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IMMUNOHISTOPATHOLOGICAL EVALUATION OF HEPATIC CD14 EXPRESSION IN MURINE SCHISTOSOMIASIS MANSONI

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Abstract

Bilharziasis is one of the most common parasitic diseases, mostly affecting the liver by causing the formation of granuloma and hepatic fibrosis**,** and historically endemic diseases in Egypt. Hundreds of studies examined the hepatic schistosomiasis pathogenesis to find out points for possible drug interference with disease progression and complications. The macrophages are the major source of CD14 in liver granulomas of *S. mansoni*-infected mice and CD14 might have regulatory roles during infection. The current study evaluated the cellular expression of CD14 in livers of *S. mansoni* infected mice in comparison with healthy controls, for assessment of its potential regulatory role in schistosome-induced hepatic fibrosis. Histopathological study of livers of *S. mansoni* infected mice after 12 weeks of infection was conducted using routine H&E stain and M.T stain for tissue fibrosis. Immunohistochemical evaluation of CD14 positive cells was also performed using DAB as a chromogen. The results showed upregulation of CD14 expression in infected mice, compared to healthy control mice, with variable percentage and different distribution in relation to granuloma type.

Key words: Egypt, CD14, Schistosomiasis *mansoni*, Liver, Immunohistochemistry, EMT

Introduction

Schistosomiasis is a waterborne disease caused by a blood fluke results from parasitization by worms of the genus *Schistosoma*; *S. haematobium, S. japonicum*, and *S. mansoni* are the most widespread species (Tang *et al*, 2018). Bilharziasis is one of the commonest parasitic diseases, mostly affecting the liver by causing granuloma and hepatic fibrosis, associated with severe morbidity (Dahesh and Farid, 2016). Schistosomiasis excessively affects people who have limited access to potable water and sanitation living in the tropics and subtropics 240 million people are infected, with 700 million people at risk of getting infected and estimated to be 50,000-100,000 died annually (Dkhile *et al*, 2015). *Schistosoma*-induced liver injury results from a granulomatous inflammatory reaction around trapped *Schistosoma* eggs in presinusoidal periportal spaces. In early infection, a predominantly hypercellular non-fibrotic granuloma response produces liver dysfunction that is not clinically detectable. Imaging studies may reveal enlargement of the left liver lobe without changes in the liver parenchyma or splenomegaly. Reversibility of these findings were expected in 12 months after chemotherapy. Development of chronicity in collagen deposition in the periportal spaces was the basis of the pathognomonic pathological feature of schistosomal associated liver fibrosis or Symmers' pipestem fibrosis (Cavalcanti *et al*, 2015).

The expression evaluation of pattern recognition receptors (PRRs) on Antigen-presenting cell (APCs) in mice infected with the helminth parasite *Schistosoma mansoni* observed an upregulation of CD14 expression on macrophages (Pearce *et al*, 2004). The highest expression of CD14 was found on liver macrophages in infected mice. Collectively, these data show the role for CD14 in regulating macrophage plasticity and CD4 T cell biasing during helminth infection. There was also an increased recruitment of alternatively activated cells in livers of CD14-deficient mice. This suggested that CD14 might have regulatory roles during infection and that macrophages are the major source

of CD14 in liver granulomas of *S. mansoni*-infected mice (Tundup *et al*, 2014).

Granulocytes (CD11b+) are recruited to egg granulomas in livers of infected mice, in contrast to what was in the livers of uninfected mice. Macrophages, eosinophils, neutrophils, and some lymphocytes are present in liver egg granulomas of *S. mansoni*-infected mice (Pearce and MacDonald, 2002). Therefore, to analyze the expression of CD14 on granulocytes in liver egg granulomas, prepared single-cell suspensions of liver granulomas and then stained them with surface markers against macrophages, eosinophils, and neutrophils as well as CD14. They found that only CD11granulocytes had high levels of CD14 expression, compared to granulocytes with low expression of CD11b (CD11b+). The experiments showed that CD14 expression was highly upregulated in schistosome-infected (Tundup *et al*, 2014).

CD14-deficient mice were associated with reduced egg burdens in the livers of infected mice compared to wt controls, suggesting that immune effector cells and/or mediators regulated via CD14 may play roles in parasite fecundity. CD14 may regulate M1 and M2 phenotypes in livers of *S. mansoni*-infected mice. CD14 expression by macrophage is a key regulator of macrophage M1/M2 plasticity. This is supported by earlier observations (Herbert *et al*, 2004).CD14 may help in recognition of glycolipids as well as dsRNAs in controlling macrophage M1 activation and immune responses during infection (Baumann *et al*, 2010). In additional studies examining the role of CD14 in the regulation of immune responses need to be performed before such a hypothesis can be confirm (Lee *et al*, 2006).

