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Measuring the Asian seabass (*Lates calcarifer*) neutrophil respiratory burst activity by the dihydrorhodamine-123 reduction flow cytometry assay in whole blood

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ABSTRACT

The neutrophil oxidative respiratory burst response is a key component of the innate immune system responsible for killing microbial pathogens. Since fish rely on the innate immune system for health, monitoring the respiratory burst activity may be an effective means of gauging fish health status. Here we report that the respiratory burst of Asian seabass neutrophils can be measured in whole blood by the dihydrorhodamine (DHR)-123 reduction assay and flow cytometry. Neutrophils responded to phorbol myristate acetate (PMA) in a concentration dependent manner with significant respiratory burst activity at 100–1000 nM. Other known neutrophil agonists, such as bacterial lipopolysaccharide, tumor necrosis factor, the tripeptide f-met-leu-phe and zymosan, did not induce a significant DHR reduction. Thus, the findings enable us to propose that the DHR-123 flow cytometry whole blood assay, incorporating PMA as a stimulator, would not only facilitate future studies into fish blood neutrophil research but provides a simple, rapid and reliable assay for gauging fish natural immunity status and health.

1. Introduction

There is increasing awareness to consume healthier food, including seafood, which contains high protein and unsaturated fatty acid by the aquaculture industry, globally. Asian seabass (*Lates calcarifer*), or Barramundi, is a marine species candidate for the aquaculture industry since the fish can be cultured in an intensive system, has fast growth rate, can accept artificial feed and has good market value [1]. To increase the productivity of the aquaculture industry, intensive aquaculture has been implemented for years. Despite the advantages of intensive aquaculture techniques, there are some possible challenges to the industry. Intensive farming systems usually increase the stress of the fish because of high stocking density and high feeding level which can decrease the water quality [2]. When fish are exposed to a variety of immunosuppressants for long time period, their immunity can be affected [3], increasing their susceptibility to microbial pathogens and disease, leading to mass mortality. Thus, to improve fish health and production there is a need to increase our understanding of immune mechanisms in fish and provide means of gauging changes in immunity during fish culture.

The innate immune system provides a rapid and early non-specific response critical to containing microbial pathogens [4–6]. The innate immune response involves both cellular and humoral components [4,7,8]. Examination of the state of innate immune responses can be useful biomarkers of fish health status as well as gauging the effects of immunomodulating agents on fish during farming [9]. Parameters of the innate immune responses such as phagocytosis, lysozyme and spontaneous haemolytic activity, and pentraxins can be used as indicators of health of the fish [10].

Phagocytosis is one of the innate immune defence mechanism that protects against a wide range of microbial pathogens by confining these in vacuoles in which microbicidal substances are released [11]. This can occur in the absence of an adaptive immune response and involves the rapid recognition of pathogens by receptors such as pattern

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recognition receptors (PRRs) on the phagocytes [10], and is of major importance in fish [12–15].

Interaction of neutrophils with microbial pathogens leads to the activation of the oxygen-dependent respiratory burst following the assembly of the NADPH oxidase in the plasma membrane of the phagocytic vacuole, considered to be an important defence system in fish [12,16]. The system generates superoxide and other oxygen derived reactive species which may either be microbicidal per se or combine with enzymes released from specific granules to generate potent microbicidal products [17]. Thus, measuring the state of the respiratory burst activity can be an indicator of the health status of organism including fish [18,19]. An increase in respiratory burst activity response can indicate improved health status. For example, Rainbow trout (*Oncorhynchus mykiss*) fed diet supplemented with 1% of powdered ginger roots for three weeks experienced a significantly higher respiratory burst activity in blood leukocytes compared to the control group [20].

Several methods have been used to measure the respiratory burst activity in fish phagocytes. This includes the nitro blue tetrazolium (NBT) assay. The NBT assay is a well-known method for monitoring respiratory burst [21]. In this method the reduction of NBT to produce blue dye formazan is measured spectrophotometrically [22]. This method has been classically used for fish leukocytes respiratory burst activity measurements [23], with some modifications [9,16,19,24,25]. However, the inconveniences associated with the NBT assay remain an issue. The original method by Secombes [23] has limitations such as the procedure requiring for the fish to be sacrificed for isolation of head kidney macrophages and requires time consuming procedures for isolating the cells, compromising viability and functionality.

Another method for measuring reactive oxygen species (ROS) production during the respiratory burst is by the chemiluminescence (CL) assay using a luminometer [26]. This assay is based on the amplification of natural luminescence emitted when ROS are released during phagocytosis [21]. The disadvantages of this method is that it requires for the fish to be sacrificed to obtain the head kidney and purifying the neutrophils or macrophage from the head kidney, which does not enable the fish to be monitored by resampling. Although neutrophils can be isolated from a fish blood sample, their isolation and purification from blood using density gradient separation is difficult [9], particularly as the sample volume is very small.

Recently, a successful flow cytometry (FC) method to assay ROS production in human whole blood neutrophils has been adapted to study fish neutrophils respiratory burst production [27,28]. The procedure required isolation, washing, and counting of granulocytes prior to loading the cells with the dye [22,28]. The assay uses dihydrorhodamine (DHR)-123 as the indicator dye of respiratory burst in isolated neutrophils, in which DHR-123 is oxidised by hydrogen peroxide to rhodamine-123 (Rho123). Rho123, which is fluorescent, and is then detected and measured in a flow cytometer [27].

Some studies on fish have used FC assay to measure the respiratory burst of fish leukocytes; Gilthead seabream (*Sparus aurata* L.) [29], Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.) [27], Lumpsucker (*Cyclopterus lumpus* L.) [12] and Wrasse (*Labrus bergylta* A.) [30]. Additionally, Velmurugan et al. [31] used flow cytometry for studying respiratory burst in Tilapia (*Oreochromis mossambicus*) under acute osmotic stress. Although FC assay is a very powerful tool for measuring fish respiratory burst activity, it is limited by the common procedure in these assays of having to isolate neutrophils from head kidney or spleen or peripheral blood by gradient separation. Here we show that the respiratory burst activity response in fish neutrophils can be monitored using whole blood FC assays using the DHR-123 method. This has the ability to use small blood samples that can be used to monitor the immune health of fish during culture.

2. Material and methods

2.1. Fish and animal ethics

The procedures/protocols used in the study were approved by The University of Adelaide Animal Ethics Committee (approval number S-2017-044). A total of 20 juvenile Asian seabass ranging in size from 15 to 25 cm in length were purchased from a local supplier (Robbara broodstock sanctuary and hatchery, South Australia). Fish were kept for 12 weeks in a circular plastic tank with a diameter of 2 m and 1 m deep, equipped with a recirculation system for maintaining water quality. Water conditions were maintained at 24 °C and pH of 7.6, with 0.25 mg/L nitrite, and 0.5 mg/L ammonia. Fish were fed on a commercial diet (Ridley, Australia) once a day at near satiation.

