High macrophage activities are associated with advanced periductal fibrosis in chronic Opisthorchis viverrini infection

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High macrophage activities are associated with advanced periductal fibrosis in chronic *Opisthorchis viverrini* infection

Running title: Innate immunity in opisthorchiasis

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Abstract

Liver fluke infection caused by *Opisthorchis viverrini* induces several hepatobiliary conditions including advanced periductal fibrosis (APF) and cholangiocarcinoma (CCA), but >25% of the infected population develops APF and 1% develop CCA. The innate immune response is the first line of defense, and macrophages are critical regulators of fibrosis. We hypothesized that macrophages from infected individuals have different capacities to either promote or suppress periductal fibrosis. We compared phagocytic activities of macrophages of healthy individuals and *O. viverrini*-infected individuals ±APF, and found that macrophages from infected individuals with APF ingested significantly higher numbers of beads compared with healthy controls and *O. viverrini*-infected individuals without APF. To further investigate proteolytic activity, we monitored real-time phagosomal proteolysis of beads conjugated to DQ BODIPY-BSA using live-cell imaging. We show that macrophages from *O. viverrini*-infected individuals with APF also have elevated phagosomal proteolysis activity, which is consistent with their increased phagocytic activity. Additionally, stimulated ROS production by blood monocytes was higher in individuals with APF compared with healthy controls and infected individuals without APF. These results suggest that during *O. viverrini* infection, macrophages with high phagocytic and proteolytic activities together with elevated ROS production, are the phenotypes that can promote tissue damage, which results in periductal fibrosis.

Keywords: *Opisthorchis viverrini*; Innate immunity; Peripheral blood mononuclear cells, Macrophages; Human; Flow cytometry

1. INTRODUCTION

Opisthorchis viverrini 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 harboring the infective stage of the parasite 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). APF provides the basis for malignant transformation to CCA. Fibrosis occurs when tissues are damaged and normal wound healing responses persist or become dysregulated (7), usually in response to repetitive tissue injury (8) such as chronic *O. viverrini* infection (5). Repeated tissue damage results from a combination of factors induced by *O. viverrini* such as 1) physical damage induced by feeding, 2) release of products such as reactive oxygen species from innate immune cells, 3) and *O. viverrini*-induced host inflammation through both innate and adaptive immunity (9). The latter mechanism, which involves host immune responses, is well supported by several findings from our group and others, which have confirmed that chronic *O. viverrini* infection promotes adaptive proinflammatory responses (10, 11), i.e. IL-6 production (11). However, to date, far less is known about *O. viverrini* effects on innate immunity.

Macrophages provide defense in innate immunity and these innate immune cells are among the earliest infiltrating cells after acute *O. viverrini* infection (9). Apart from host defense, macrophages also play a pivotal role in tissue homeostasis and wound repair. In chronic *O. viverrini* infection that usually leads to persistent inflammation, macrophages produce large

amounts of mediators that are potentially toxic to the parasites but these may also cause bystander damage to surrounding host tissues. To compensate for this injury, a small subset of macrophages induce tissue repair, which results in the formation of fibrosis or fibrogenesis (12).

We hypothesized that different rates of development of APF may be due to the consequence of different levels of innate immune responses that vary among individuals. As macrophages are part of the innate immune system and critical regulators of fibrosis (13), we sought to determine if *O. viverrini*-infected individuals with APF possess active innate immune responses (e.g. phagocytic, proteolytic activities and ROS production), and hence have a higher risk of developing APF compared with individuals without APF. Unraveling this association should take us a step closer toward identifying a high-risk group and possibly providing data for early and effective diagnosis or primary prevention of APF and CCA.

2. MATERIALS AND METHODS

2.1 Sample recruitment

The current study is an analysis of baseline data collected from a community-based, case-control study of risk factors associated with the development of APF using high resolution ultrasonography, as previously described (14), from ten villages including Bankae, Banbor, Nongtu, Nonmakum, Saadsomsri, Jikngam, Songplui, Nhonkho, Huahad, and Somhong in Kalasin province (Thailand). Overall, 332 individuals aged between 20 and 60 years old were recruited into this study. The control group consisted of 102 *O. viverrini*-infected individuals (60 males, 42 females) without APF (APF-), while the case group consisted of 104 *O. viverrini*-infected individuals (58 males and 46 females) with APF (APF +). A group of healthy donors

consisted of 126 *O. viverrini*-negative participants (68 males and 58 females) (Ov-). Written informed consents were obtained from all participants. This study complied with the standard good clinical practice (GCP) guideline and was approved by the Ethics Committee of Khon Kaen University, Khon Kaen, Thailand, reference number HE591185 and HE480528.