The present work aimed to monitor the role of CD14 in macrophage activation, pathology, and immune responses in vivo during helminth infection. Given that activation and recruitment of macrophages are dominant features of many inflammatory diseases, such as cancer and autoimmune, cardiovascular, metabolic, and allergic diseases, CD14 may serve as a potential, broadly immunotherapeutic target for numerous pro-inflammatory diseases.

Materials and Methods

Ethics statement: The guide for the Care and Use of Laboratory Animals was in accordance with the American Association for Accreditation of Laboratory Animal Care (AALAC) and the Animal Welfare Act (AWA). The study was approved by Theodor Bilharz Research Institute Ethical committee.

Mice, parasites, and infection: C57BL/6 female albino mice were housed at the animal house (Theodor Bilharz Research Institute) under specific-pathogen-free conditions. Two groups of mice (10 for each) were segregated in special cages, fed with conventional food pellets and drinking tap water. G1 constituted Egyptian strain of *S. mansoni* infected mice. G2 represented healthy control (non-infected) mice. G1 mice were subcutaneous infected with 50 to 60 cercariae. They were sacrificed at 12 weeks post infection, livers were removed for histopathological and immunohistochemistry studies

Paraffin sections from hepatic lesions undergo de-paraffinization, rehydration. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide. Antigen retrieval was performed by microwaving the sections in citrate buffer, pH 6.0. Sections were incubated overnight at 4^{o}C in humid chamber with the primary antibodies: Anti-CD14 (monoclonal antibody, Beckman Coulter, clone RMO52). Next day, sections washed twice in PBS, then the second biotinated antibody was applied for 20min, washed, followed by application of Envision detection system (Universal Detection Kit, Dako, Denmark). Antigen was localized by adding 3, 3'diaminobenzidine tetrahydrochloride (DAB) substrate chromogen solution. Slides were counterstained with hematoxylin, dehydrated in ethanol and mounted.

For each setting, positive and negative control slides were included. As a negative control, hepatic tissue was processed in mentioned sequences but without primary antibodies instead adds non-immune immunoglobulin G (IgG; DAKO, Glostrup, Copenhagen, Denmark).

Paraffin sections were stained by hematoxylin and eosin for histopathological examination, counting & size of granulomas, as well as identification of inflammatory and other types of cells. Counting of granulomas was done in 5 successive low power (100X) fields. Granuloma size was represented as the mean diameter of each granuloma with central ova, non-amalgamated and away from the portal tract. This was dome through the software (VISION) supplied by Zeiss Company. Liver sections were also stained by Masson's Trichrome stain, to assess hepatic fibrosis and identification of types of granuloma (cellular or fibrocellular).

Interpretation of immunostaining and scoring analysis: **H**epatic sections were blind-quantified by two pathologists. Sections were examined by light microscope and. photographs were taken using a microscope-camera (Axio-Cam, MRc5, Zeiss, Germany). CD14 positive cells were identified by cytoplasm brown coloration. Cells with these criteria were matched with corresponding cells in stained sections to identify their nature. CD14 positive cells were counted with other inflammatory cells %. Localization of CD14 positive cells were in control and in *S. mansoni* infected livers.

Statistical Study: Data were processed on SPSS program version 20. Comparison between means were done by student T-test, with a significant values with ($p<0.05$).

Results

Histopathological examination of liver sections from infected mice revealed many lobular and portal egg-granulomas, surrounded by cellular or fibrocellular tissue response. There was expansion of portal tracts by inflammatory cells with vascular proliferation and fibrous tissue deposition. Hepatic lobule showed Kupffer cell hyperplasia, pigmentation, increase in sinusoidal monocytes and mild sinusoidal fibrosis. These were absent in liver sections of healthy controls. Only few mononuclear cells were detected in some portal tracts.

Table 1: Control and *S. mansoni* infected mice as to granuloma count, granuloma size & $CD14^+$ cellular expression:

group		Granuloma count	Granuloma Size	$CD14^+$ cells	Localization of $CD14^+$ cells
		Mean in 5 successive LPF	Mean diameter (µm)	Mean percentage	(+++): Mostly (++): moderately (+): Infrequently
Control (10 mice)	Mean	0.0	-	3.50	Portal tracts (+)
	S.D.	0.0	-	1.19	
Infected (10 mice)	Mean	66.18	171.63	7.09	Granulomas (+++) Portal tracts (++) Lobular (+)
	S.D.	3.15	35.53	2.54	
P Value		H.S.	-	H.S	

S.D.: Standard Deviation H.S: High significance ($p<0.0001$)

$CD14^+$ cells were infrequently recognized within portal tracts of control mice, while presented at peripheral zones of egg granulomas and in portal tracts and hepatic lobules of *S. mansoni* infected mice. Difference in $CD14^+$ cellular expression between both was highly significant ($p<0.0001$).

