Enhanced neutrophil functions during *Opisthorchis viverrini* infections and correlation with advanced periductal fibrosis

Kanin Salaoa, Edward M. Spoffordd, Charlotte Priced, Eimorn Mairiangb, Sutas Suttiprapac, Helen L. Wrighte, Banchob Sripac,1,\* and Steven W Edwardsd,1,\*

a *Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand*

b *Department of Radiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*

c *Tropical Disease Research Laboratory, Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*

d *Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK*

e *Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool L69 7ZB, UK*

1These authors contributed equally.

\*Corresponding authors.

Professor Steven W. Edwards, Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, L69 7ZB, UK. Tel.: +44 (0)151 7945815.

*E-mail address*: s.w.edwards@liv.ac.uk

Professor Banchob Sripa, Tropical Disease Research Laboratory, Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

*E-mail address*: Banchob@kku.ac.th

**Abstract**

Millions of people are infected with the liver fluke, *Opisthorchis viverrini* (OV), but only ~25% of those infected develop liver disease and even fewer develop cholangiocarcinoma. The reasons for these differential outcomes following infection are unknown but it has been proposed that differential immune responses to the parasite may play a role. We therefore measured granulocyte (neutrophil) function in OV-infected individuals, with and without advanced periductal fibrosis (APF), to determine if these cells have a “pro-inflammatory” phenotype that may contribute to liver disease post-infection.A case-controlled study (*n*=54 in each cohort) from endemic OV-infected areas of northeastern Thailand measured neutrophil functions in whole blood from non-infected (healthy controls) and OV-infected individuals with and without APF. We measured reactive oxygen species (ROS) production, phagocytosis, receptor expression and apoptosis.Secreted products from OV cultures (obtained after in vitro culture of parasites) stimulated ROS production in non-infected healthy controls, but levels were two-fold greater after OV infection (*P*<0.0001); neutrophil ROS production in individuals with APF was double that observed in those without APF (*P*<0.0001). OV-infected neutrophils had elevated CD11b expression and greater phagocytic capacity, which was even three-fold higher in those with APF (*P*<0.0001). This “activated” phenotype of circulating neutrophils was further confirmed by the observation that isolated neutrophils had delayed apoptosis ex vivo. We believe this is the first study to show that **c**irculating blood neutrophil function is enhanced following OV infection and is more activated in those with APF. We propose that this activated phenotype could contribute to the pathology of liver disease. These data support the hypothesis of an activated innate inflammatory phenotype following OV infection and provide the first evidence for involvement of neutrophils in disease pathology.

*Keywords*: Neutrophil; Liver fluke; Opisthorchiasis; Cholangiocarcinoma; Helminth; Inflammation; Respiratory burst; Apoptosis

**1.** **Introduction**

Liver fluke infection by *Opisthorchis viverrini* and related species is a major health and economic problem in southeastern Asia, with tens of millions of people infected worldwide (Sripa et al., 2011; Furst et al., 2012). It is endemic in northeastern Thailand and countries in the lower Mekong region (Lao-People’s Democratic Republic (Lao PDR), Cambodia and southern Vietnam), with an estimated 6 million people in Thailand infected, and infection rates >65% in some regions: in Lao PDR, an estimated 37% of the country’s entire population of 6.5 million inhabitants are infected (Sayasone et al., 2011; Sripa et al., 2011). A major complication of opisthorchiasis is its association with bile duct cancer (cholangiocarcinoma, CCA), an untreatable disease with most patients dying <1 year post-diagnosis (Sripa et al., 2007, 2012). The World Health Organization (WHO) have classified *O. viverrini* as a group I carcinogen (Bouvard et al., 2009), and the Mekong region has the highest global incidence of CCA worldwide (Sripa et al., 2011; Furst et al., 2012). This represents a significant healthcare and social issue, and *O. viverrini*-induced CCA accounts for ~20,000 deaths/year in Thailand alone (Sripa and Pairojkul, 2008).