2.2 Sample size calculation

A statistical power analysis was performed for sample size estimation based on data from a published study (14). The effect size in this study was 0.5, which is considered medium by Cohen's (1988) criteria. With an alpha = 0.05 and power = 0.80, the projected sample size needed for this effect size (G*Power 3.1 analysis) is approximately n= 51 per group for the simplest between-group comparison. Thus, our sample size of >100 per group was more than adequate for the main objective of this study.

2.3 Ultrasonography

A detailed description of the ultrasonography methods used in this study can be found in previous publications (14, 15). Using a mobile, high-resolution ultrasound (US) machine (GE model LOGIQ Book XP, GE healthcare, WI), hepatobiliary abnormalities including portal vein radical echoes, echoes in 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 *O. viverrini* infection (e.g., cirrhosis from HBV or HCV) were also excluded from this study.

2.4 Blood collection

A total of 20 mL of venous blood collected from the participants by venipuncture, 8 mL of which was collected in sodium heparin sprayed coated tubes (cat# 367871, Becton Dickinson, NJ) and used for monocyte isolation.

2.5 Monocyte isolation for subsequent macrophage generation

The monocyte-derived macrophages (MDMs) used in this study were from peripheral blood monocytes isolated using a double gradient centrifugation method (16). Briefly, 8 mL of blood was overlaid on top of 6 mL of Ficoll solution (1.077 g/ml)(cat# 17-1440-02, GE Healthcare Life Sciences, Singapore) in a 15 mL Falcon tube (cat# 352097, Becton Dickinson, NJ) before centrifuging at 400x *g* without braking for 45 min at room temperature. The white band of peripheral blood mononuclear cells (PBMCs) at the interface was collected and washed 3 times with PBS and resuspended in 9 mL of RPMI-1640 medium (Cat #11875-093, Thermo Fisher Scientific, CA). For the second density gradient, 9 mL of the RPMI-resuspended PBMCs were placed on top of 6 mL of Percoll solution (1.131 g/mL) (cat# 28-9038-33, GE Healthcare Life Sciences, Singapore) in a 15-mL Falcon tube and centrifuged at 550x *g* without braking for 45 min at room temperature. The band of monocytes at the interface between the two phases was collected and washed 3 times with PBS. Monocytes were allowed to differentiate into macrophages by culturing in RPMI medium supplemented with 10% autologous serum on a 35-

mm petri dish (cat# CLS430165, Corning Inc., NY) for 7 days. The MDMs were then cultured in complete medium (RPMI medium containing 100 µg/mL streptomycin (Cat #15140-122, Thermo Fisher Scientific, CA), 2 mM L-glutamine (Cat #25030-081, Thermo Fisher Scientific, CA), 50 µM 2-mecaptoethanol, and 10% heat-inactivated fetal calf serum (Cat #14190-250, Thermo Fisher Scientific, CA).

2.7 Preparation of Alexa Fluor 594 dye and bodipy-BSA conjugated silica beads

The preparation of silica beads was adapted from the protocol described by Yates and Russell (17). Briefly, 3.0-µm carboxylate-modified silica particles (Cat #PSi-3.0COOH, Kisker Products for Biotechnologies, Germany) were conjugated with Alexa Fluor 594 Succinimidyl Esters (mixed isomers) (Alexa594-SE, Cat #A20004, Thermo Fisher Scientific, CA) or DQ green BODIPY bovine serum albumin (DQ-BODIPY BSA, Cat #D-12050, Thermo Fisher Scientific, CA). For opsonization, 0.5 mL of silica beads (14 mg/mL) was mixed with 0.5 mL purified goat-IgG (5 µg/mL) (Cat # I9140, Sigma-Aldrich, MO) and incubated at 37°C in 5% CO_2 for 30 min. The opsonized silica beads were then washed, and resuspended in PBS at a concentration of 1 mg/mL.

2.8 Synchronized phagocytosis

MDMs that adhered to a 35-mm petri dish were washed 3 times with PBS and resuspended in complete medium and then placed on ice for 10 min. Five µL ($2x10^6$ particles/mL) silica beads conjugated with red Alexa Fluor 594 fluorescent dye were added to the petri dish containing adhered MDMs and mixed gently. The MDMs were then incubated on ice for a further 10 min to

allow the beads to attach to the cell surface. Synchronised phagocytosis was then triggered by warming the dish to 37 ^{o}C.

