.07[2]		

1: Comparison between 10 weeks and 6 weeks, 2: Comparison between 10 weeks and 8 weeks, *Significant

Table 3: Egg load per gram of intestinal tissue among the studied groups

	Studied groups					
Duration PI	G1 (X±SD)	G2 (X±SD)	G3 (X±SD)	G4 (X±SD)	K-test	p-value
6 wpi	18803.3±2803.6	14106.7±3365.4	1631.3±376.5	10422.3±1319.5	9.51	0.02*
8 wpi	21255.3±921.4	17880.3±1895.0	2558.7±212.4	18997.3±1373.9	8.90	0.03*
10 wpi	23757.7±3247.0	12522.0±2029.5	5110.7±36.9	13294.0±1824.5	9.46	0.02*
F-test	2	4.67	6.0	6.0		
p-value	0.37	0.10	0.05*	0.05*		

K: Kruskal Wallis test, F: Freidman test, *Significant

Regarding 6th week post infection, the mean diameters of *Schistosoma mansoni* granuloma found in liver tissue of infected mice in G1 (control group), G2, G3 and G4 were 149.6, 101.6, 140.0 and 121.6, respectively (Table 4) and the difference was statistically significant (p = 0.02). At 8th week post infection, the mean diameters of *Schistosoma mansoni* granuloma found in liver tissue of infected mice in G1 (control group), G2, G3 and G4 were 156.0, 126.0, 152.0 and 137.6, respectively (Table 4) and the difference was statistically significant (p = 0.03).

Table 4: Mean diameter of *Schistosoma mansoni* in liver tissue among the studied groups

	Studied groups					
Duration PI	G1 (X±SD)	G2 (X±SD)	G3 (X±SD)	G4 (X±SD)	Kruskal wallis	p-value
6 wpi	149.60±23.1	101.6±14.2	140.0±21.9	121.6±25.4	9.74	0.02*
8 wpi	156.0±18.17	126.0±13.4	152.0±10.9	137.6±15.1	8.76	0.03*
10 wpi	126.40±12.1	119.6±10.5	149.6±19.3	124.0±21.9	7.75	0.05*
Wilcoxon signed rank	0.14	1.63	0.68	1.46		
	0.82	1.83	0.18	2.02		
p-value	0.89[1]	0.10[1]	0.50[1]	0.14[1]		
	0.41[2]	0.07[2]	0.85[2]	0.04[2]		

1: Comparison between 10 weeks and 6 weeks, 2: Comparison between10 weeks and 8 weeks, *Significant

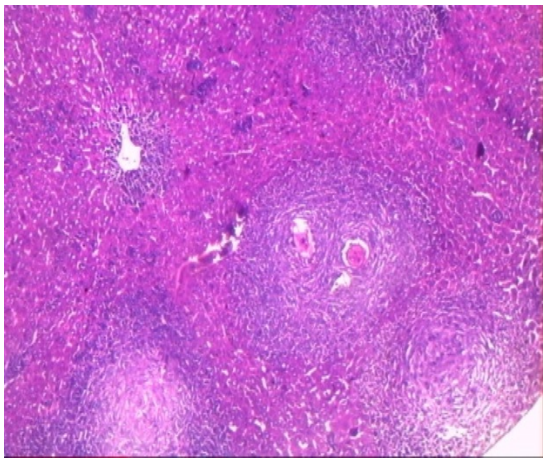

Fig. 5: Liver tissue of mice in G1 showing multiple Schistosoma granulomas with three amalgamated *Schistosoma mansoni* eggs. The surrounding reaction contains cellular components composed of fibroblasts and collagen. The cellular infiltrates mainly surround ova (H and E stain×100)