Table 2: Difference in % and localization of $CD14^+$ cells in cellular & fibrocellular granulomas of infected mice:

Granuloma Type	Granuloma count (in 5 LPF)	Granuloma Diameter (µm)	Percentage of $CD14^+$ cells	Localization of Granulomatous $CD14^+$ cells
	Mean (%)	M±S.D	M±S.D	
Cellular	18.00 (27.28%)	209.00±30.05	6.63±2.67	Peripheral (++)
Fibrocellular	48.00 (72.72%)	157.63±26.89	8.33±2.08	Peripheral(++) Diffuse (+)
P value	<0.0001	<0.0001	<0.05	

S.D.: Standard Deviation

Histopathological results showed higher percentage of fibrocellular granulomas compared to cellular egg granulomas in livers of *S. mansoni* infected mice (group1) with significant difference ($p<0.0001$). Meanwhile, cellular granulomas were of larger diameters compared to fibrocellular ones, with statistically significant differences ($p<0.0001$).

The mean percentage of $CD14^+$ cells was higher in the fibrocellular granulomas compared to cellular granulomas with significant difference ($p<0.05$), however, as cellular granulomas have a greatly higher absolute number of inflammatory cells compared to fibrocellular ones, the absolute number of $CD14^+$ cells is higher in cellular granulomas. On the other hand, the distribution of $CD14^+$ cells differs in both granuloma types; they are mostly peripheral in cellular granulomas, while these cells located both at the periphery and scattered throughout the whole fibrocellular granulomas.

Discussion

Many Studies showed that schistosomes caused small proportion of individuals presents severe clinical disease (as periportal fibrosis (PPF)) that may lead to death. Severe PPF results from an abnormal deposition of extracellular matrix proteins in the periportal spaces due to a chronic inflammation triggered by Schistosomes eggs (Zaiss *et al*, 2015). In the present study, histopathological examination of liver sections from infected mice revealed many lobular and portal egg-granulomas, surrounded by cellular or fibrocellular tissue response. Also, portal tracts showed expansion by inflammatory cells with vascular proliferation and fibrous tissue deposition. In infected mice, hepatic lobule showed Kupffer cell hypeplasia and pigmentation, increase in the sinusoidal monocytes and mild sinusoidal fibrosis. These features were absent in liver sections from the healthy control mice, where only few mononuclear cells were detected in some portal tracts. The present results showed that 73% of the egg-granulomas of the infected group were fibrocellular while only 27% of the granulomas were cellular. Early cellular granulomas consist mostly of neutrophils with some eosinophils and lymphocytes; while later-on they consisted mostly of mononuclear inflammatory cells including macrophages at their peripheral zones. Fibrocellular granulomas consist of central zones of fibroblasts and deposited collagen fibers, surrounded by mononuclear inflammatory cells including macrophages. Previous studies showed that granulocytes (CD11b+) are recruited to egg granulomas in livers of infected mice, in contrast to what is seen in the livers of uninfected mice. In addition macrophages, eosinophils, neutrophils, and some lymphocytes are present in liver egg granulomas of *S. mansoni*-infected mice (Pearce and MacDonald, 2002). The present results showed that CD14 positive expression was infrequently recognized within monocytes in the portal tracts of the healthy control mice (group2) and occasionally within scattered hepatocytes, while there were obvious upregulation of CD14 in monocytes at the peripheral zones of egg granulomas as well as in the portal tracts and hepatic lobules of S. mansoni infected mice (group1). The difference in CD14 cellular expression between both groups is highly significant ($p<0.0001$).

In addition, the endotoxin-responsive monocytes/macrophages (CD14-positive) were potential sources of profibrogenic factors. The number of the hepatic $CD14^+$ cells increased in advanced fibrosis in subjects with primary biliary cirrhosis and hepatitis C. In addition, the increased CD14 positive cells were associated with high inflammatory activity (Leicester *et al*, 2006).

In the present study, cellular granulomas were of larger diameters compared to fibrocellular ones, with statistically significant differences ($p<0.0001$). The mean percentage of $CD14^+$ cells was higher in fibrocellular granulomas compared to cellular granulomas with significant difference ($p<0.05$), however, as cellular granulomas have a greatly higher absolute number of inflamma-

tory cells compared to fibrocellular ones, the absolute number of $CD14^+$ cells is higher in cellular granulomas. Also, studies showed that liver fibrosis is a wound healing response to chronic liver injury and inflammation in which macrophages and infiltrating monocytes participate in both the development and resolution phase. In humans, three monocyte subsets were identified: the classical CD14++CD16-, intermediate CD14++ CD16+, and non-classical CD14+ CD16++ monocytes, & intermediate CD14++ CD16+ monocytes accumulate in chronically inflamed human liver as a consequence of enhanced recruitment from blood and local differentiation from classical CD14++ CD16- monocytes (Liaskou *et al,* 2013).