2.9 Rate of Phagocytosis

After 60 min of synchronized phagocytosis of the beads by the MDMs at 37°C in a 5% CO_2 incubator, phagocytosis was stopped by 3 washes with PBS before adding 2 mL of 4% paraformaldehyde (Cat # P6148, Sigma-Aldrich, MO) and incubated at room temperature for 10 min for fixation. The fixed MDMs were then washed 3 times with PBS. Randomized images of a total of 100 images were captured using scanning modes for red Alexa Fluor 594 fluorescent dye (excitation 490 nm, emission 594 nm) using BioStation IM/IM-Q software version 2.21 available from BioStation IM-Q Time Lapse Imaging System (Nikon, Japan). Images were acquired using a x40 objective with randomized automated acquisition following the manufacturer's instructions. The phagocytosed beads were manually counted by an investigator blinded to the group assignment.

2.10 Rate of Proteolysis

MDMs that adhered to the petri dish after undergoing synchronized phagocytosis were placed into a 37 °C chamber containing 5% CO_2 on BioStation IM. The fluorescence intensities of green reporter DQ BODIPY dye (excitation 490 nm, emission 525 nm) were acquired in 60 sec intervals for 60 min. Approximately 100 cells were analyzed per sample. The fluorescence intensity was measured using an ImageJ (64-bit) software (NIH, imagej.nih.gov/ij/download/)

and plotted against time using Prism 6 (GraphPad Software Inc., CA) for ratiometric data analysis of intraphagosomal proteolysis.

2.11 Measurement of ROS production

50 µl of whole blood was incubated ± GM-CSF (5 ng/mL) in a total volume of 100 µl in phosphate-buffered saline. After 30 min incubation at 37°C, samples were treated with 5 µM dihydrorhodamine 123 followed by addition of either 1µM fMet-Leu-Phe or 0.1 µg/mL PMA for a further 5- or 15 min, respectively at 37°C. Flow cytometry was used to measure the production of ROS.

2.12 Statistical analysis

All data were expressed as the mean ± SEM. Statistical comparisons were performed using the *t* test between groups. When serial measurements were taken over time, groups were compared using two-way repeated-measures ANOVA with Bonferroni correction for multiple comparisons. A *P value* <0.05 was considered statistically significant. All data were analyzed using GraphPad Prism 6.0 statistical software.

3. RESULTS

3.1 MDMs from *O. viverrini*-infected individuals with APF had high phagocytic activity. MDMs from *O. viverrini*-infected individuals with APF phagocytosed more beads per cell than MDMs from healthy controls (Figures 1, A-D: 3.50 ± 0.09 vs 1.58 ± 0.87; n=102/group with >100 cells analyzed per sample, p=0.01, unpaired t test) and *O. viverrini*-infected individuals

without APF (Figures 1, A-D: 3.50 ± 0.09 vs 1.48 ± 0.05; n=102/group with >100 cells analyzed per patient, $p<0.001$, unpaired t test). We then calculated the percentage of macrophages that phagocytosed the beads and found that the percentage of phagocytic cells was increased in individuals with OV infection and increased further in APF+ individuals (Figure 1E). These data provided the first indication of a possible association between macrophages with high phagocytic activity and the presence of APF in chronic *O. viverrini* infection.

3.2 MDMs from *O. viverrini*-infected individuals with APF had elevated phagosomal proteolysis.

We then measured the rate of intraphagosomal proteolysis of individual ingested beads, as the rate of increased fluorescence of individual particles (increased fluorescence per ingested bead) by fluorescence imaging microscopy. The rate of proteolysis of BSA protein after phagocytosis (measured by BODIPY fluorescence intensity) by MDMs from *O. viverrini*-infected individuals with APF (Figure 2C) was greater than that from non-APF individuals (Figure 2B) and healthy controls (Figure 2A) at 30 min and 60 min after phagocytosis. The time course of BSA proteolysis clearly indicated that MDMs from patients with *O. viverrini*-induced APF proteolysed BSA faster than MDMs from the healthy controls (Figure 2D, n=102/group with 10-15 individual beads measured for each sample, $p<0.001$, two-way repeated-measures ANOVA) and *O. viverrini*-infected individuals without APF (Figure 2D, $p<0.001$, two-way repeated-measures ANOVA).