2.2. Blood sampling

Fish were anaesthetised using AQUI-S® (AQUI-S New Zealand Ltd, Australia) at 200 mg/L prior to sampling and blood collected from the caudal vein using a 1 ml syringe with a 25G needle. Approximately 500 µl of blood was collected from each fish and placed into a heparinised tube (1.5 ml Eppendorf tube containing 10 µl of heparin, 1,000 IU, DBL™ Heparin Sodium BP, porcine mucous, Hospira, Illinois, USA). The samples were transported on ice and in the main used within 3 h, but in some cases they were processed within 5 h of collection.

2.3. Dihydrorhodamine-123 flow cytometry assay of Asian seabass neutrophil respiratory burst

The respiratory burst of neutrophils in heparinised blood from individual Barramundi was assessed using the DHR-123 assay, adapted from methodology described previously [32,33]. In separate 12 × 75 mm round bottom polystyrene tubes, 25 µL blood was mixed with 225 µL phosphate buffered saline (PBS) supplemented with the DHR-123 (Sigma-Aldrich) to a final concentration of 25 µg/ml. Then incubated at either 37 °C or at 23 °C in a water bath for 15 min in the presence of the neutrophil stimulating agents; PMA, lipopolysaccharide (LPS) from *Escherichia coli*, N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLF), zymosan (Sigma-Aldrich) or human recombinant TNF (Prospec-Tany TechnoGene Ltd), known to stimulate fish leukocytes [34]. An unstimulated control tube was included with every assay. One mL of ammonium chloride lysis solution (0.15 M NH_2Cl, 10 mM $NaHCO_3$, 1 mM disodium EDTA, pH 7.4) was added to all tubes, briefly vortexed, and incubated at ambient temperature for 10 min. The tubes were then centrifuged at 500 × *g* for 3 min, and the supernatant discarded. The cells were washed and resuspended in 100 µL of PBS and then acquired on a BD FACSCanto I with BD FACSDiva v8.0 and FlowJo v10.4 (FlowJo, LLC) for analysis. Doublets were excluded by forward scatter height (FSC-H) and forward scatter area (FSC-A), and the neutrophil population gated by forward scatter (FSC) and side scatter SSC, where at least 2000 events were recorded. The median fluorescence of the FL-1 (FITC) channel was examined for increased Rho123 fluorescence representing respiratory burst activity in activated cells against unstimulated cells. Rho123-positive cells were gated using the profile of the control stimulation as a reference for setting the positive threshold (e.g. in Fig. 1d). Delta-Rho123 fluorescence intensities (ΔRho123 FI) were calculated by subtracting the geometric mean (gMean) FI or median FI of the stimulated neutrophils from that of the control neutrophils.

2.4. Data analysis

Graphing and statistical testing of the data was performed using Graphpad Prism 8.00 software (Graphpad Software Inc.). Graphs present the data points or the mean and standard deviation unless otherwise stated. For comparison between multiple concentrations of