 Infection results in an inflammatory response in both humans and experimentally-infected animals, with neutrophils amongst the first cells recruited into the liver, followed later by macrophages and eosinophils (Wongratanacheewin et al., 2003; Jittimanee et al., 2007; Surapaitoon et al., 2017). These phagocytes can produce reactive oxygen- and nitrogen-species, and release other toxic molecules (e.g. proteases, Wright et al., 2010) that fail to clear the parasite, instead inducing host tissue damage e.g. DNA damage (Pinlaor et al., 2006; Yongvanit et al., 2012), which may be compounded by dietary factors (e.g. nitrosamines, Thamavit et al., 1978; Boonmars et al., 2011). Physical processes, e.g. mechanical damage caused by the parasite feeding on host tissues, plus parasite-derived factors (Chaiyadet et al., 2017), may also contribute to tissue and DNA damage, and development of periductal fibrosis (Chaiyadet et al., 2015). Parasite-derived factors (e.g. thioredoxin) can delay apoptosis of damaged epithelial cells and block tissue repair (Suttiprapa et al., 2008, 2012; Matchimakul et al., 2015) leading to advanced periductal fibrosis (APF); these factors are also immunogenic and can trigger inflammation (Sripa and Kaewkes, 2000; Sripa et al., 2002; Ninlawan et al., 2010; Nair et al., 2011). This persistent inflammation, mechanical damage and altered apoptosis results in a cycle of continued tissue damage and repair, creating an environment stimulating periductal fibrosis that leads to malignant transformation of biliary epithelial cells and eventually CCA (Smout et al., 2015).

 A major, unsolved puzzle is the heterogeneity of pathological outcomes following *O. viverrini* infection. Only ~25% of infected individuals develop APF, and only ~1-2% develop CCA (Mairiang et al., 2012), with some infected for their entire lifetime without clinical symptoms. Heterogeneous immune responses have been proposed as one likely explanation as to whether or not a patient develops APF and CCA following infection (Sripa et al., 2007, 2012). For example, a recent study showed that IL-6 production by peripheral blood mononuclear cells (PBMC) stimulated in vitro with a crude (lipopolysaccharide-depleted) supernatant from *O. viverrini* cultures (*O. viverrini*-ES), was elevated in PBMCs from infected patients with APF and was even higher in PBMCs from patients with CCA, compared with infected patients with neither of these clinical symptoms (Sripa et al., 2009, 2012). Plasma levels of IL-6 mirrored this pathology, with median levels 58x higher in APF+ patients and 221x higher in patients with CCA, compared with APF- controls.

 In view of the ability of activated neutrophils to generate pro-inflammatory and tissue damaging molecules, the aim of this study was to measure the function of these cells in the blood of individuals infected with *O. viverrini* ± APF. We sought to determine if infection with this parasite resulted in systemic activation of granulocytes in vivo and if infection-driven liver disease was associated with a more aggressive and more damaging phenotype of these cells. We show that circulating neutrophil function is elevated following OV infection and that these cells exhibit even higher activity, in terms of receptor expression, ROS production and phagocytosis, in those with APF and are also sensitized to OV products. These novel data provide the first known evidence of a role for neutrophils in the immunopathology of opisthorchiasis and liver disease.

**2. Materials and methods**

*2.1. Reagents and materials*

The monoclonal antibodies, anti-CD11b and anti-CD62L were purchased from Immunotools, Germany; HetaSep solution was from StemCell (Cambridge, UK); Ficoll-Paque was from GE Healthcare (Little Chalfont, UK); RPMI 1640 medium was from Life Technologies (Paisley, UK). fMet-Leu-Phe, (N-formylmethionine-leucyl-phenylalanine) PMA, (phorbol 12-myristate 13-acetate) latex beads, annexin V, propidium iodide, GM -CSF (granulocyte-macrophage colony-stimulating factor), TNFα (tumor necrosis factor-alpha) , Dihydrorhodamine 123 were from Sigma Aldridge (UK).

*2.2. Samples and diagnosis*

This study was approved by the Ethics Committee of Khon Kaen University, Khon Kaen, Thailand, reference numbers HE591185 and HE480528.