3.3 ROS production is elevated in monocytes from *O. viverrini*-infected individuals with APF.

Because macrophages used in previous two experiments are derived from peripheral blood monocytes, we then measured the production of ROS from circulating monocytes in these three cohorts. We found that there were no differences between groups if the cells in unprimed cells regardless of the stimulants. For example, there were no differences in ROS production in GM-CSF primed or unprimed cells when stimulated by the protein kinase C activator, PMA (data not shown). However, significant differences in ROS production were observed in response to the peptide, fMet-Leu-Phe which activates receptor-mediated stimulation of ROS, particularly after exposure to priming agents. In the absence of the priming agent, GM-CSF, there were no differences in ROS production between the three cohorts and fMet-Leu-Phe did not activate ROS in the absence of GM-CSF (Figure 3A-C). However, monocytes from *O. viverrini*-infected individuals with APF produced significantly more ROS than healthy control monocytes and monocytes from *O. viverrini*-infected individuals without APF (Figure 3D). These data suggest that circulating monocytes from APF+ individual are capable of generated higher levels of ROS after priming and stimulation.

4. DISCUSSION

Liver fluke infection has been widely known for more than two decades as a major cause of several hepatobiliary diseases including APF (4). The immunopathogenesis of APF is well described and it is partly induced by host adaptive immune responses through interaction of several immune cells (9) such as T cells (18), B cells (18), cytokines (11) and reactive oxygen

species (19). However, far less is known about the role of host innate immune responses on APF generation. Our studies demonstrate for the first time that MDMs from *O. viverrini*-infected individuals who have APF have high phagocytosis (Figures 1) and high proteolysis activity (Figure 2) when compared with both non-*O. viverrini*-infected controls and *O. viverrini*-infected individuals without APF. Furthermore, circulating monocytes from infected individuals with APF have elevated ability to generate ROS, compared to infected individuals without APF or healthy controls. This suggests that the presence of APF may associate with the pro-inflammatory phenotype of MDMs, consistent with several findings from our group and others (11, 14).

Phagocytosis is one of several ways for uptake of antigen by macrophages (20). This process involves the vesicular internalization of solid insoluble particles and can subsequently activate NF-κB signal transduction to produce pro-inflammatory cytokines and antimicrobial peptides (21). These pro-inflammatory products are toxic to invading pathogens, but could also damage host tissues when deregulated, especially in chronic infection/inflammation. Thus, enhanced phagocytosis found in MDMs from APF+ individuals could result in an excessive production of pro-inflammatory mediators such as reactive oxygen species. Indeed, we measured enhanced ROS production by monocytes in whole blood and found that this was significantly elevated in infected individuals with APF. Reactive oxygen species could damage host tissues surrounding the bile ducts, which in turn, undergo repair processes, which result in the development of fibrosis (22). In support of this hypothesis, a previous study showed that a subset of MDMs promotes fibrogenesis after chronic *O. viverrini*-induced tissue damage (23).

Another possible mechanism by which pro-inflammatory MDMs could lead to more advanced fibrosis is by elevated proteolytic activity. Proteolysis activity is tightly regulated by a

number of protease enzymes (24) and phagosomal pH (25) because effective proteolysis determines the fate of antigens, influencing antigen processing and presentation, which determines whether an efficient adaptive immune response is generated. As such, the elevated proteolysis that observed in this study in MDMs of APF+ individuals could induce stronger adaptive immune responses, which in turn, could promote robust inflammation, resulting in more severe host tissue damage and repair.

Chronic infection leads to inflammation and the production of inflammatory mediators that are toxic to invading pathogens (25). In the case of helminth infection, the helminthes could modulate host immune responses and switch immune responses from proinflammatory (Th1) to anti-inflammatory (Th2) responses (26). The key innate immune cells that play a significant role in this switching are MDMs, which can switch from M1- to M2-like phenotypes that can induce Th1 and Th2, respectively. Once recruited to chronic inflamed sites in response to local stimuli, M1 macrophages drive Th1 responses for pathogen clearance, whereas M2 macrophages drive Th2 responses for tissue remodeling and fibrosis (12). Therefore, in future studies, identification of MDM subsets from these populations of *O. viverrini* infected individuals will provide a better understanding of the mechanisms underlying the development of APF.

In conclusion, we measured and compared the phagocytic and proteolytic activities of MDMs between non-*O. viverrini*-infected control subjects, and *O. viverrini*-infected individuals with and without APF. We also showed elevated ROS production in those with APF. We propose that in the case of chronic *O. viverrini* infection, tissue resident macrophages with high phagocytic and proteolytic activities are the phenotypes that could damage bile duct epithelial and surrounding tissues, which results in the development of APF. Excessive production of

proinflammatory mediators from these macrophages might have effects on the parasites, but could also lead to excessive injury of surrounding tissue and hence result in extensive periductal fibrosis.

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AUTHOR CONTRIBUTION

Conceived and design experiments: KS BS. Performed the experiments: KS EM BS. Analysis and/or data interpretation: KS ST SS BS. Contributed reagents/materials/analysis/technical expertise tools: EM SS ST SWE BS. Wrote the paper: KS SS ST SWE BS.