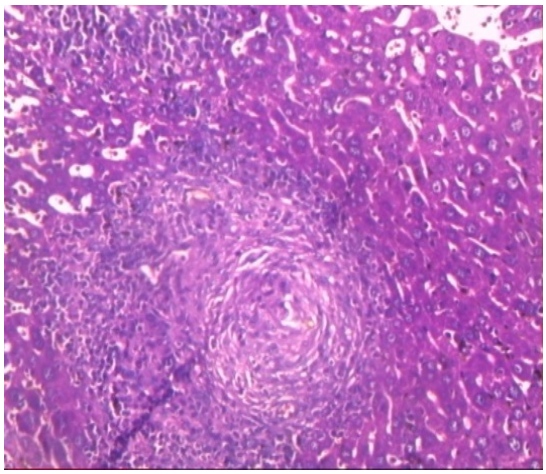

Fig. 6: Liver tissue of mice in G2 granuloma surrounding *Schistosoma mansoni* egg consisting of chronic mononuclear inflammatory cells mainly lymphocytes and plasma cells (H and E stain×200)

Regarding 10th week post infection, the mean diameters of *Schistosoma mansoni* granuloma found in liver tissue of infected mice in G1 (control group), G2, G3 and G4 were 126.4, 119.6, 149.6 and 124.0, respectively (Table 4) and the difference was statistically significant ($p \leq 0.05$). Histopathological examination showed that the cellular infiltrates mainly surround the periphery of ova with frequent inflammatory cells infiltrating the granuloma and the surrounding liver tissue. There is also hypertrophy of the wall of large portal vessel filled with RBCs and adult worms may be seen inside. Deposition of collagen and fibrin was recorded. Histopathological examination of liver tissue in G1 showed multiple granulomas with three amalgamated *S. mansoni* eggs. The surrounding reaction contains less cellular components and is mainly composed of fibroblasts and collagen. The cellular infiltrates mainly surround the periphery of ova (Fig. 5 and 6). Examination of liver tissue in G2 showed granuloma surrounding *S. mansoni* egg consisting of chronic mononuclear inflammatory cells mainly lymphocytes and plasma cells (Fig. 7). Examination of liver tissue of mice in G4 showed two *S. mansoni* scattered granulomas with varied diameters with lymphocytic infiltration and normal liver tissue in between and periportal fibrosis (Fig. 8).

At 6th week post infection, the mean number of leukocytes found in blood of infected mice in G1 (control group), G2, G3 and G4 were 8050, 6000, 1750 and 6075, respectively (Table 5) and the difference was statistically significant ($p = 0.004$). At 8th week post infection, the mean number of leukocytes found in blood of infected mice in G1 (control group), G2, G3 and G4 were 9500, 5600, 2500 and 7075, respectively (Table 5) and the difference was statistically significant ($p = 0.003$). At 10th week post infection, the mean number of leukocytes found in blood of infected mice in G1 (control group), G2, G3 and G4 were 6700, 4225, 4000 and 6025, respectively (Table 5) and the difference was statistically significant ($p = 0.005$). The WBCs count was least in G3.

Table 5: Total leucocytic count among the studied groups

	Studied groups					
Duration PI	G1 (X±SD)	G2 (X±SD)	G3 (X±SD)	G4 (X±SD)	Kruskal wallis	p-value
6 wpi	8050±300	6000±81.7	1750±420.3	6075±50.0	13.18	0.004*
8 wpi	9500±81.7	5600±81.7	2500±163.3	7075±629.2	14.18	0.003*
10 wpi	6700±476.1	4225±434.9	4000±141.4	6025±28.9	12.85	0.005*
Wilcoxon signed rank	1.83	2.0	1.84	1.83		
	1.83	1.83	1.83	0.0		
p-value	0.07[1]	0.04[1*]	0.07[1]	0.07[1]		
	0.07[2]	0.07[2]	0.07[2]	1.0[2]		

1: Comparison between 10 weeks and 6 weeks, 2: Comparison between 10 weeks and 8 weeks, *Significant