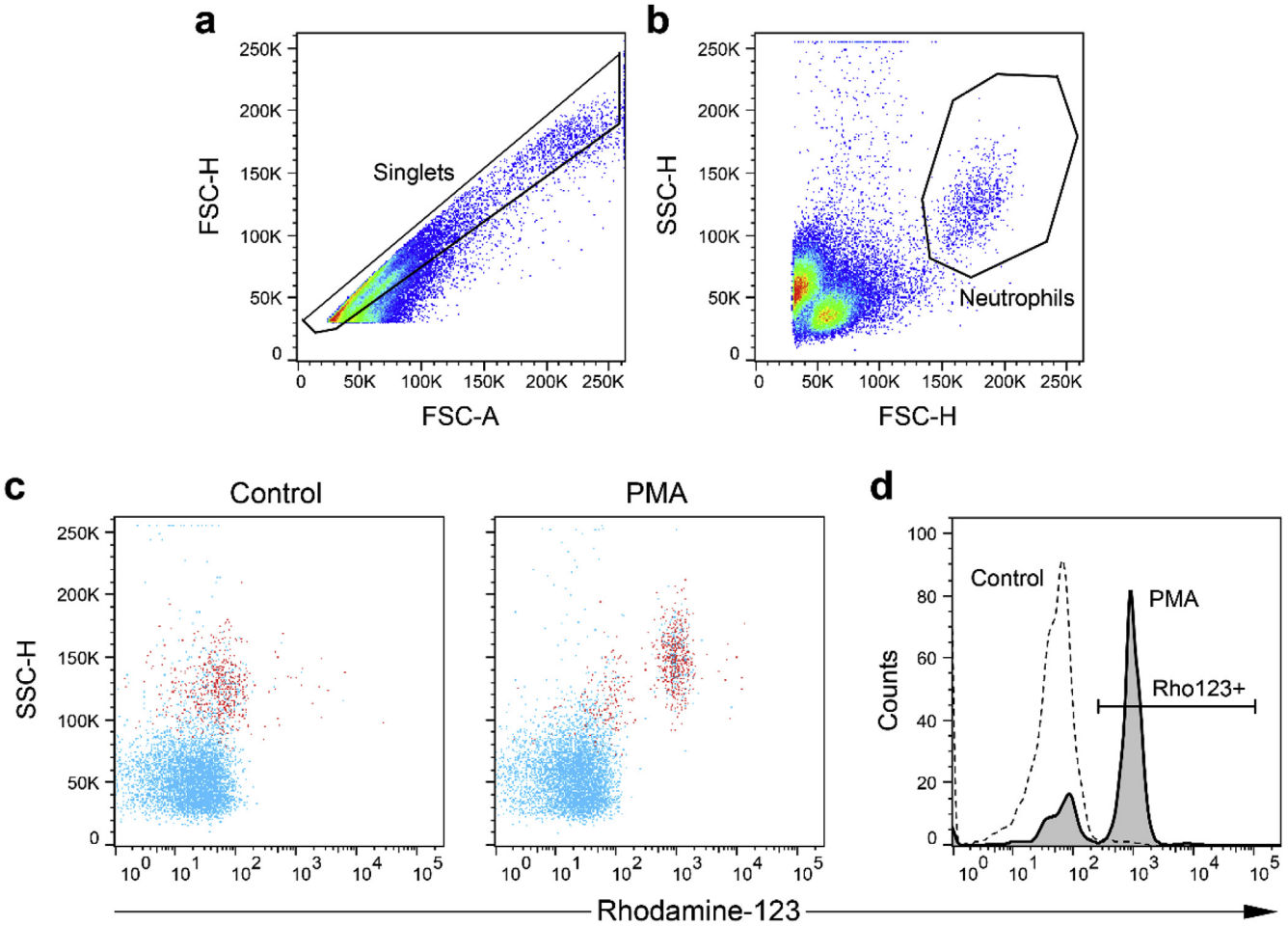

Fig. 1. Flow cytometric gating strategy for the assessment of the neutrophil respiratory burst in Asian seabass whole blood. (a) Doublets were excluded using a dot plot of FSC-A vs FSC-H. **(b)** Neutrophils were gated by their higher FSC and SSC properties than other leukocyte/nucleated populations. **(c)** Rho123 fluorescence was ascertained on singlet neutrophils (red populations) in control/unstimulated and stimulated (100 nM PMA) conditions, with histogram overlay **(d)** of the fluorescence profiles allowing resolution of the Rho123$^+$ (oxidase$^+$) neutrophil population. The positive gate was generated using the control profile as a reference for setting the threshold (Rho123$^+$% ~0.1%). Increased fluorescence resulting from ROS production is indicated by a shift to the right on the x-axis. The presented plots are all from an individual fish, representative of three tested under the same conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stimulant, one-way analysis of variance with post hoc Dunnett's Multiple Comparisons Test was used. Statistical significance was defined as $P < 0.05$. Significance levels are indicated by asterisks; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3. Results

In the first set of experiments the neutrophil assays were conducted under the same conditions as human neutrophil diagnostic testing in our Laboratory which has been accredited by the National Association of Testing Authorities, including maintaining incubation temperature at 37 °C. Fig. 1 shows representative fluorescence dot plots and histograms from Asian seabass neutrophil respiratory burst activity results. Since doublet cells can affect the analysis and could lead to inaccurate conclusions, a forward scatter height (FSC-H) vs forward scatter area (FSC-A) density plot were used to exclude doublets as shown in Fig. 1a. In order to identify cells of interest, forward scatter (FSC) vs side scatter (SSC) gating strategy was used as shown in Fig. 1b. This gating can also be useful to exclude debris and dead cells. From the gated granulocytes, the Rho123 fluorescence was measured in the FITC channel. Fig. 1c left side (control no PMA) shows fluorescence of the unoxidized DHR-123, while the PMA stimulated cells (Fig. 1c right side) show a dramatically increased amount of Rho123 fluorescence as a result of DHR-123 oxidation. Fig. 1d shows the histogram overlay of Rho123 fluorescence of control/unstimulated and PMA-stimulated Asian seabass granulocytes. The histogram shows relative cell count against fluorescence of granulocytes in unstimulated and PMA stimulated cells. A peak with very low basal fluorescence prior to stimulation was observed, and after stimulation with PMA the peak shifted to the right, indicating increased cellular fluorescence corresponding with increased respiratory burst activity.

PMA caused the activation of the respiratory burst in a concentration dependent manner (Fig. 2a). Different concentrations of PMA resulted in different fractions of activated cells. Fig. 2b shows that in the absence of PMA, the percentage of positive cells was very low with Rho123-positive cells was 2.97 ± 1.29% (mean ± standard deviation), while treatment with 1 nM of PMA could not activate the neutrophils, with the % Rho123-positive cells was 2.3 ± 1.01%. However, higher concentrations of PMA at 20, 100, 200 and 500 nM increased the % Rho123$^+$ cells to 42.03 ± 22.29%, 65.70 ± 4.88%, 66.43 ± 6.01% and 61.77 ± 8.88% respectively. The percentage of positive cells peaked at a PMA concentration of 100 nM, with no further increase seen with higher concentrations, but the geometric mean/median Rho123 fluorescence intensity (FI) measurements continued to climb at concentrations beyond 100 nM (gMean: 315.7 ± 64.2; Median: 871 ± 118.4), although seemingly approaching a plateau by