CONFLICT OF INTERESTS STATEMENT

The authors declare no conflict of interests.

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FIGURE LEGENDS

FIGURE 1 MDMs from individuals with *O. viverrini*-induced APF phagocytosed more beads.

Numbers of phagocytosed opsonized silica beads covalently coupled to Alexa Fluor 594 were manually counted after synchronized phagocytosis for 60 min with MDMs from healthy individuals **(A)**, *O viverrini*-infected individual with no APF **(B)** and *O viverrini*-infected individual with APF **(C)**. Average number of phagocytosed beads per cell was calculated by manual counts **(D)**. The % of phagocytic cells was also calculated **(E)**. Data represents mean ± SEM analyzed using the *t* test. * indicates a p value of <0.05, and mean values are of at least 20 individual cells (chosen blinded) for each of the 102 samples in each cohort.

FIGURE 2 Proteolysis of MDMs from individuals with *O. viverrini*-induced with and without APF and healthy controls. Live MDMs that had undergone synchronized phagocytosis of silica beads covalently coupled with DQ-BODIPY BSA were continuously monitored for 60 min using an inverted live cell BioStation IM microscope. Representative images are shown in Figures **A-C**. Upper panels are bright-field images while lower panels are fluorescence images. The time course of proteolytic activity within the phagosome, measured as gain of fluorescence, is shown in Figure **D**. Data are mean ± SEM analyzed using two-way repeated-measures ANOVA of 10-15 individual beads measured in each of the 102 samples in each cohort. * indicates a *p* value of <0.05

Figure 3 ROS production of monocytes in whole blood

Whole blood from healthy controls (OV-) or OV-infected individuals without (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+) were incubated in the absence (unprimed) or presence of 5 ng/mL GM-CSF for 30 min. DHR 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 monocyte responses. Figure 3A-C show mean fluorescent intensities of DHR123 from OV-, OV+/APF-, OV+/APF+ respectively. Figure 3D, ( ) shows fMet-Leu-Phe responses in unprimed cells, ( ) GM-CSF responses in the absence of fMet-Leu-Phe and ( ) ROS production by fMet-Leu-Phe in GM-CSF cells. N = 51 per group for GM-CSF primed responses. Data represents mean ± SEM analyzed using the *t* test. * indicates a p value of <0.05.