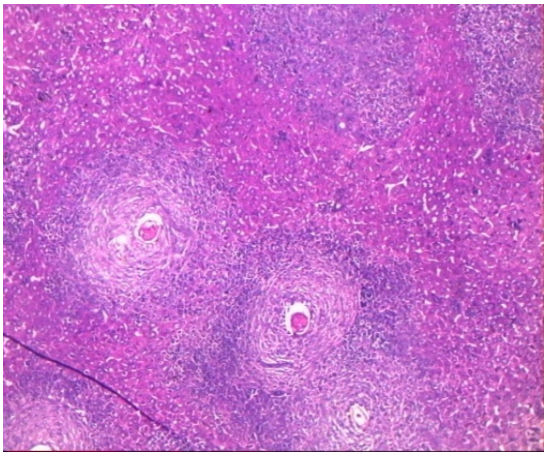

Fig. 7: Liver tissue of mice in G3 showing multiple granulomas with one *Schistosoma mansoni* egg evident in each one of them. The granulomas are composed mainly of fibroblasts with only a rim of plasma cells and lymphocytes at the periphery (H and E stain ×100)

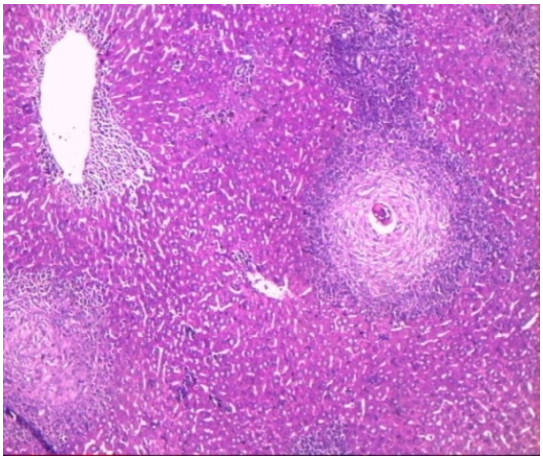

Fig. 8: Liver tissue of mice in G4 showing two *Schistosoma mansoni* scattered granulomas with varied diameters with lymphocytic infiltration and normal liver tissue in between and periportal fibrosis (H and E stain ×100)

Fig. 9: Liver tissue of mice in G1 showing macrophage cells in Schistosoma granuloma (Immunestain reaction for $CD68^+$ ×100)

Evaluation of used TLRs ligands on expression of macrophages: Expression of $CD68^+$ cells (macrophage) by immunohistochemical staining was recorded as brown pigmentations of the nucleus and cell membrane. Regarding G1 expression of $CD68^+$ cells (macrophage) by immunohistochemical staining was recorded at all tested weeks showing mean number of macrophage/microscopic field of 8.0, 12.0 and 11.4 at 6th, 8th and 10th weeks post infection, respectively (Table 6 and Fig. 9) ($p = 0.02$). Expression of $CD68^+$ cells in G2 by immunohistochemical staining was recorded at all tested weeks showing mean number of macrophage/microscopic field of 21.0, 19.6 and 15.8 at 6th, 8th and 10th weeks post infection, respectively (Table 6 and Fig. 10) ($p = 0.09$). Expression of $CD68^+$ cells in G3 by immunohistochemical staining was recorded at all tested weeks showing mean number of macrophage/microscopic field of 8.4, 11.2 and 12.6 at 6th, 8th and 10th weeks post infection, respectively (Table 6 and Fig. 11) ($p = 0.08$). Expression of $CD68^+$ cells in G4 by immunohistochemical staining was recorded at all tested weeks showing mean number of macrophage/microscopic field of 11.4, 16.4 and

Table 6: Mean number of macrophage cells ($CD68^+$) in liver tissue among the studied groups(mean/10 hpf)

	Studied groups					
Duration PI	G1 (X±SD)	G2 (X±SD)	G3 (X±SD)	G4 (X±SD)	K-test	p-value
6 wpi	8.0±0.7	21.0±0.7	8.4±1.5	11.4±2.41	15.33	0.002*
8 wpi	12.0±2.6	19.6±1.1	11.2±0.5	16.4±2.41	15.33	0.002*
10 wpi	11.4±2.88	15.8±0.8	12.6±1.9	14.4±2.1	9.10	0.03*
F-test	7.89	9.33	9.58	6.78		
p-value	0.02*	0.009*	0.008*	0.03*		