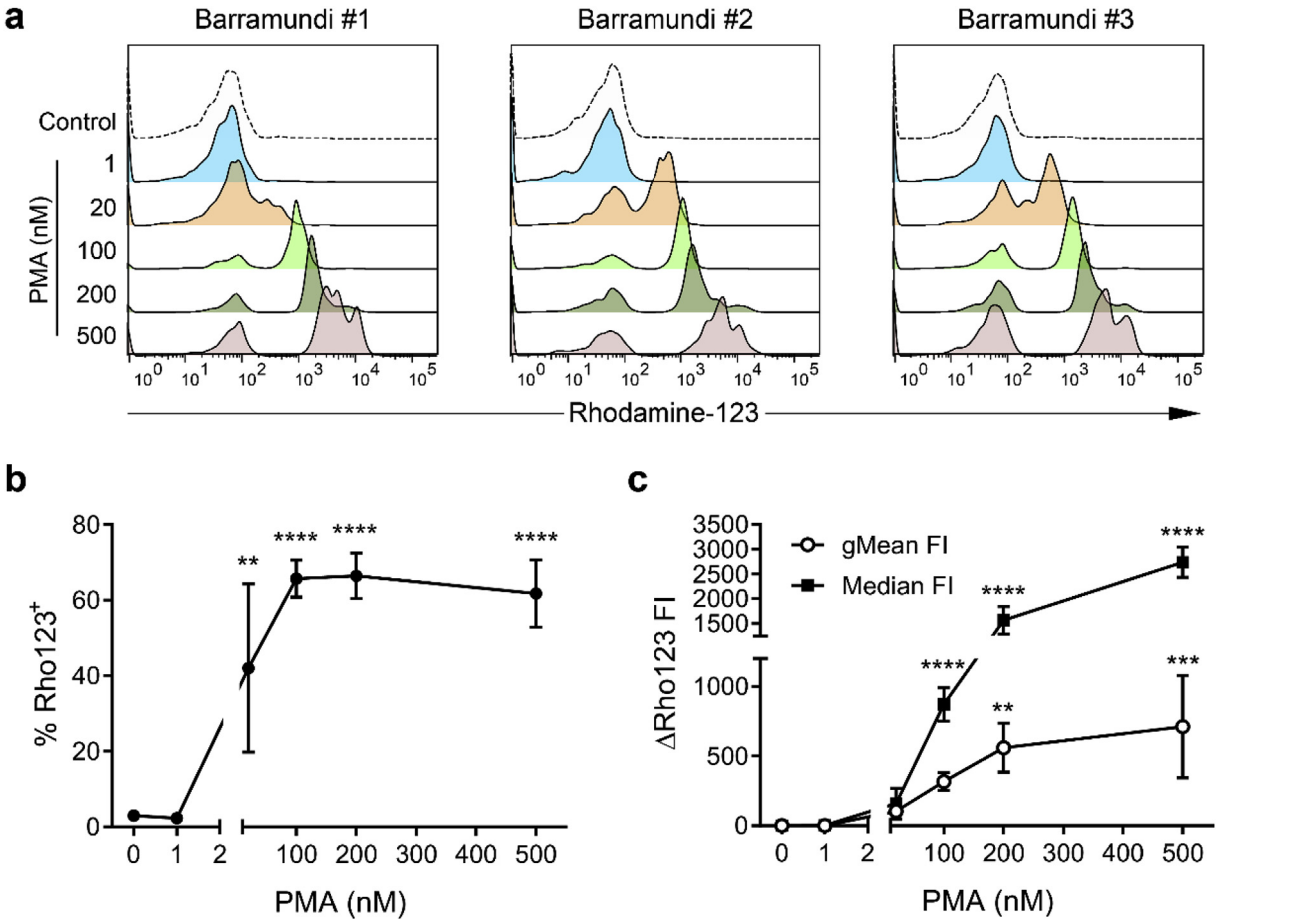

Fig. 2. PMA concentration related effects on respiratory burst activity of Asian seabass neutrophils at 37°C. (a) Aligned Rho123 histogram profiles of control and PMA treated neutrophils in three individual fish. **(b)** The Rho123$^+$% of neutrophils under each stimulation concentration. and **(c)** the median and gMean FI of the Rho123 channel from neutrophils under each stimulation concentration. Data is presented as mean ± standard deviation of three fish.

500 nM (gMean: 711.1 ± 368.7; Median: 2737 ± 306.4), indicating increased level of activity per cell (Fig. 2c). However, we selected 100 nM PMA for use as a positive control in the remaining experiments to stimulate the Asian seabass oxidative burst, as we were only interested in detecting the fraction of responsive granulocytes.

Stimulating neutrophils with different concentration of LPS to induce respiratory burst was compared to PMA as the positive control. LPS did not stimulate respiratory burst activity in Asian seabass. Fig. 3a shows there were no differences in % Rho123$^+$ cells between control (unstimulated cells) and LPS treated cells. Similarly, stimulation of Asian seabass neutrophils with TNF or fMLF, did not stimulate the cells (Fig. 3b and c). The experiment in which the fish neutrophils were treated with zymosan as the stimulating agent for respiratory burst assay in Asian seabass did not stimulate a respiratory burst (Fig. 3d).

To determine responses of Asian seabass neutrophil respiratory burst assay at a temperature comparative to aquatic conditions, we repeated the experiments with incubations at ambient laboratory temperature (23 °C). At this temperature, we were able to resolve two distinct cellular populations of higher FSC and SSC (Fig. 4a), as opposed to a single population at 37 °C. The larger and slightly less granular population was evidently neutrophils, as it demonstrated a clear capacity to reduce DHR-123 to Rho123 in response to increasing concentrations of PMA, whereas the cells of the other population were not responsive (Fig. 4a and b). We deduced this second population to be monocytic, as it is also consistently lesser in proportion than neutrophils in untreated whole blood, at a mean neutrophil-to-monocyte ratio of 1.27 ± 0.32 (n = 6), which is consistent with previously reported differential cell counts [35]. We also found that at lower incubation temperature, a higher minimal concentration of PMA of 200 nM was required to induce and allow detection of the maximal fraction (79.23 ± 3.77%) of Rho123$^+$ gated neutrophils with an active respiratory burst, with higher concentrations inducing lower Rho123$^+$ fractions (500 nM PMA: 74.1 ± 3.32%, 1000 nM PMA: 67.63 ± 5.33%) (Fig. 4c). We also found that 500 nM PMA achieved peak Rho123 FI measures (gMean: 346.2 ± 86.6; Median: 1039.1 ± 66.5) without a further increase at 1000 nM (gMean: 319.5 ± 206; Median: 1055.4 ± 367.3) (Fig. 4c). Similarly to 37 °C, the responses of the Asian seabass neutrophils and monocytes treated with arrange of concentrations of LPS, TNF, zymosan, and fMLF, under the lower temperature assay conditions, did not induce any activation of the respiratory burst (Fig. 5).

As our experimentation was limited by the number of fish specimens and also the specimen size per fish, this did not permit technical replicates. As such, we specifically examined intra-assay variation associated with the DHR-123 assay of Asian seabass neutrophils. We assessed three individual fish in triplicate assay reactions (technical replicates) for control and PMA stimulation at a concentration of 500 nM at 23 °C. We selected this specific PMA concentration as it was demonstrated to give clear resolution of Rho123$^+$ and Rho123$^-$ PMA-stimulated neutrophils and gave peak measurement of Rho123 FI (Fig. 4). The data revealed that there was minimal variation in the technical replicates of PMA-stimulated Rho123$^+$% or control and PMA-stimulated FI measurements from neutrophils of individual fish (Fig. 6a), with coefficient of variation (CV) not exceeding 10% (Fig. 6b). Across the different fish specimens, the PMA-stimulated Rho123$^+$% was the least variable measure (CV = 1.43 ± 0.86%), whilst for FI, the