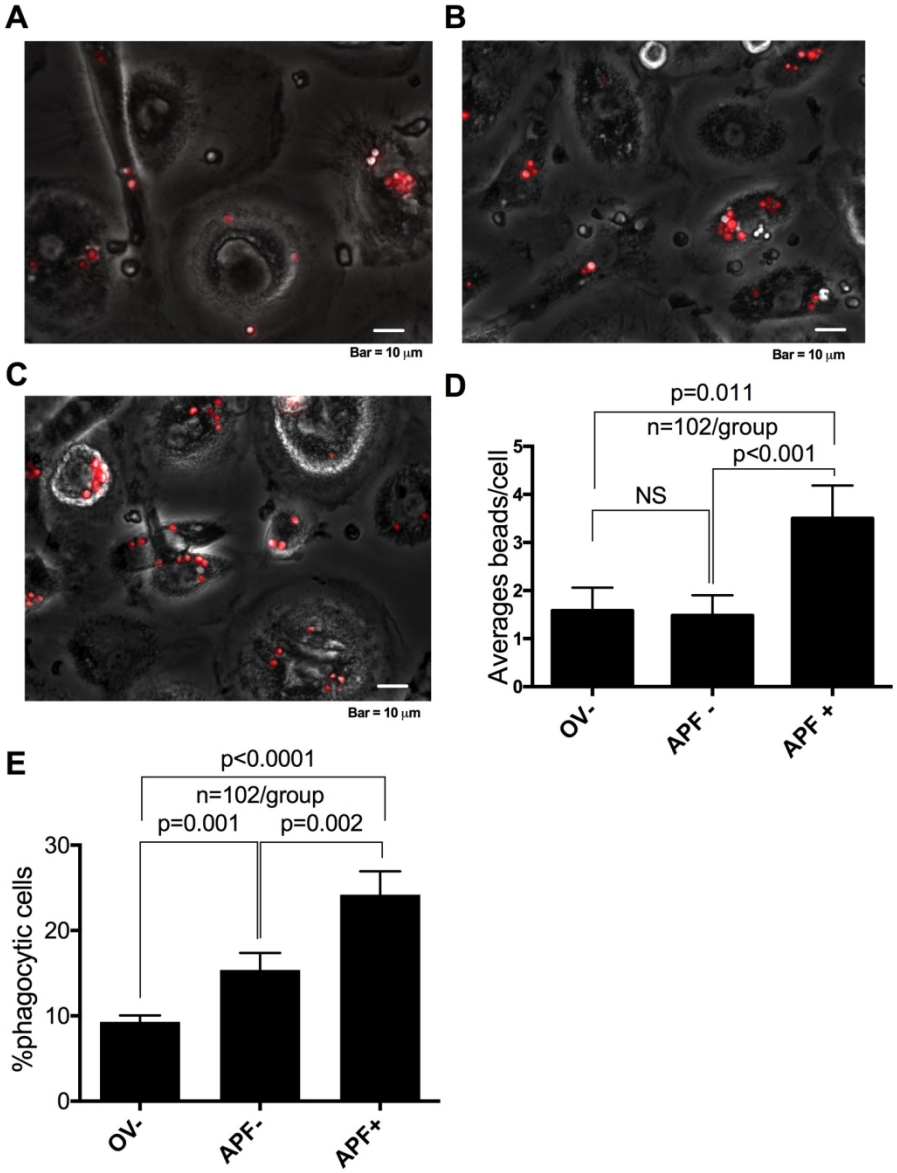

FIGURE 1 MDMs from individuals with O. viverrini-induced APF phagocytosed more beads. Numbers of phagocytosed opsonized silica beads covalently coupled to Alexa Fluor 594 were manually counted after synchronized phagocytosis for 60 min with MDMs from healthy individuals (A), O viverrini-infected individual with no APF (B) and O viverrini-infected individual with APF (C). Average number of phagocytosed beads per cell was calculated by manual counts (D). The % of phagocytic cells was also calculated (E). Data represents mean ± SEM analyzed using the t test. * indicates a p value of <0.05, and mean values are of at least 20 individual cells (chosen blinded) for each of the 102 samples in each cohort.

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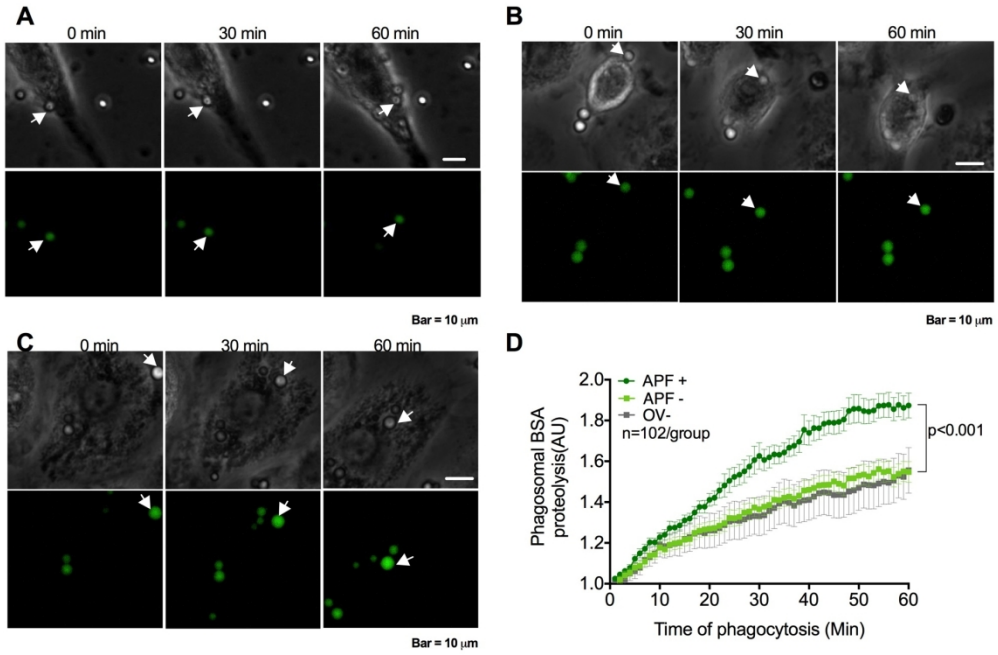

FIGURE 2 Proteolysis of MDMs from individuals with O. viverrini-induced with and without APF and healthy controls. Live MDMs that had undergone synchronized phagocytosis of silica beads covalently coupled with DQ-BODIPY BSA were continuously monitored for 60 min using an inverted live cell BioStation IM microscope. Representative images are shown in Figures A-C. Upper panels are bright-field images while lower panels are fluorescence images. The time course of proteolytic activity within the phagosome, measured as gain of fluorescence, is shown in Figure D. Data are mean ± SEM analyzed using two-way repeated-measures ANOVA of 10-15 individual beads measured in each of the 102 samples in each cohort. * indicates a p value of <0.05

