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Original Article

Elevated circulating TLR4$^+$ monocytes in patients with liver fluke *Opisthorchis viverrini* is associated with advanced periductal fibrosis

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Abstract

Introduction: *Opisthorchis viverrini* (*Ov*) infection can lead to several disease manifestations of the bile duct including advanced periductal fibrosis (APF) and the most severe complication, cholangiocarcinoma (CCA). Monocytes migrate to the infection site and differentiate into tissue macrophages to express and release molecules such as cytokines, reactive oxygen species, and growth factors. TLR4$^+$ monocytes are classified as having a pro-tumor phenotype and secrete tumor-promoting factors. The aim of this study is to investigate the role of monocytes in the pathogenesis of opisthorchiasis.

Methodology: We used flow cytometry to measure the number of TLR4$^+$ monocytes in the circulating blood of *Ov* infected patients with or without APF compared to healthy, non-*Ov*-infected controls.

Results: We found, for the first time, that patients with AFP have elevated numbers of circulating TLR4$^+$ monocytes when compared to patients without fibrosis and healthy individuals. Intriguingly, when we measured ROS from these monocytes, we found increased ROS production in patients with APF.

Conclusions: We propose that excessive production of ROS from these TLR4$^+$ monocytes may lead to excessive injury of surrounding tissue and hence contribute to the pathological processes that lead to the development of advanced periductal fibrosis.

Key words: monocytes; *Opisthorchis viverrine*; toll-like receptor 4; reactive oxygen species; advanced periductal fibrosis.

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Introduction

Opisthorchis viverrini (*Ov*) or liver fluke infection is endemic in the Lower Mekong regions of Southeast Asia, including Thailand, with approximately 8 million people infected, especially in the northeast of Thailand where consumption of raw freshwater fishes is common [1]. Infection can cause several disease manifestations of the bile duct including cholangitis, cholelithiasis, advanced periductal fibrosis (APF), and the most severe complication, cholangiocarcinoma (CCA) [2–6]. Fibrosis occurs when tissues are damaged and normal wound healing responses are dysregulated [7], usually in response to repetitive tissue injury [8] such as *Ov* infection [9]. To date, it has been proposed that the tissue damage caused by *Ov* can arise from 1) physical damage e.g., during parasite feeding; 2) reactive oxygen species (ROS) release by infiltrating immune cells; 3) persistent inflammation. However, evidence to support the latter is scarce or sometimes contradictory.

Macrophages are recruited to the infection site to protect the host from intruding extracellular pathogens and utilize a wide range of effector mechanisms such as phagocytosis, ROS, proteases, or release cytokines and growth factors. It was initially thought that macrophages are derived from four sources 1) yolk sac 2) spleen 3) bone marrow and 4) monocytes. Until now there is no clear cut on an exact mechanism for these sources, especially with regard to *Ov* infection.

TLR4 is a pattern recognition receptor that recognizes LPS of Gram-negative bacteria and it has been reported that TLR4 can also recognize the intracellular parasite *Leishmania major* [10]. Recently,

our group has shown that TLR4 is highly expressed on the *Ov* gut epithelium and may serve as a reservoir for Gram-negative bacteria such as *H. pylori* [11]. Also, our group has recently reported that macrophage functions were enhanced in patients infected with *Ov* and further enhanced in those with APF [12]. This suggests a "double-edge sword" role of macrophages in the liver fluke, with a pathogen-protective role and inflammation-inducing activities that may promote fibrosis and liver disease. Understanding the mechanisms by which human monocytes and macrophages become activated during *Ov* infection is important to determine the processes that lead to APF and ultimately cholangiocarcinoma.

In this study, we have measured the numbers of circulating $TLR4^+$ monocytes and compared these numbers in *Ov* infected patients with or without APF, compared to healthy participants who had no infection. We then determined if these $TLR4^+$ monocytes also released molecules that could promote tissue dames and so we measured ROS production from these monocytes within the three cohorts of samples.

Methodology

Reagents and materials

Anti-CD14 and anti-TLR4 antibodies were from Biolegend, USA; RPMI 1640 media was from Life Technologies (Paisley, UK). fMLP, Dihydrorhodamine 123 were from Sigma Aldridge.