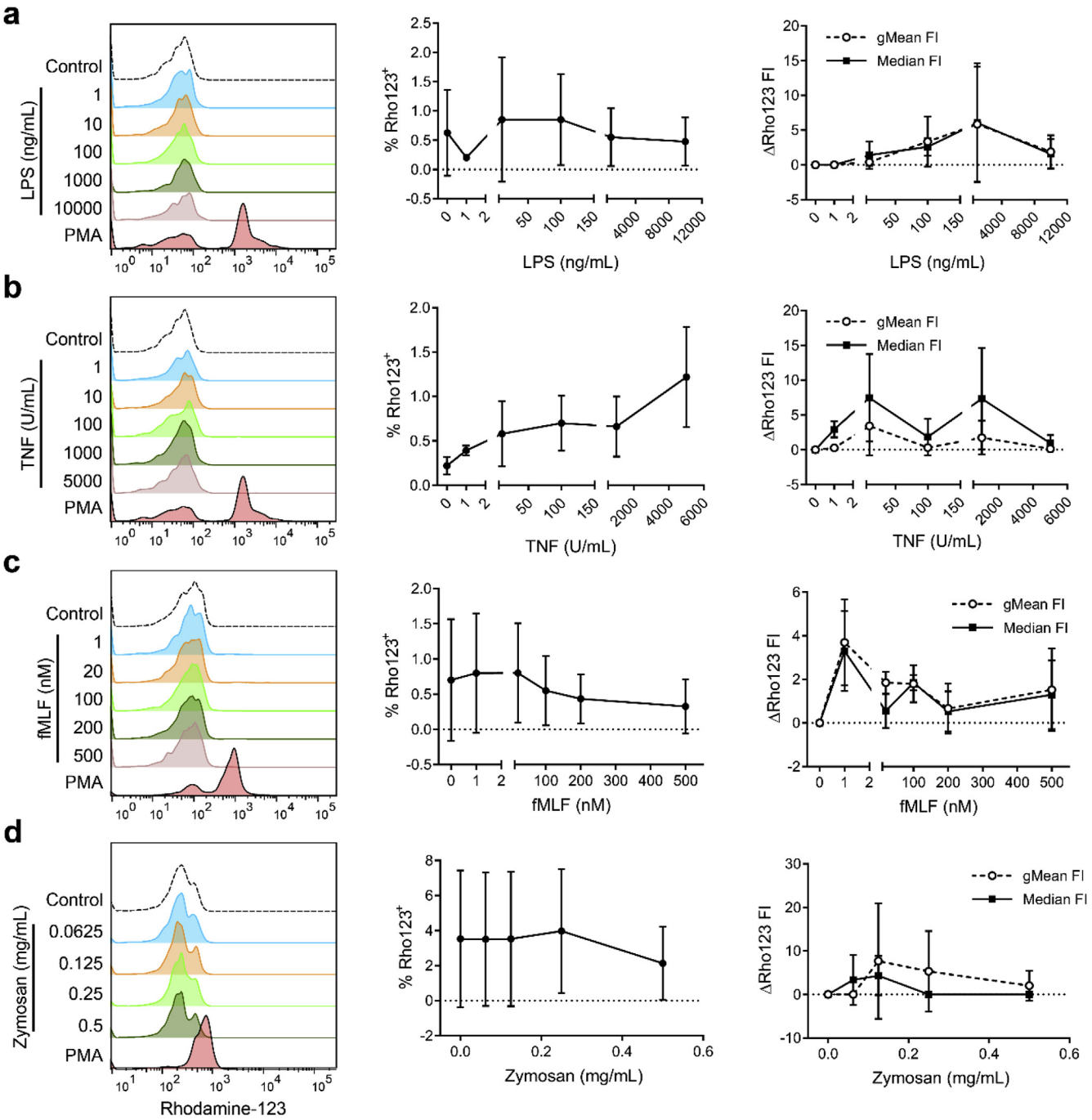

Fig. 3. Concentration related effects of LPS, TNF, zymosan and fMLF on Asian seabass neutrophils respiratory burst activity at 37°C. The representative aligned Rho123 histogram profiles of control and stimulated neutrophils with varying concentration of stimulant, are presented with Rho123^{+}% and median/gMean FI graphs of data from three individual fish: **(a)** LPS, **(b)** TNF, **(c)** fMLF and **(d)** zymosan. Note that the aligned Rho123 histogram profiles includes the PMA-stimulated profile, a treatment that was included as a positive control for respiratory burst activity in each sample.

median for PMA-stimulated neutrophils (2.42 ± 1.28%) was less variable than gMean (5.36 ± 3.83%).

4. Discussion

Respiratory burst is an important mechanism of fish innate immunity. There are several assay methods for measuring respiratory burst activity such as NBT, chemiluminescence (CL) and FC assay [11]. Flow cytometry has been used intensively to investigate respiratory burst activity in human and animal neutrophils. It provides useful data of respiratory burst activity [27]. To our knowledge, there is no information regarding the direct measurement of respiratory burst activity in whole blood by FC in Asian seabass or other fish. Tumbol et al. [8] studied the production of hydrogen peroxide (H_2O_2) in Asian seabass. However, they used isolated leucocytes from head kidney. This method has disadvantages because it is unsuitable for studies where repeat examinations of individual fish is required. In addition, isolation of phagocytic cells can lead to loss of viability and functional responses [32,36]. While Masterman and Barnes [35] have used highly enriched neutrophils preparations isolated from peripheral blood of Asian seabass in flow cytometric or chemiluminescence assays, this method is time consuming since it requires dextran sedimentation, followed by