Also, the results showed $CD14^+$ cells distribution differed in both granuloma types; they were mostly peripheral in cellular granulomas, while these cells located both at the periphery and scattered throughout whole fibrocellular granulomas Studies evaluated expression of PRRs on APCs in mice infected with *S. mansoni* and observed an up-regulation of CD14 expression on macrophages. Highest CD14expression was on liver macrophages in infected mice. These data identify a previously unrecognized role for CD14 in regulating macrophage plasticity and CD4T cell biasing during helminthiasis (Vanhoutte *et al,* 2007). This showed that CD14 expression increased in livers of infected mice suggested that CD14 might have regulatory roles during infection. Macrophages were CD14 major source in liver granulomas of *S. mansoni*-infected mice (Han *et al,* 2010). So. to analyze the expression of CD14 on granulocytes in liver egg granulomas, prepared single-cell suspensions of liver granulomas and then stained with surface markers against macrophages, eosinophils, neutrophils and CD14 found that only CD11 granulocytes had high levels of CD14 expression, compared to granulocytes with the low expression of CD11b- (Tundup *et al,* 2014). These experiments demonstrated that CD14 expression was highly upregulated in schistosome-infected. CD14 may regulate M1 & M2 phenotypes in livers of S. mansoni-infected mice. CD14 expression by macrophage is a key regulator of macrophage M1/M2 plasticity, which agreed with Layland *et al.* (2007). Role of CD14 in the regulation of immune responses need to be performed before such a hypothesis can be confirmed. CD14 may help in recognition of glycolipids as well as dsRNAs in controlling macrophage M1 activation and immune responses during infection (Sackesen *et al,* 2011)

Comparably, many studies showed the relationship between soluble types of CD14 (sCD14) and histological features in patients with other chronic liver diseases like non alcoholic fatty liver disease (NAFLD). Also, the increased sCD14 levels in nonalcoholic steato-hepatitis (NASH) patients were highly correlated with increased hepatic CD14 expression and liver inflammation (Ogawa *et al,* 2013). Polymorphism in human CD14 promoter region caused in higher levels of CD14, associated with an increase in severity of alcoholic liver disease (Jarvelainen *et al,* 2001). Animal studies, strongly supported involvement of CD14 in alcoholic liver disease and that CD14 was a risk factor for ethanol-induced pathology (Yin *et al,* 2001). Up-regulation of CD14 sensitization showed that CD14 levels are elevated in liver following chronic exposure to ethanol (Kono *et al,* 2000; Kishore *et al,* 2002). Also the role of CD14 in liver inflammation after chronic hepatitis B virus infection is essential for hepatocellular carcinoma (HCC) development. The individuals with HCC outcome had statistically higher serum levels of IL-23 than controls. HCC tissues analysis showed that $CD14^+$ inflammatory macrophages were major IL-23 producers (Zang *et al,* 2018).

Conclusions

The outcome data proved that $CD14^+$ cells played a regulatory role in the pathogenesis of *S. mansoni* induced liver fibrosis through modulation of the epithelial-mesenchymal transition (EMT).

A point is the use of more tissue markers, to evaluate the effect of different anti-helminthic, anti-inflammatory and anti-fibrotic therapeutic agents on schistosomiasis or other liver diseases. Such studies are ongoing and will be published in due time elsewhere.

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Explanation of figures

Fig. 1: Sections in liver of healthy control mice (G2); showed preserved hepatic architecture and unremarkable pathological changes of liver parenchyma and portal tracts (a) H & E stain, X100; (b) Masson trichrome stain X100; (c) Few hepatocytes and mononuclear cells showed positive expression for CD14 antigen (arrows). 73% of granulomas of infected mice were fibrocellular with only 27% of granulomas cellular. Early cellular granulomas consist mostly of neutrophils with some eosinophils and lymphocytes, later on they consist mostly of mononuclear inflammatory cells including macrophages at peripheral zones. Fibrocellular granulomas of central fibroblasts zones, and deposited collagen fibers, surrounded by mononuclear inflammatory cells including macrophages.

Fig. 2: Sections in livers of *S. mansoni* infected mice showed distorted hepatic architecture by many egg-granulomas (a&c)H&E stain, X200; (b&d)MT stain, X200, with upregulation of CD14 expression in egg-granulomas(e) and portal tracts (f).