Participants

This study is an analysis of baseline data from a community-based, case-control study of risk factors associated with *Ov* infection and the development of APF, from participants from ten villages including Bankae, Banbor, Nongtu, Nonmakum, Saadsomsri, Jikngam, Songplui, Nhonkho, Huahad, and Somhong in Kalasin province (Thailand). Overall, individuals aged between 20 and 60 years old were recruited into this study. Ov infection was determined by faecal egg counts, while APF was diagnosed by ultrasonography (see below). The study groups were identified as (i) healthy controls (*Ov* negative (*Ov*-) without advanced periductal fibrosis (APF-); (ii) *Ov*-positive individuals (*Ov*+) without APF (APF-) and (iii) *Ov*-positive individuals (*Ov*+) with APF (APF+). Written informed consent was obtained from all participants. This study complied with the standard good clinical practice (GCP) guidelines and was approved by the Ethics Committee of Khon Kaen University, Khon Kaen, Thailand, reference numbers HE591185 and HE480528.

Sample size calculation

A statistical power analysis was performed to calculate the sample size. With an alpha = 0.05 and power = 0.80 (G*Power 3.1.9.2 analysis). This analysis revealed that a cohort size of 12.7 was required.

Ultrasonography

A detailed description of the ultrasonography methods used in this study can be found in previous publications [13,14]. Using a mobile, high-resolution ultrasound (US) instrument (GE model LOGIQ Book XP, GE healthcare, WI), hepatobiliary abnormalities including portal vein radical echoes, echoes in the liver parenchyma, indistinct gallbladder wall, gallbladder size, sludge, and suspected CCA were graded and recorded. Individuals were classified as having "non-advanced periductal fibrosis" or "APF-" if the US grade was 0 or 1, and "advanced periductal fibrosis" or "APF+" if the US grade was 2 or 3. Individuals with alcoholic liver disease, which is seen as fatty liver by US examination, were excluded. Individuals with marked hepatic fibrosis not related to *Ov* infection (e.g., cirrhosis from HBV or HCV) were also excluded from this study.

Cell surface staining and flow cytometry

50 µL of whole blood was stained for surface receptor expression using anti-CD14 and anti-TLR4 antibodies, by incubation with 1 µL of these antibodies, as per the manufacturer's instructions. Erythrocytes were then lysed using BD FACS Lysing Solution (ratio of 10:1) and samples were analysed by flow cytometry using a Beckman-Coulter FC500 flow cytometer measuring a total of 20,000 events/sample.

ROS measurement

50 µL of whole blood was incubated with *Opisthorchis viverrini* excretory/secretory products (OvES) for 1 hour at 37 °C. After the incubation respiratory burst: 5 µM dihydrorhodamine (DHR) 123 was added.

Statistical analysis

For data analysis, GraphPad Prism 7 (v. 7.03 h, GraphPad Software, Inc.) was used. Statistical analysis was performed with GraphPad Prism 7. The one-way ANOVA with Turkey's multiple comparison test was used. All data was presented as mean ± SD and statistical significance was set as: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ and NS = not significant.

Table 1. Baseline characteristics of participants in this study.

Characteristics	Population (%)			*p* value
	$Ov^- APF^-$ (n = 15)	$Ov^+ APF^-$ (n = 15)	$Ov^+ APF^+$ (n = 15)	
Gender				
Male	7 (46.7%)	9 (60%)	10 (66.7%)	0.76
Female	8 (53.3%)	6 (40%)	5 (33.3%)	
Age				
Years of age (mean ± SD)	46.2 ± 9.2	48.5 ± 7.3	50.6 ± 6.3	0.37

Results

Participant demographics

Fifteen healthy controls and thirty patients participated in this study (Table 1). There was a gender balance among these three groups ($p = 0.76$) and the average age of patients was also comparable ($p = 0.37$). All subjects were under 60 years of age.