K: Kruskal Wallis test, F: Freidman test, *Significant

Table 7: Correlation between granuloma size and other tested parameters

	Granuloma size (6 weeks)		Granuloma size (8 weeks)		Granuloma size (10 weeks)	
	R	p-value	R	p-value	R	p-value
Worm count	-0.19	0.49	-0.10	0.71	-0.30	0.25
WBCs	-0.06	0.81	-0.24	0.36	-0.26	0.33
Egg load (intestine)	+0.02	0.96	-0.19	0.56	-0.50	0.10
$CD68^+$ count/10 hpf	-0.42	0.07	-0.54	0.01*	+0.23	0.33

*Significant, r: Pearson correlation factor

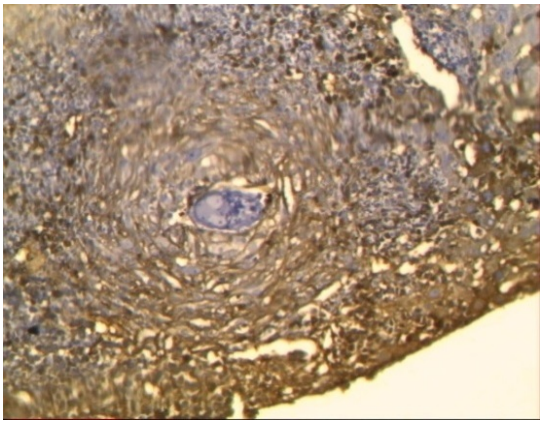

Fig. 10: Liver tissue of mice in G2, showing increased macrophage cell number in Schistosoma granuloma (immunestain reaction for $CD68^+$ ×100)

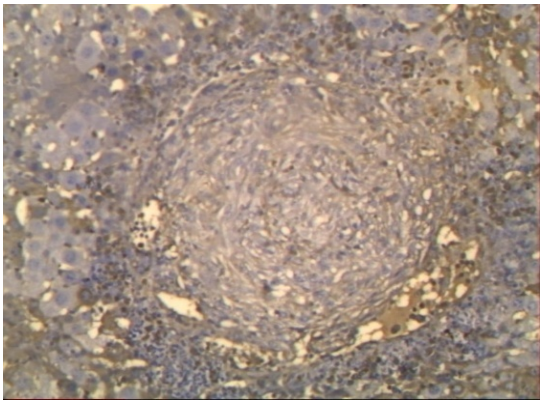

Fig. 11: Liver tissue of mice in G3 showing decreased macrophage cell number in Schistosoma granuloma (immunestain reaction for $CD68^+$ ×100)

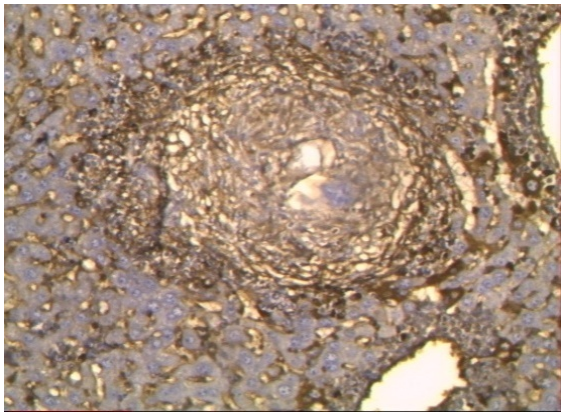

Fig. 12: Liver tissue of mice in G4, showing increased macrophage cell number in Schistosoma granuloma (immunestain reaction for $CD68^+$ ×100)

14.4 at 6th, 8th and 10th weeks post infection, respectively (Table 6 and Fig. 12) (p = 0.03). The differences between groups were significant at all tested weeks (p = 00.002, 0.002 and 0.003).