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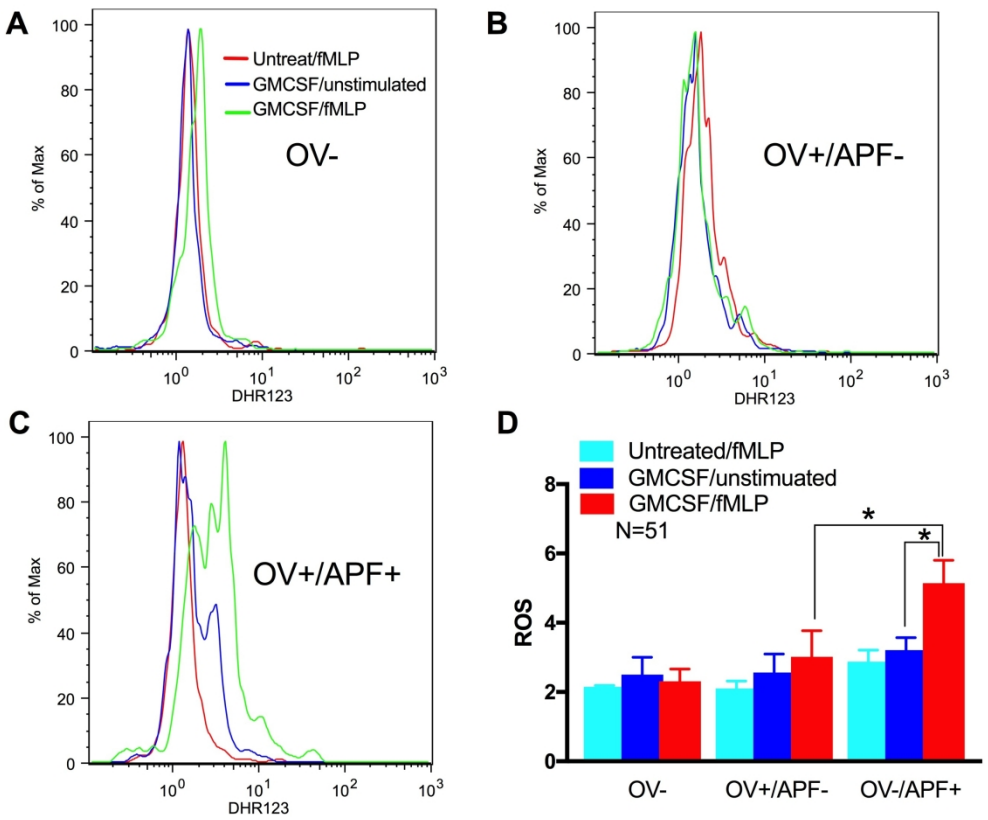

Figure 3 ROS production of monocytes in whole blood
Whole blood from healthy controls (OV-) or OV-infected individuals without (OV+/APF-) or with advanced periductal fibrosis (OV+/APF+) were incubated in the absence (unprimed) or presence of 5 ng/mL GM-CSF for 30 min. DHR 123 (at 5 μM) was then added 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 monocyte responses. Figure 3A-C show mean fluorescent intensities of DHR123 from OV-, OV+/APF-, OV+/APF+ respectively. Figure 3D, ( ) shows fMet-Leu-Phe responses in unprimed cells, ( ) GM-CSF responses in the absence of fMet-Leu-Phe and ( ) ROS production by fMet-Leu-Phe in GM-CSF cells. N = 51 per group for GM-CSF primed responses. Data represents mean ± SEM analyzed using the t test. * indicates a p value of <0.05.

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Response to reviewers' comments

Reviewer: 1

Comments to the Author
Review on "Association between macrophages phagocytosis activities and advanced periductal fibrosis in chronic opisthorchiasis"

The presented manuscript analyses in vitro generated macrophages (MDM) from patients infected with O.viverrini exhibiting (APF+) or free from (APF-) signs of advanced periductal fibrosis. The authors report an enhanced uptake of opsonised silica beads by MDM from APF+ patients. The study seems for the most part technically sound and is well described. However the conclusions drawn seem rather speculative and are only loosely based on the presented data. Overall the extent of the presented findings is very limited and would need further experimental work to corroborate the authors claims.