Patients with APF and Ov infection have an increased number circulating TLR4+ monocytes

To compare the numbers of circulating monocyte that expresses TLR4 on their cell surface, we stained the whole blood of healthy individuals (Figure 1A), patients with Ov infection without APF (Figure 1B) and with APF (Figure 1C) with anti-CD14 and anti-TLR4 antibodies. We found that there was an increase in $TLR4^+$ monocytes in *Ov* infected patients without APF when compared to healthy controls (Figure 1D: 2.26 ± 0.56 vs 0.71 ± 0.43, respectively, $p < 0.0001$). Of note, when we compared two groups of *Ov* infected patients, we found that there was a significant increase in the numbers of $TLR4^+$ monocytes in patients with APF compared to those infected but without APF (Figure 1D, 5.59 ± 0.77 vs 2.26 ± 0.56, respectively, $p < 0.0001$).

Increased ROS production by monocytes of Ov infected patients with and without APF

$TLR4^+$ monocytes are known to produce ROS. To measure ROS from these cells, we incubated whole blood with OvES and stained for intracellular H_2O_2 produced in the cells. We first gated for $TLR4^+$ monocytes and then measured DHR123 fluorescence (Figure 2A). We found that $TLR4^+$ monocytes from patients with *Ov* infection produced more H_2O_2 after treatment with OvES when compared to noninfected individuals (Figure 2B). Interestingly when we compared between *Ov* infected patients with or without APF, we found even more ROS production in the patients with APF (Figure 2B: 7.39 ± 1.62 vs 2.81 ± 0.53, $p > 0.0001$).

Figure 2. ROS production from TLR4+ monocytes among three populations. TLR4+CD14+ monocytes were treated with OvES (time and concentration) and ROS was measured as shown: green = healthy controls (Ov-/APF-); blue = Ov+/APF- ; orange = Ov+/APF+ (A). The average geometric means of fluorescence intensity of DHR123 detection for H2O2 production for each sample were calculated and presented in (B).

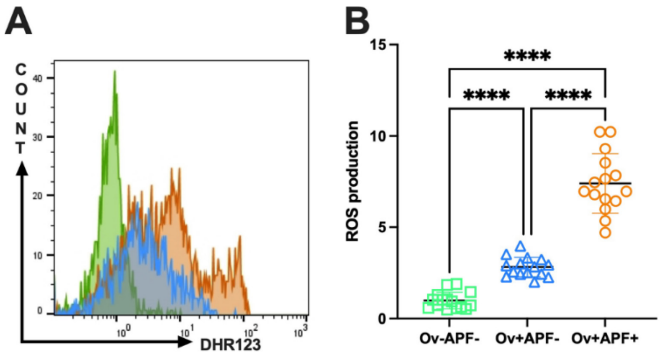
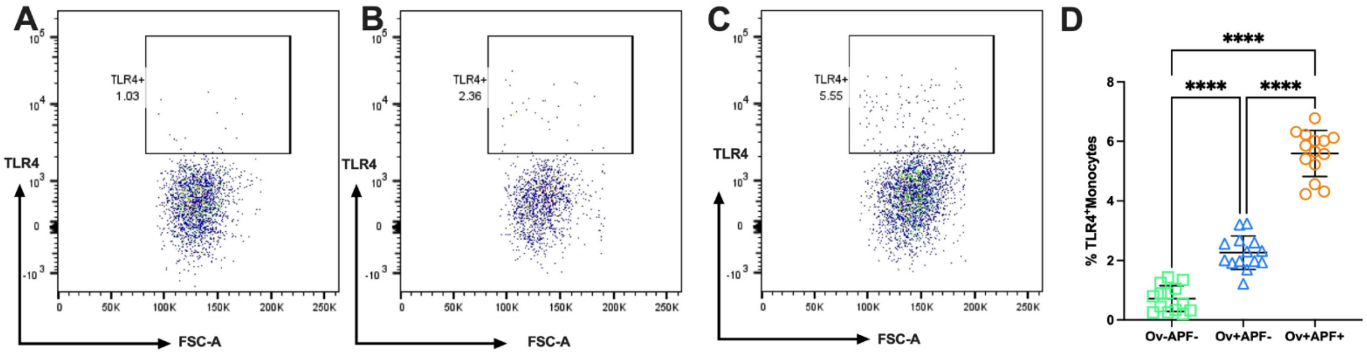


Figure 1. Numbers of TLR4+ monocytes in circulating blood. Whole blood monocytes were gated on CD14 and TLR4 and representative populations that are positive for both markers are shown for Ov-APF- (A), Ov+APF- (B) and Ov+APF+ (C). The percentage of TLR4+CD14+ monocytes are shown in D as mean values, ± SD (n = 15 for each cohort group) (D). **** is $p < 0.0001$