Correlation between the tested parameters with granuloma size: Correlation between worm count, egg load in intestine, WBCs count and $CD68^+$ vs granuloma size was nonsignificant at all tested weeks (Table 7) except negative correlation between $CD68^+$ count and granuloma size at 8th week post infection (p = 0.01) (Table 7).

DISCUSSION

Schistosoma infections are fourth most prevalent worldwide, with about 200 million people infected (Riner *et al*.,

2013). *Schistosoma mansoni* infection is orchestrated by two Th phases: First Th1 (IFN-γ) response, followed by a stronger Th2 (IL-10, IL 5, IL-13) one. Granuloma was known to be induced by microbial molecules, which activate pattern recognition receptors such as the TLR family and leads to the synthesis of pro-IL-1β (p35) in an NF-κB-dependent manner (Yu and Finlay, 2008). Schistosomal antigens could suppress the secretion of TNF-α and IL-6 by dendritic cells after TLR triggering. The granuloma depends on a TLR-mediated priming signal to increase pro-IL-1β (p35), which is then cleaved into the biologically active IL-1β (p17). Parasite antigens does not induce IL-1β without a TLR stimulus (Goodridge *et al*., 2007).

As *S. mansoni* infection causes similar immune responses and pathology in human and mice. Schistosome eggs induce alternatively activated macrophage-rich granulomas and Th2-biased immunity that prevents acute mortality but ultimately causes liver fibrosis (Wynn *et al*., 2004).

This data showed that treatment of G2 with LPS reduced worm count, egg burden and granuloma size WBCs count, but increased macrophage count significantly. All the previous measured parameters shared in better immune response with increased mice survival. This can suggest LPS as an adjuvant therapy for schistosomiasis. The TLR4 plays an important role in the recognition of helminthes products by DCs and macrophages and in the development of Th2 responses (Kane *et al*., 2008). Production of cytokines from macrophage was shown to depend on TLR4 stimulation. The TLRs mice infected with *S. japonicum* showed lower mRNA expression of IL4, IL12 and IFNγ early in infection. After 6 weeks of infection TLRs mice showed increased egg burden (Zhang *et al*., 2011).

However, chronic LPS treatment in *S. mansoni* infected mice resulted in downregulation of proinflammatory cytokines as Tumor Necrosis Factor (TNF) and this restores parasite development (Riner *et al*., 2013). Systemic host and microbial-derived TLR ligands can modify B-cell responses in a pro or antiinflammatory manner. This was evident through differential responses of human B cells to TLR4 ligands (McDonnell *et al*., 2011). This may be related to the type of LPS in the bloodstream. The number of acyl chains on lipid A, the inflammatory moiety of LPS, can dictate whether LPS is agonistic or antagonistic (Coats *et al*., 2003). Systemic LPS may stimulate B cells through lipid A acetylation burden (McDonnell *et al*., 2011) Evidence suggests that many schistosome-derived molecules, particularly TLR4 ligands and several glycoproteins, are immunosuppressive and down-regulate Th1 responses, while actively promoting Th2-like immunity (Marshall and Pearce, 2008).

Treatment of G3 with flagellin decreased worm count, egg burden, WBCs count and macrophage significantly, however, granuloma size was increased and subsequently mice survival was reduced significantly. These can explain a relationship between survival, granuloma size and macrophage count approved by Pearson's correlation test. Toll-like receptor (TLR) ligands instruct human DCs to induce distinct Th cell responses by differentially modulating mitogen-activated protein kinase signaling. Thus, flagellin, which triggers TLR5, instruct DCs to stimulate Th1 responses via IL-12p70 production, which depends on the phosphorylation of p38 and c-Jun N-terminal kinase 1/2 (Agrawal *et al*., 2003).