 Eight hundred and sixty-four individuals (adults) from 10 villages in Bankae, Banbor, Nongtu, Nonmakum, Saadsomsri, Jikngam, Songplui, Nhonkho, Huahad, and Somhong in Kalasin province (Thailand), northeastern Thailand gave written consent, completed questionnaires and provided blood (for immune functions) and stool samples for detection of *O. viverrini.* Fecal samples were processed as described previously [Elkins et al., 1991], and *O. viverrini* (OV) infection was determined by microscopic detection and quantification using the formalin/ethyl acetate technique (Elkins et al., 1991). Two hundred and thirty-four individuals (27%) were infected (designated as OV+), referred for praziquantel treatment, and examined by ultrasonography for APF.

 Of these 234 OV+ individuals, 52 were diagnosed as APF+ by ultrasonography as previously described in detail (Sripa et al., 2009). Hepatobiliary abnormalities e.g. portal vein radial echoes, echoes in liver parenchyma, indistinct gall bladder wall, gall bladder size, sludge and suspected cholangiocarcinoma (Mairiang et al., 2012) were graded as non-APF (APF-) if this grade was 0 or 1; or APF (APF+) if their grade was 2 or 3. Individuals with alcoholic liver disease (seen as fatty liver), or hepatic fibrosis that was not related to OV infection (e.g. cirrhosis from Hepatitis B Virus or Hepatitis C Virus) were excluded from this study.

 Blood samples were collected as follows: 52 x OV+/APF+; 182 x OV+/APF-; 106 x OV-/APF- (age- and sex-matched healthy controls) and used for serology, hematology and immunological assays. For the experiments shown in Fig. 1, blood was obtained from healthy individuals attending the blood donation centre at Srinagarin University Hospital, Khon Kaen, Thailand.

*2.3. Measurements on whole blood*

Whole blood (50 µl) was incubated ± GM-CSF (5 ng/mL), TNFα (10 ng/mL) or supernatant from OV cultures (OVES, see below) in a total volume of 100 µl in PBS. After 30 min incubation at 37oC, samples were treated as follows:

(i) Respiratory burst: 5 µM dihydrorhodamine 123 was added followed by addition of either 1 µM fMet-Leu-Phe or 0.1 µg/mL of PMA for a further 5 or 15 min, respectively, at 37oC. These two agents activate neutrophils via receptor-mediated and non-receptor-mediated activation pathways, respectively.

(ii) Phagocytosis: 1.0 µm diameter latex beads (at a beads:neutrophil ratio of 10:1) were added and incubated for a further 60 min at 37oC.

(iii) Receptor expression: 1 µl of anti-CD11b or anti-CD62L was added and incubated for 15 min as per the manufacturer’s instructions.

 Erythrocytes were then lysed using BD FACS Lysing Solution (BD Biosciences, UK) (ratio of 10:1) and samples analysed by flow cytometry using a Beckman-Coulter FC500 flow cytometer measuring a total of 20,000 events/sample. Responses of granulocyte, monocyte and lymphocyte sub-populations were measured by gating (see Fig. 1).

*2.4. Measurements on isolated neutrophils*

Heparinsed blood was mixed with HetaSep (StemCell Technologies, UK) (5:1 ratio of blood: HetaSep) and incubated for 30 min at 37oC. The buffy coat was removed and layered onto Ficoll-Paque (1:1), and centrifuged for 30 min at room temperature at 500 *g* (Wright et al., 2017). The upper layer was removed, while the monocyte layer at the interface and the granulocyte pellet were retained in fresh tubes. The granulocyte pellet was suspended at a ratio of 9:1 in ammonium chloride lysis buffer (13.4 mM KHCO3, 155 mM NH4Cl, 96.7 μM EDTA) for 3 min before centrifuging at 500 *g* for 3 min. The pellet was then suspended in RPMI 1640 medium, and purity and viability measured by cytospin (Romanowsky staining) and trypan blue exclusion, respectively. Cells were suspended in RMPI 1640 medium at 107 cells/mL.

*2.5. Measurements of apoptosis*

Isolated neutrophils were incubated in RPMI 1640 medium plus 10% (v/v) FBS and incubated at 37oC for 24 h. Apoptosis was determined by flow cytometry and FITC-Annexin V/propidium iodide staining (Moulding et al., 1998).