Discussion

Monocytes migrate to the site of infection and become tissue macrophages in response to inflammatory signals. These monocytes can polarize to become either M1 or M2 phenotype dependent upon the factors in the microenvironment milieu to acquire different properties [15]. In this work, we first measured the numbers of circulating $TLR4^+$ monocytes and then their ability to produce ROS in healthy individuals and compared these parameters in individuals who were infected with the parasite *Ov* with or without advanced periductal fibrosis (AFP). Our study shows for the first time that there is an increase in circulating $TLR4^+$ monocytes in the blood of *Ov* infected patients and this increase is greater in those infected and even higher in those with APF, when compared to healthy individuals. Intriguingly, when we measured ROS from these monocytes, we found that monocytes from *Ov* infected patients produce more ROS than healthy controls and notably that monocytes from those with APF produce the highest amount of ROS when compared to those infected but with no APF.

TLR4 is a member of the family of pattern recognition receptors (PRR), recognizing the lipopolysaccharide (LPS) of Gram-negative bacteria [16]. Previous results from our group have shown that OvES can induce cholangiocytes to upregulate TLR4 to activate NF-kB and upregulate secretion of IL-8 and IL-6, suggesting a role for TLR4 in the induction of an inflammatory cascade in *Ov* pathogenesis [17]. In line with these results presented here, we observed that *Ov* infected patients with APF have elevated $TLR4^+$ monocytes in their circulating blood, together with enhanced ROS production, inferring that TLR4 may also act as the receptor for OvES in the monocytes and further extend the inflammatory cascade in *Ov* pathogenesis. In contrast to our results, another study has shown that the mouse macrophage cell line, RAW 264.7, treated with OvES had increased expression of surface TLR2 but not TLR4 [18]. This apparently contradictory may be explained by the fact that the latter study used a mouse cell line that may not represent the primary human monocytes or macrophages and the events that occur during infections *in vivo*.

One intriguing observation from our study is that the monocytes from Ov infected individuals had increased expression of a PRR that is associated with activation by bacterial lipopolysaccharide. There is now a great deal of evidence to show that *Ov* may serve as a reservoir of *H. pylori*, a Gram-negative bacterium, resulting in coinfection with the two pathogens in endemic areas [19]. Furthermore, a long-term follow-up study following praziquantel treatment to clear Ov infection has shown that those with relapsed or persistent APF have a statistically-significant higher incidence of infection with *cagA*-positive *H. pylori* [20]. However, the mechanism by which *cagA*-positive *H. pylori* drives APF is still undefined. Our data in this report suggests a plausible mechanism that increased $TLR4^+$ monocytes and their elevated ROS production may be driven by *H. pylori* coinfection with *Ov*. However, it is unclear whether these $TLR4^+$ monocytes are actively recruited to the site of *Ov* infection, but it is likely that most of tissue-resident macrophages are derived from constant replacement from blood monocytes especially in inflammation and diseases [21]. It is possible that similar mechanisms may also function to activate other immune cells such as neutrophils, which are also activated by *H. pylori* and can also contribute to the release of ROS and other tissue-damaging molecules.

ROS has been reported to be involved in the mechanisms responsible for the development of APF in chronic *Ov* infection [22]. Previously, we also showed enhanced neutrophil functions including ROS production during *Ov* infection, that were further increased in cells from Ov-infected individuals with APF. Thus, these results further support our discovery that $TLR4^+$ monocytes may also contribute to this event by migrating to the site of *Ov* infection and polarizing to become inflammatory or M1 macrophages. A study on whether $TLR4^+$ monocytes migrate to the *Ov* infection site and their polarization to tissue-resident macrophages is now merited.

Conclusions

In this work, we investigated the numbers of circulating monocytes and their ROS production from non-infected or *Ov* infected patients with or without APF. We found an increase in $TLR4^+$ monocytes and ROS production in *Ov* infected patients. The increase was highest in patients with APF. We propose that excessive production of ROS from these $TLR4^+$ monocytes may lead to excessive bystander injury to surrounding tissues and hence result in extensive periductal fibrosis.

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