In G4, decreased worm count, granuloma size and WBCs and increased macrophage count significantly led to significant increase in mice survival in this group. The CpG inhibited Treg immunosuppressive function, upregulated the production of interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, interleukin (IL)-4, IL-10, IL-2 and IL-6 and decreased $CD4^+$ $CD8^+$ $foxp3^+$ expression *in vitro* (Lu *et al*., 2013). The CpG increases the proliferation of splenocytes and IgG levels and improves disease protection after immunization with the *S. japonicum* vaccine pVAX1-Sj26GST. This enhancement of protection is related to the inhibition of Treg expansion and function, upregulating the secretion of proinflammatory cytokines and decreasing Foxp3 expression (Wang *et al*., 2013). The TLR9 mice showed increased pulmonary granuloma size, augmented collagen deposition, increased Th2 cytokine phenotype and impaired accumulation of DCs. The BM-derived DCs from TLR9 mice induced impaired Th1 cytokine compared with DCs from Wild Type (WT) mice. Macrophages from TLR9 mice expressed a significantly higher alternatively activated (M2) phenotype characterized by increased "Found in inflammatory zone-1" (FIZZ1) and arginase-1 expression. The adoptive transfer of BM-derived DCs from syngeneic WT mice into TLR9 mice restored the granuloma phenotype seen in WT mice (Ito *et al*., 2009).

The lower expression of CCL20 and CCL22 during granuloma development in TLR9-deficient lungs may contribute to the decreased DC numbers observed during pulmonary granuloma formation. In addition, lower levels of CXCL9, CXCL10 and CXCL11 that act as ligands for CXCR3 on Th1 cells (Liu *et al*., 2005). These chemo can contribute in impaired DC migration and decreased Th1 production (Ito *et al*., 2009).

The presence of CpG in a conventional *in vitro* suppression assay induced a panel of inflammatory cytokines, including IFN-γ, TNF-α, IL-4, IL-10, IL-2 and IL-6 and these elevated cytokines may inhibit the Treg suppressive function, since a variety of cytokines have been reported to inhibit Treg function in autoimmune reactions, including TNF-γ, IL-4, IL-6 and IL-12 (Buckner, 2010).

From the present study, it can find that decreased granuloma size and increased macrophage count were accompanied by increased host survival. However, as known parasite burden (worm and egg), WBCs count may have little effect on host survival.

Microbial products plus IFN-γ 'Classically' activate macrophages to induce microbicidal activity during Th1-dominant responses macrophages have the capability to promote, restrict, or resolve inflammation and fibrosis and their role depends on their activation status (Wynn and Barron, 2010).

However, the addition of TLR ligands as adjuvants induces proinflammatory setting, which acts either by direct inhibition of Treg to allow expansion of antigen-specific T cells against *S. japonicum*. Therefore, modulation of Tregs by adjuvant TLR ligand combinations may represent an attractive strategy to enhance the efficacy of vaccination against pathogens (Wang *et al*., 2013).

Induction of immunoregulatory M2 macrophages (alternatively activated) is critical for parasite development (Riner *et al*., 2013). There is evidence that *Schistosomes* require M2 macrophages early and later on in infection as they are critical for the egress of Schistosome eggs from the body of the host (Herbert *et al*., 2004).

Napolitani *et al*. (2005) reported that synergistic TLR stimulation mimics pathogens that contain several TLR ligands and induces enhanced and sustained T helper type 1 responses in DCs. Furthermore, several studies have shown that certain TLRs enhance T cell-mediated immune responses through synergistic activation of DCs with their ligands (Zhu *et al*., 2008) or through induction of high levels of proinflammatory cytokines by simultaneously activating different signaling pathways (Raman *et al*., 2010).

CONCLUSION

Decrease worm load and egg count in tissues cannot judge survival of host unless accompanied with proper immune response with increased macrophage and decreased granuloma size. The TLR ligand may increase protective efficacy against schistosomiasis, which may be through impairing Treg development and function, upregulating the secretion of pro-inflammatory cytokines and type of macrophage activation. These factors provide the effector response to generate protective immunity by eliciting the appropriate immune response. TLR ligands coordinate innate, adaptive and regulatory immune responses.

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