*2.6. Preparation of secreted products from O. viverrini cultures (OVES).*

Male Syrian (golden) hamsters (*Mesocricetus auratas*) were reared at the animal facilities of the Faculty of Medicine, Khon Kaen University. Protocols for the experiments were approved by the Animal Ethics Committee of Khon Kaen University, approval number AEKKU25/2554, according to the Ethics of Animal Experimentation of the National Research Council of Thailand. Mature worms, following removal from infected Syrian Hamsters (Sripa et al., 2000) were cultured in RPMI-1640, penicillin (100 U/ml), streptomycin (100 μg /ml) and protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM leupeptin and 0.1 mM E-64 (N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucine]-agmatine)) and maintained in vitro at 37°C for up to 7 days. Dead worms were periodically removed. The culture fluid was collected every 12 h and centrifuged at 1000 *g* for 10 min to remove any eggs. Supernatants were pooled, dialyzed in PBS, concentrated, absorbed with Triton-X114 to remove residual LPS (Aida and Pabst, 1990) followed by Bio-Beads SM2 (Bio-Rad) to remove Triton-X114, filtered through a 0.2 μm membrane and then aliquoted and stored at −80°C. The protein concentration was measured by the Bradford method (Bio-Rad). Its effects on neutrophil functions were determined after a 30 min incubation period

*2.7. Statistics*

All data were expressed as the mean ±S.D. Statistical comparisons were performed using the Student’s *t* test between groups. *P* <0.05 was considered statistically significant. All data were analyzed using GraphPad Prism 6.0 statistical software.

**3. Results**

*3.1. OVES primes ROS production in control blood neutrophils*

Control blood from healthy individuals (obtained from the blood donation centre, Srinagarin University Hospital, Khon Kaen) was incubated without (untreated) or with GM-CSF or TNFα for 30 min and ROS production was measured by flow cytometry. ROS production by the granulocyte population was determined by gating, as shown in Fig. 1A, and priming by these two cytokines greatly increased the magnitude of the response. We then incubated whole blood with OVES at 20 and 30 µg/mL, for 30 min prior to stimulation of ROS production in response to stimulation by fMet-Leu-Phe (concentrations of 10 µg/mL and lower had more variable effects). These experiments showed that under these experimental conditions both concentrations of OVES maximally primed fMet-Leu-Phe-stimulated ROS production by both granulocytes and monocytes, but not lymphocytes (Fig. 1B).

*3.2. Neutrophil ROS production is enhanced in OV-infected individuals with and without APF*

Uninfected individuals from the study site (OV-) acted as age- and sex-matched controls for this study and their whole blood responses were compared with those of infected individuals with (OV+/APF+) and without (OV+/APF-) APF. We first compared cellular responses to the protein kinase c activator (PKC), PMA, which revealed that PMA activated ROS increases above unstimulated controls, but there were no significant differences between groups (Fig. 2). PMA activates granulocytes via PKC independently of surface receptors and independently of the primed status of the cells: it was therefore used to measure the total capacity of neutrophils to generate ROS. These data indicate that the total capacity of granulocytes to generate ROS was unaffected by the infection status of the individuals or their liver disease.

We next measured the ability of fMet-Leu-Phe to stimulate ROS. Unlike PMA, levels of fMet-Leu-Phe-stimulated ROS are highly dependent on the priming status of neutrophils and their prior exposure to priming agents, as shown in Fig. 1A. The data shown in Fig. 3A and B show that levels of ROS production in response to fMet-Leu-Phe alone (in the absence of ex vivo priming), was low in all groups with no significant differences between groups. Similarly, GM-CSF and TNFα alone, in the absence of fMet-Leu-Phe, did not activate ROS production. However, after GM-CSF or TNFα priming, fMet-Leu-Phe activated ROS production in all groups. There was a slight increase in primed fMet-Leu-Phe ROS production in OV+/APF- individuals (compared with OV- controls) after both GM-CSF (Fig. 3A) and TNFα (Fig. 3B) priming, but this increase did not reach statistical significance. However, primed responses were significantly higher (*P*<0.0001) in the OV+/APF+ group, particularly after GM-CSF priming which was approximately three-fold elevated above levels measured in uninfected controls (Fig. 3A). This suggests that infection “sensitizes” the cells to exhibit higher ROS responses following ex vivo priming and stimulation, and that in APF+ individuals, this level of sensitization is even greater.