Response: Thank you for your comments! We agree that the work presented in the previous submission was limited as only phagocytotic and proteolytic assays were included. We have therefore performed more experiments to support our claims and conclusions. We have now measured and compared ROS among these three groups. The results can be found from lines 230-245 and Figure 3.

Individual comments:

The conclusions drawn by the authors seem speculative and one-sided. Phagocytosis per se is not pro-inflammatory but can be anti-inflammatory and prevent chronic inflammation (e.g. phagocytosis of apoptotic cells during cystic fibrosis). Thus, the authors would need to include further data on cytokine production or other inflammatory mediators to clarify whether the enhanced rate of phagocytosis promotes or protects from APF.

Furthermore, the authors repeatedly imply that the altered phagocytosis rate observed in macrophages from APF+ patients may be causative for the formation of fibrosis. However, this is not supported by the presented data. Indeed, since none of the APF- patients (some of whom will develop APF eventually) show altered macrophage phagocytosis it would seem this is a consequence rather than a cause of fibrosis.

Response: In order to address this, we have now performed ROS assays. We found that ROS production by blood monocytes was higher in individuals with APF compared with healthy controls and infected individuals without APF. ROS is a key inflammatory mediator that could cause tissue damage if overexpressed. We believe that elevated ROS production can therefore contribute to tissue damage and promote persistent inflammation. Also, we have re-examined the phagocytosis data and have measured the % of monocytes that underwent phagocytosis (new Figure 1E). This new figure shows that in infected individuals without APF, a greater % of the population of macrophages were capable of phagocytosis, compared to non-infected controls. Therefore, infection *per se* leads to a great phagocytic activity of these macrophages.

The data presented in figure 3 is inconclusive and does not reflect on the rate of proteolysis in MDM. If I understand the experimental procedure correctly macrophages were incubated with BSA-Bodipy coupled silica beads and the release of Bodipy measured over time. However, the authors have already shown that MDM from APF+ patients take up more beads in a similar assay. Thus, the increased fluorescent signal might not reflect an altered rate of proteolysis but rather a higher uptake of substrate by the APF+ MDM. Therefore this data cannot be interpreted beyond what has already been shown in figure 1&2 and should be removed. Alternatively the authors would need to provide evidence of substrate normalisation.

Response: This is a very good point raised by the referee but unfortunately, we did not adequately explain the experiment or how the data were obtained. In fact, we actually measured the increase in fluorescence of individual ingested beads. We tracked the release of bodiopy from individual engulfed beads over the first 60 min of phagocytosis, measuring the fluorescence of 10-15 individual beads per patient sample (n= 102 patient samples). We apologise for not making the nature of this measurement clear in our first submission. We added a clearer explanation in the manuscript in line 218-220.

The flowcytometry data presented in figure 2 and the microscopy pictures shown in figure 1 seem to contradict each other. Whereas the microscopy suggests virtually all macrophages do phagocytose opsonised silica beads only about 1% of cells do so according to the flow cytometry data. Is this due to variation in donors and the specific datasets chosen? If so it might be more prudent to chose more representative images / flow-plots.

Also, please include the flow cytometry data as % positive cells rather than or in addition to the MFI of the whole population.

Response: We agree with these comments. We believe that the phagocytic capacity of the cultured macrophages was grossly perturbed when we scraped them from the culture plates prior to flow cytometry, which explains their very low activity. Therefore, we have removed this figure from the revised manuscript as we believe that the data are not representative of the true phagocytic activity of these cells. The images of phagocytosis shown in Figure 1A-C are single images but we have quantified the number of ingested particles and (new Figure 1E) the number of cultured macrophages (expressed as a % of the total number), that could undergo phagocytosis. We analysed at least 100 cells/patient and believe that these are a truer representation of the phagocytic capacity of these cells.

Since the authors use autologous serum to generate MDMs in vitro they should include data on the differentiation and activation status (i.e. MHC-expression, costimulatory molecules etc.) of the macrophages from the three groups. Does serum from APF+ patients induce altered differentiation / pre-activation of MDM?

Response: These are extremely helpful comments, but we now cannot do this retrospectively. We will include such measurements in a new study.

Thank you for your invaluable comments.

Reviewer: 2

Comments to the Author
The manuscript entitled "Association between macrophages phagocytosis activities and advanced periductal fibrosis in chronic opisthorchiasis" addresses a major gap in the knowledge on the pathogenesis of chronic opisthorchiasis, namely the role innate immune cells such as macrophages. The authors demonstrate that elevated macrophage phagocytic and proteolytic activities are associated with chronic OV-induced liver fibrosis. This study will establish the basis for more mechanistic studies on the role of macrophages in OV-induced liver disease.

Response: Thank you for your comments!