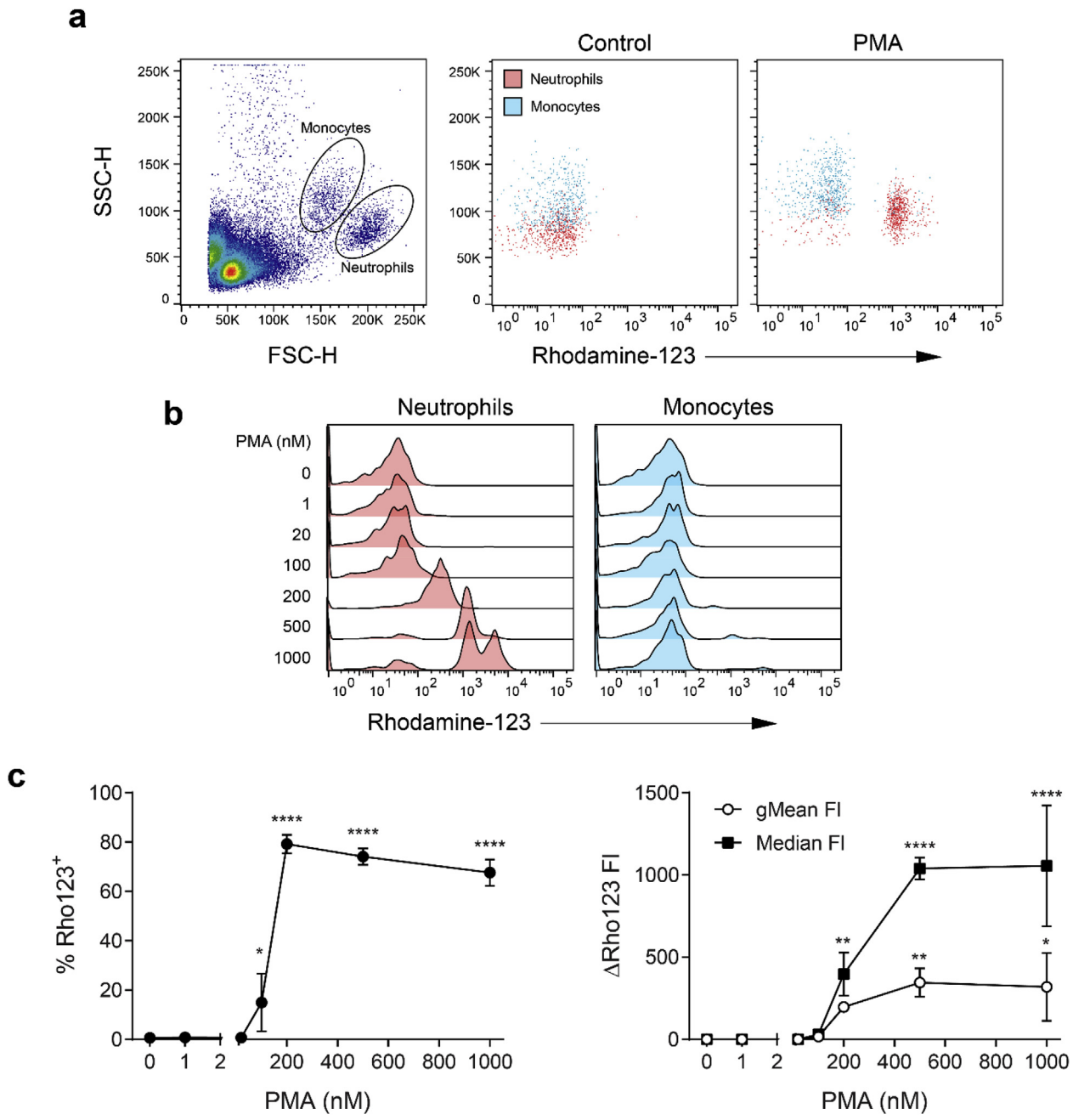

Fig. 4. Assessment of PMA-induced respiratory burst activity of Asian seabass neutrophils at 23°C. (a) At this lower temperature, two cell populations higher FSC and SSC properties than other leukocytes were resolved, with one effective in reducing DHR-123 to Rho123 (neutrophils), and the other ineffectual (monocytes), in response to 500 nM PMA. **(b)** Representative Rho123 histograms showing concentration effects of PMA on neutrophils and monocytes. **(c)** The Rho123$^+$%, median and gMean FI of neutrophils under each stimulation concentration with data representative of three individual fish.

density gradient separation on an eight step Percoll density gradient.

Our results demonstrate the effective measurement of the neutrophil respiratory burst in Asian seabass, using whole blood assays and flow cytometry. The key advantages of directly assaying whole blood by FC is that it only requires very small sample volumes (25 μL), making the method ideal for studies of the respiratory burst in small fish, since it is not practicable to collect adequate sample from small fish for neutrophil isolation. It is also possible to re-asses the same fish at different times for oxidative burst activity. Moreover, since there are no isolation steps in the procedure, it will minimize the manipulation of the blood which may modify the functional responses and/or viability of neutrophils [32]. Papp et al. [37] stated that whole blood phagocytic assays would reflect better the physiological state of the host than isolated leukocytes.

The present study used DHR-123 as the probe for assessing oxidative burst in granulocytes, since it is reported to work quicker and to be the most effective and sensitive probe for flow cytometry compared to other probes such as 2′,7′-dichlorofluorescine-diacetate (DCFH-DA) or hydroethidine (HE) [38,39]. DCFH-DA and HE are not very sensitive probes which require strong stimulation of the cells for detecting responses. In addition, DCFH-DA and HE can be oxidised by a wide range of oxidants making the method less specific [40]. The DHR-123 is responsive to H_2O_2 accumulation [38,41]. The detection of increasing fluorescence in stimulated cells is an indication of H_2O_2 production in peripheral blood neutrophils which can be detected and measured by FC [38,42]. Moreover, since the neutrophils represent a very small