 Finally, we determined if parasite products in OVES had any differential effects on ROS production in these cohorts. In contrast to the effects of GM-CSF and TNFα, which alone did not activate ROS production, OVES alone increased ROS production of granulocytes in whole blood of the (OV-) uninfected controls from this study site (Fig. 4). ROS levels were approximately two-fold greater in infected (OV+/APF-) individuals and even higher in the OV+/APF+ cohort, compared with the healthy control cohort. fMet-Leu-Phe-activated responses after OVES priming were two-fold higher in OV+/APF- and four-fold greater in the OV+/APF+ cohort, compared with uninfected controls (*P*<0.0001). This again indicates that infection sensitizes granulocytes to OV products and enhanced ROS production, and that those with liver disease have significantly elevated responses.

*3.3. Neutrophil receptor expression and phagocytosis are enhanced during OV infection*

Expression of many neutrophil plasma membrane receptors can be up- or down-regulated during neutrophil activation or priming (Wright et al., 2010). Fig. 5A shows low levels of expression of surface CD11b in control healthy granulocytes which were unaltered following in vitro incubation of whole blood with OVES. However, in OV-infected individuals, levels of expression of this receptor in circulating granulocytes were significantly elevated, approximately two-fold, and four-fold in individuals that were OV+/APF+ compared with uninfected control samples (*P*<0.0001, Fig. 5A). In all samples, in vitro incubation with OVES did not further increase expression levels. No changes were detected in the expression of CD62L (which is shed upon neutrophil activation) between the groups (data not shown).

 Similar patterns were seen when we measured phagocytosis, which was low in healthy controls (and unaffected by in vitro treatment with OVES), but three-fold higher in circulating granulocytes from infected individuals (*P*<0.01) and 8-10-fold higher in those with APF (*P*<0.0001, Fig. 5B). Again, in vitro treatment with OVES did not enhance phagocytosis in any of these groups.

*3.4. Neutrophil apoptosis is delayed after OV infection*

Neutrophils normally rapidly undergo apoptosis, but survival is increased upon exposure to pro-inflammatory mediators (Moulding et al., 1998). Enhanced survival is necessary for the cells to perform their roles in infections, but can also lead to persistent/unresolved inflammation. We therefore measured rates of apoptosis in isolated neutrophils from these cohorts of control and infected individuals (*n*=12 per group). In line with previous observations (Moulding et al., 1998), neutrophils underwent extensive apoptosis when cultured in vitro over a 24 h period. As shown in Fig. 6, >80% of healthy control neutrophils underwent apoptosis after 24 h in culture. However, neutrophils from OV-infected individuals were much more resistant to apoptosis, with only ~50% undergoing apoptosis by this time (*P*<0.001). There was no significant difference in apoptosis in OV-infected individuals with and without APF.

**4. Discussion**

While there is emerging evidence implicating the immune system in the development of liver disease and CCA following OV infection (Sripa et al., 2007, 2012; Thanan et al., 2013; Surapaitoon et al., 2017), we report here, to our knowledge for the first time, that circulating neutrophils are activated in vivo following OV infection. This activated phenotype includes an enhanced ability to generate ROS, increased surface receptor expression, enhanced phagocytosis and delayed apoptosis. This finding that circulating blood cells are activated indicates that these cells are responding to systemic factors that are generated either directly by the parasite or indirectly via parasite effects on other immune cells that generate neutrophil-activating factors. In view of the ability of these cells to release tissue-damaging molecules (e.g. ROS and proteases) and their emerging roles as regulators of immune responses via the generation of a wide range of chemokines, cytokines, angiogenic factors and growth factors (Mantovani et al., 2011; Wright et al., 2014), our novel data reveal a previously unrecognized role for these cells in the immuno-pathology of opisthorchiasis.

 A major aim of this study was to further explore the hypothesis that a “pro-inflammatory phenotype” (Sripa et al., 2009, 2012) might explain why only a proportion of OV-infected individuals (~25%) develop APF or CCA (1-2%) following infection (Mairiang et al., 2012). There is considerable evidence that inflammation can be instrumental in the development of a number of cancers (Shalapour et al., 2015) and there is emerging evidence that OV-infected individuals with APF and CCA have elevated levels of pro-inflammatory markers such as IL-6 (Sripa et al., 2009, 2012). Our data presented here add further weight to this ‘pro-inflammatory’ hypothesis, but for the first time directly implicates neutrophils in this phenomenon. OV infection per se increased some neutrophil functions such as delayed apoptosis, enhanced expression of surface markers and increased capacity for phagocytosis and ROS production, and some of these activities were further enhanced in those OV-infected individuals who had developed APF. These observations lead to two conclusions: first, OV infection itself leads to pro-inflammatory events that can enhance neutrophil functions, and second, when OV-infected individuals develop APF, the levels/activity of these neutrophil-activating agents is increased further. As enhanced neutrophil function may be associated with tissue damage and events that may lead to carcinogenesis (Coffelt et al., 2016; Powell and Huttenlocher, 2016), these observations strongly support the theory of a pro-inflammatory response, at least in part, determining pathological outcomes following OV infection. Further work is needed to establish whether activated immune cells drive liver disease or if systemic neutrophil activation is a consequence of liver disease.

In our experiments, we measured ROS production, phagocytosis and receptor expression in gated granulocyte populations in whole blood assays, which contained ~90-95% neutrophils and ~2-10% eosinophils (plus ~1% basophils) (Table 1). Eosinophilia has been reported following helminth infection including OV infection, but we did not detect significant differences in circulating eosinophil counts in the three cohorts in this study, which accounted for 5.4- 6.9% of the total circulating white blood cell count and did not differ between control and patient groups (Table 1). Perhaps this is because our healthy control group were non-infected (and did not have APF) age-and sex-matched individuals from the same villages as the recruited OV+/APF- and OV+/APF+ cohorts. In response to fMet-Leu-Phe, human neutrophils generate 3-4 times more ROS than eosinophils (Sedgwick et al., 1988). Therefore, the granulocyte responses measured in this study come predominantly from neutrophils, with a minor contribution from eosinophils; contributions from eosinophils are therefore highly unlikely to contribute to the disease-specific differences and functions that we detected. It has recently been reported that *Helicobacter pylori* infection may play a role in liver carcinogenesis in a similar way to its role in gastric cancer (Boonyanugomol et al., 2012; Deenonpoe et al., 2015, 2017; Sripa et al., 2017). Indeed, *O.viverrini* is a reservoir for *Helicobacter* spp. (Deenonpoe et al., 2015) and liver fluke infection is associated with a higher frequency of CagA (cytotoxin-associated gene A)-positive *H. pylori* (64.6%) compared with non-infected controls (29.6%, Deenonpoe et al., 2017). *Helicobacter pylori* and its products are known activators of neutrophils (reviewed in Edwards et al., 2018) and it would be interesting to establish whether co-infection of OV+ individuals with *H. pylori* explains the elevated neutrophil responses. *Helicobacter pylori* and its secreted products are potent activators of neutrophils and there is evidence to implicate neutrophil-mediated activation processes in the pathogenesis of *H. pylori*-induced gastric complications (Wroblewski et al., 2010; Carrasco et al., 2013; Fu et al., 2016). Further work is clearly needed to establish both (i) the role of *H. pylori* infections in the pathogenesis of APF and CCA and (ii) whether *H. pylori* and its products are responsible for neutrophil activation in OV infection. If either of these theories is confirmed, then therapeutic co-targeting of OV (with praziquantel) and *H. pylori* (with antibiotics) would be a logical approach.