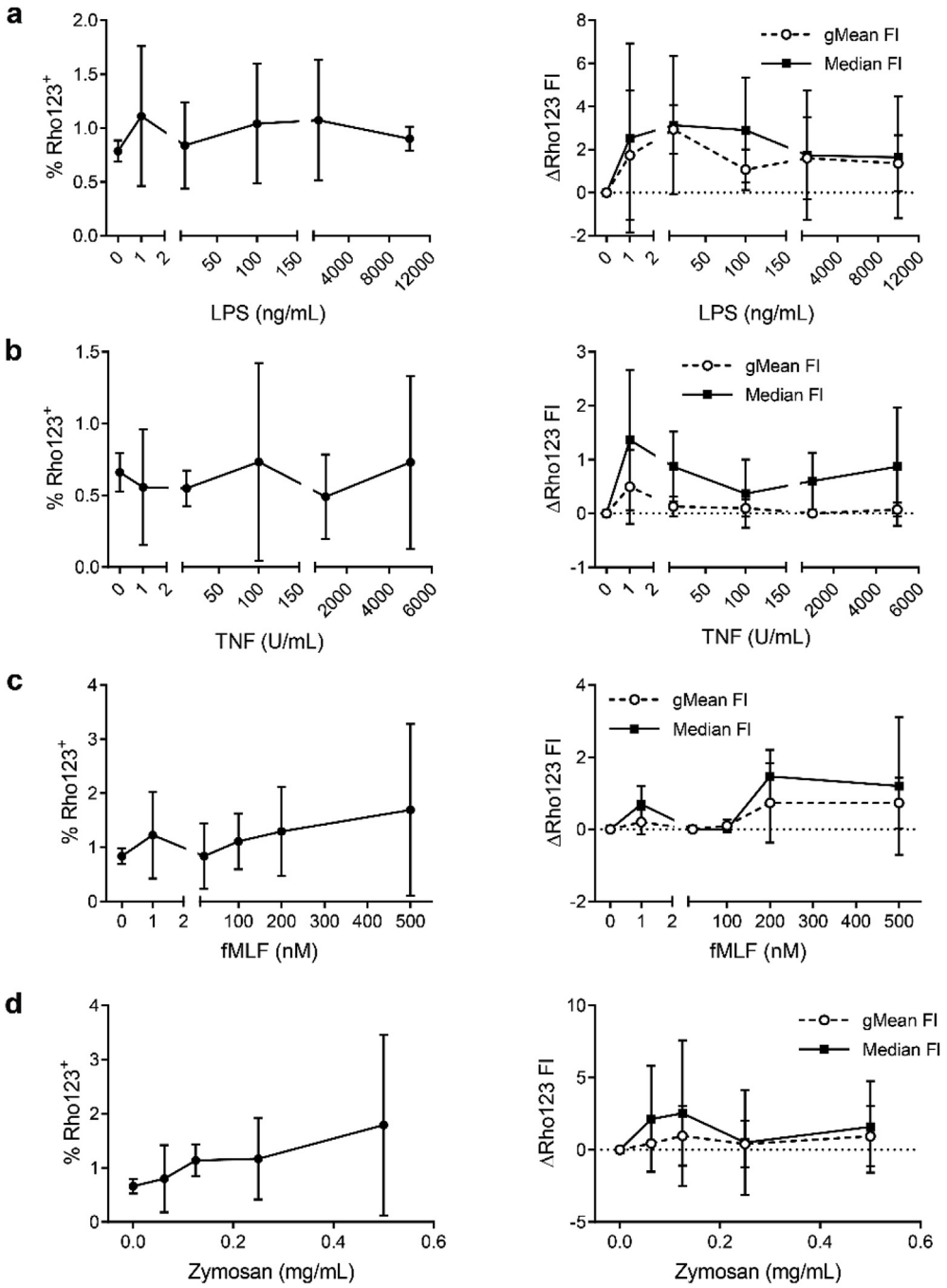

Fig. 5. Concentration related effects of LPS, TNF, zymosan and fMLF on Asian seabass neutrophils respiratory burst activity at 23°C. Rho123^{+}% and median/gMean FI graphs are presented for each stimulant with data representing three individual fish: **(a)** LPS, **(b)** TNF, **(c)** fMLF and **(d)** zymosan.

proportion of the fish leucocytes compare to what has been found in human and other mammals, the DHR flow cytometry assay becomes very valuable for measuring this cell function. It is reported that the proportions of Teleostei neutrophils is less than 5% of circulating leucocytes compare to humans which have 40–70% neutrophils in the circulation [43]. Asian seabass neutrophils represent about 9% of the blood leucocytes [35,44].

In this study we investigated the ability of the agonist PMA to stimulate a respiratory burst in Asian seabass neutrophils in whole blood assay. PMA is a well-known potent stimulating agent for activating leucocytes. The phorbol ester was found to stimulate the respiratory burst in a concentration dependent manner, using the DHR-123 assay. Tumbol et al. [8] reported that PMA stimulated the respiratory burst in the isolated neutrophils from the Asian seabass by the chemiluminescence method. Comparing the DHR-123 assay results of incubation at 37 °C (as traditionally performed on human blood) against that of 'aquatic-like' 23 °C, demonstrated the importance of using the appropriate temperature to resolve the monocyte and neutrophil populations to effectively and their differing respiratory burst capacities in Asian seabass blood. The elevated temperature affected the morphology of these cells, thus interfering with the proper gating of neutrophils, and also increased immune activity, as noted by the higher level of fluorescence detected in the Rho123^{+} population of PMA-activated neutrophils at 37 °C. The Rho123^{-} population observed in our PMA work could be partially accounted for by the presence of monocytes in the neutrophil gate (specifically at 37 °C), but the remainder were likely

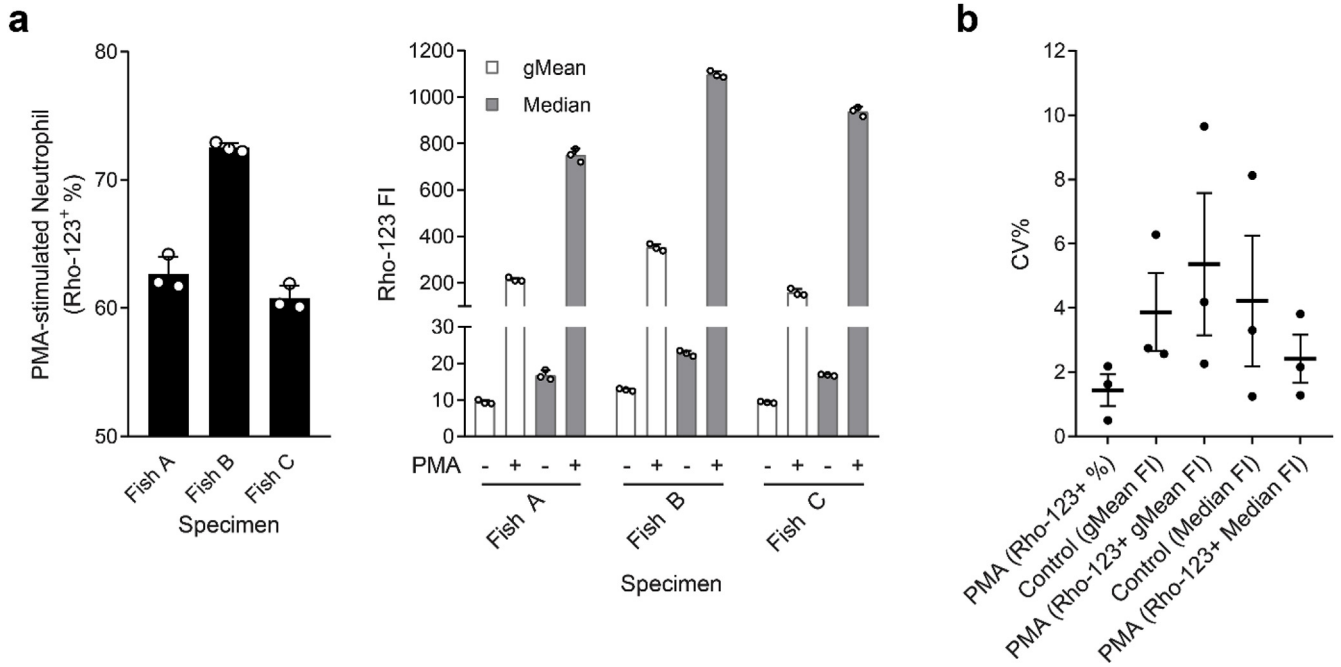

Fig. 6. DHR assay reproducibility, precision and intra-specimen variability in PMA-stimulated Asian seabass neutrophils. Three Asian seabass blood specimens were assayed for the neutrophil respiratory burst in triplicate tubes (technical replicates) for with assessment of control or PMA-stimulation (500 nM) at 23 °C. **(a)** Graphs of the Rho123^{+}% of PMA-stimulated neutrophils; and Rho123 gMean and median fluorescence intensities of control and PMA-stimulated neutrophils, with points representing each technical replicate and bars showing their mean and standard deviation. **(b)** Coefficient of variation (CV) of each triplicate measure from (a) with each point representing one fish, along with mean ± standard deviation for each Rho123 measure across the three individual fish specimens.

due to neutrophil decay or early apoptosis, due to either: (1) delayed assay due to sampling location being distant from the testing laboratory (which we minimised by expediting transport as soon as possible post-collection), where we have observed a natural decay in neutrophils has been observed in DHR assay of human blood over a few hours [33]; and/or (2) suboptimal PMA stimulation, where inadequate PMA concentration will only activate some neutrophils, whilst excessive PMA concentration increases the level of activated neutrophils that enter into an early apoptotic state.