 OVES has been reported to activate PBMCs to generate inflammatory cytokines and it contains factors, e.g. thioredoxin, that regulate cholangiocyte apoptosis (Suttiprapa et al., 2008, 2012). We show here that when added to whole blood from healthy controls, OVES primed and activated the ability of granulocytes and PBMCs to generate ROS, in much the same way as the pro-inflammatory cytokines GM-CSF and TNFα (Fig. 1). Whether this is a direct effect of OVES on granulocyte (neutrophil) function or an indirect effect of a PBMC-derived product itself stimulated by OVES, remains to be determined. Notwithstanding whether this is a direct or indirect effect of OVES, it shows that in an inflammatory environment containing neutrophils, eosinophils and macrophages, parasite products greatly enhance the ability of neutrophils to generate ROS which could contribute to oxidative tissue damage and ultimately carcinogenesis. One the most striking effects of OVES was that it activated neutrophils isolated from individuals from the study site, even in the absence of a priming agent (Fig. 4). This result was somewhat surprising because OVES only acted as a priming agent for neutrophils in whole blood from blood donors attending Srinagarin Hospital, Khon Kaen (Fig. 1). In contrast, OVES itself activated neutrophils from OV-/healthy controls from the study site; OVES activated higher levels of ROS in OV+/APF- individuals and even higher levels in OV+/APF+ individuals. The reasons from these contrasting effects of OVES in the two control groups is not known, but it strongly suggests that OV- individuals in the study site have an activated innate immune system, perhaps as a result of: (i) past OV infections; (ii) a current OV infection not detected by the stool examination; (iii) *Helicobacter* infection; (iv) genetic factors; (v) other unknown environmental factors or (vi) combinations thereof.

 In conclusion, circulating neutrophils had enhanced functional responses following OV infection and even higher responses in infected individuals with liver disease. Moreover, these cells were sensitized to OV products in vivo, which enhances their functional capacity and potential to cause tissue damage and generate pro-inflammatory molecules. These new data are in line with our recent discovery of enhanced macrophage function in OV infected individuals with APF (Salao et al, 2019), implicating a broad activation of innate immune systems in this disease. We propose that enhanced neutrophil function contributes to the pathological events that can lead to bile duct damage and possibly CCA following infection with this liver fluke.

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**Figure legends**

**Fig. 1.** Effects of priming on Reactive oxygen species (ROS) production by granulocytes and monocytes in whole blood. Whole blood (from healthy volunteers attending the blood donation centre at Srinakarin Hospital, Khon Kaen, Thailand) was incubated in vitro in the absence (control) and presence of GM-CSF (granulocyte-macrophage colony-stimulating factor) (5 ng/mL), Tumor necrosis factor-alpha (TNFα) (10 ng/mL) or secreted products from *O. viverrini* cultures(OVES) at 20 or 30 µg/mL, as described in Section 2. After 30 min incubation, 5 µM DHR 123 was added and cells were stimulated by the addition of 1 µM fMet-Leu-Phe. ROS production was then measured by flow cytometry. ROS generation of granulocytes, monocytes and lymphocytes was then determined by application of suitable gates, as indicated in the figure. (A) Effects of GM-CSF and TNFα priming on granulocyte, monocyte and lymphocyte ROS production (red is after fMet-Leu-Phe addition to non-primed controls, which was identical to no additions; blue is after fMet-Leu-Phe addition to primed cells). (B) Whole blood was incubated in the absence (control) or presence of OVES, followed by the additions of dihydrorhodamine (DHR) 123 and fMet-Leu-Phe. Responses of granulocytes, monocytes and lymphocytes are shown after no pre-incubation with OVES (red trace) or additions of 20 µg/mL (blue trace) or 30 µg/mL (green trace) of OVES. Typical results from at least five separate experiments. PMN = neutrophils; Mo = monocytes; T/B = T- or B-cells.

**Fig. 2.** Stimulation of reactive oxygen species (ROS) production by phorbol 12-myristate 13-acetate (PMA). Whole blood from healthy controls (OV-, not infected with *O. viverrini*) or OV-infected individuals without advanced periductal fibrosis (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+) were incubated with Dihydrorhodamine 123 and then stimulated with 0.1 µg/mL of PMA for 15 min and analysis by flow cytometry. Responses of the granulocytes were determined by gating, as described in Figure. Non-PMA- stimulated responses (white bars) of each group of individuals and the PMA stimulated responses (grey bars) are shown. *n* = 54 per group and \**P* < 0.001. NS, not significant

**Fig. 3.** Effect of priming on Reactive Oxygen Species production. Whole blood from healthy controls (not infected *with O. viverrini*, OV-) or OV-infected individuals without advanced periductal fibrosis (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+) were incubated in the absence (unprimed) or presence of 5 ng/mL of Granulocyte-macrophage colony-stimulating Factor (GM-CSF) (A) or 10 ng/mL of tumor necrosis factor-alpha (TNFα) (B) for 30 min. Dihydrorhodamine 123 (at 5 µM) was then added and then primed or unprimed cells were incubated with or without fMet-Leu-Phe for 5 min before analysis by flow cytometry and gating of granulocyte responses. fMet-Leu-Phe responses in unprimed cells (white bars), GM-CSF or TNFα responses in the absence of fMet-Leu-Phe (grey bars) and ROS production by fMet-Leu-Phe responses (black bars) of GM-CSF or TNFα primed cells are shown. *n* = 51 per group for GM-CSF primed responses and *n* = 21 for each group of TNFα primed responses. \* *P* <0.01, \*\**P* <0.0001,

**Fig. 4.** Effects of secreted products from *O. viverrini* cultures(OVES) on granulocyte reactive oxygen species (ROS) production. Whole blood from healthy controls (OV-) or OV-infected individuals without advanced periductal fibrosis (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+) were incubated in the absence (unprimed) or presence of 20 µg/mL of OVES for 30 min. Dihydrorhodamine 123 (at 5 µM) was then added followed by addition of 1 µM fMet-Leu-Phe for 5 min. ROS production was determined in gated granulocytes as follows: unprimed + fMet-Leu-Phe (white bars); OVES minus fMet-Leu-Phe (grey bars); OVES + fMet-Leu-Phe (black bars). *n* = 54 for each measurement group. \**P* < 0.0001.

**Fig. 5.** Receptor expression and phagocytosis of granulocytes during *O.viverrini* (OV) infection. Whole blood from healthy controls (OV-) or OV-infected individuals without advanced periductal fibrosis (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+) were incubated in the absence (unprimed, white bars) or presence (grey bars) of 20 µg/mL of secreted products from *O. viverrini* cultures(OVES) for 30 min. (A) Surface expression of CD11b was measured in gated granulocytes. (B) Whole blood was incubated with latex particles (10:1 ratio of beads:neutrophils) for 60 min and phagocytosis determined in gated granulocytes by flow cytometry. *n* = 51 for each measurement group. \**P* < 0.01, \*\* *P* < 0.0001.

**Fig. 6.** Apoptosis of isolated neutrophils. Neutrophils were purified from blood of healthy controls (O.viverrini negative, OV-) or OV-infected individuals without advanced periductal fibrosis (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+). They were then incubated for 24 h in RPMI 1640 medium containing 10% FBS and apoptosis was determined by Annexin-V binding and flow cytometry. Viable cells were measured as Annexin V negative cells. *n* = 12 per group. \**P* < 0.001.

|  |  |  |  |
| --- | --- | --- | --- |
|  | OV- Healthy Controls | OV+/APF- | OV+/APF+ |
| Total WBC (106/ml) | 7.48±1.67 | 7.33±1.58 | 7.50±2.09 |
| Neutrophils (%) | 52.00±8.72 | 52.24±10.84 | 54.23±11.32 |
| Lymphocytes (%) | 34.03±6.88 | 33.28±7.80 | 32.01±8.63 |
| Monocytes (%) | 7.55±2.28 | 6.64±1.68 | 7.21±2.30 |
| Eosinophils (%) | 5.47±4.23 | 6.89±5.64 | 5.40±5.27 |
| Basophils (%) | 0.93±0.94 | 0.91±0.71 | 0.99±0.75 |

Table 1. White blood cell counts of *O. viverrini* negative (OV-) healthy controls, and OV+ individuals with and without advanced periductal fibrosis (APF). *n* = 54 for each cohort.

WBC, white blood cells.