We also examined agonists which stimulate neutrophils by engaging cell surface receptors. N-formyl-L-methionyl-L-leucyl-phenylalanine is a potent chemotactic peptide which is produced and released in tissues by bacteria during infection. It attracts and activates neutrophils by binding to specific G protein coupled receptors, the formyl peptide receptors (FPRs). The complex binding of fMLF to its specific cell surface receptor facilitates the migration of neutrophils to the site of microbial pathogen invasion and subsequently eliminating the infiltrating microbes [41,45,46]. fMLF stimulates the respiratory burst in neutrophils [47]. There are only limited reports on the activation of the respiratory burst in fish neutrophils. Our data demonstrated that fMLF over a concentration range of 1–500 nM failed to induce a respiratory burst in Asian seabass neutrophils. This result is similar to that found in Carp (*Cyprinus carpio*) head kidney neutrophils [48]. Failure of fMLF to induce migration of enriched neutrophils isolated from Barramundi has also been reported [49]. The different result in the respond of fish neutrophils to fMLF could be caused by the differences in fish species. Thus in mammalians, not all neutrophils respond to fMLF. Syrt [50] explained even though fMLF is a strong chemoattractant for most of mammalian neutrophils, neutrophils from some species such as cow, pig, dog and cat did not respond to fMLF. Since not many studies have been done using fMLF as a stimulating agent for activating fish respiratory burst and no information is available regarding the FPR receptors in fish, the underlying mechanism pathways of fMLF on Teleostei oxidative burst are still unclear and warrant further investigation.

Zymosan is a component of baker's yeast cell walls (*Saccharomyces cereviceae*) which is composed of proteins, lipids, β-glucan and chitins [28,51,52]. Insoluble β-glucan and mannan which are contained in zymosan are capable of inducing the oxidative burst. It is reported that zymosan is able to generate ROS production in human neutrophils by binding to Toll-like receptor (TLR2) and Dectin1, which is known as the receptors for zymosan in human neutrophils. Under our experimental conditions, we found that zymosan over a concentration range did not stimulate the neutrophil respiratory burst in whole blood assays compared to positive control PMA. This result is similar to that in the study of Goldfish (*Carrasius auratus* L.) in isolated neutrophils from kidneys [53]. In contrast, Pietretti et al. [51] found that zymosan can stimulate the release of reactive oxygen radicals in Carp macrophage. It has, however, been recognised that in the case of neutrophils the strong degranulation induced by zymosan may mask the respiratory burst response measurement (52). It would be interesting to follow this up using fish pathogens as the stimulators.

LPS a component of the outer membrane cell wall of gram-negative bacteria, consists of a polysaccharide region, an O-polysaccharide of variable length and a lipid part called “lipid A”, which is the primary immunostimulatory centre of LPS, responsible for the activation of the innate immune response [54]. It is reported that LPS could enhance phagocytic activity of leucocytes as occurred in eels injected with LPS. Our data demonstrated that LPS, over a concentration range, did not induce an oxygen dependent respiratory burst in fish neutrophils, compared to PMA. This result is in agreement with a previous report in gold fish neutrophils [53]. Although a respiratory burst induced by LPS was found in leukocytes from head kidney of Barramundi, this was a mixed population of neutrophils and macrophages [8]. Similar for other studies with Gilthead seabream [55], Atlantic salmon and Rainbow trout [56], Goldfish [57], and Sea bass [58]. However, LPS has been shown to induce the neutrophil respiratory burst (by NBT reduction) of Common carp by stimulation *in vitro* [59]. This information indicates that different species of fish have different capacity for respiratory burst in response to LPS, and it was of interest to examine this in Asian seabass as it has yet to be reported. Indeed, since LPS diet

supplementation also demonstrated a reduction in mortality of rainbow trout challenged with *Aeromonas hydrophila* experimental infection through the boost of immune activity [60], the exploration of the potential for similar application in Asian seabass was warranted. However, since we did not observe a significant respiratory burst induction by LPS and the other non-PMA stimulants in our experiments, including with the neutrophil stimulating cytokine, TNF [61], despite fish neutrophils having TNF receptors [62], we suggest that other potential stimulators of the burst in Asian seabass can be tested by this DHR assay prior to experiments testing its application in boosting immunity to handle experimental pathogen challenge. It would also be interesting to further elaborate on activation of the fish neutrophil respiratory burst using fish microbial pathogens.

Despite the limited number of neutrophils found in Asian seabass compared to mammalians neutrophils, the data presented supports the use of DHR-123 flow cytometry analyses using fish whole blood as a useful tool to study respiratory burst. The whole blood methodology is the advantage of DHR over other ROS detection techniques such as NBT and chemiluminescence, as it circumvents the requirement of neutrophils to be isolated to enable detection of neutrophil specific responses. Isolation of neutrophils where counts are very low is susceptible to cell loss, and not perfected yet with Asian seabass neutrophils as there has only been demonstration of the ability for enrichment, rather than purification [35]. The DHR concentration we have used was the same as that used for the human blood assay, where we have successfully detected small populations of functional neutrophils which were clearly Rho-123$^+$ (i.e. in a X-linked chronic granulomatous disease (XL-CGD) carrier case with skewed lyonisation) [33], and it has been shown by Vowells et al. [63] that samples with lower functional neutrophil content can be effectively tested with minimal loss of Rho123 FI in *in vitro* dilution of functional neutrophils with XL-CGD neutrophils. The percentage of neutrophils of the total events acquired for the samples (RBCs lysed and removed) in our experiments were consistent between individual fish (1–2%), and the CV (< 10%) of Rho123$^+$% and FI observed in the PMA responses of individual fish, indicate the methodology is robust, and a negligible effect of the phenomenon described by van Pelt et al. [64] where the varying amounts of non-neutrophil populations can influence Rho123 FI of activated neutrophils. This versatile method assures a faster, simpler and reliable way to measure neutrophil function, which could be implemented as a routinely biomarker to monitor health status of the fish in aquaculture system. PMA is a reliable stimulator in this assay to gauge the neutrophil respiratory burst activity. Indeed commercially available kits are available for this assay.

Conflicts of interest

None.

Acknowledgments

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