Characterisation of rainbow trout peripheral blood leucocytes prepared by hypotonic lysis of erythrocytes, and analysis of their phagocytic activity, proliferation and response to PAMPs and proinflammatory cytokines

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Abstract

Rapid and high quality preparation of peripheral blood leucocytes (PBL) is important in fish immunology research and in particular for fish vaccine development, where multiple immune parameters can be monitored on the same fish over time. Fish PBL are currently prepared by density separation using Percoll or Hispaque-1.077, which is time consuming, costly and prone to erythrocyte contamination. We present here a modified PBL preparation method that includes a 20 seconds hypotonic lysis of erythrocytes and a subsequent separation of PBL from cell debris by a cell strainer. This method is simple, rapid and cost effective. The PBL obtained are similar in cellular composition to those prepared by density separation but have less erythrocyte contamination as demonstrated by FACS analysis and the expression of cell marker genes. Marker gene analysis also suggested that PBL prepared by hypotonic lysis are superior to those obtained by the gradient method in that some high-density cells (certain B cell types and neutrophils) might be lost using the latter. The PBL prepared in this way can proliferate in response to the T cell mitogen PHA, and both lymphoid and myeloid cells can phagocytose fluorescent beads and bacteria, with the latter enhanced by treatment with pro-inflammatory cytokines (IL-1β and IL-6). Furthermore, the PBL can respond to stimulation with PAMPs (LPS, poly I:C) and cytokines (IL-1β and IFNγ) in terms of upregulation of proinflammatory cytokine gene expression. Such data demonstrate the utility of this approach (hypotonic lysis of erythrocytes) for PBL isolation and will enable more studies of their role in disease protection in future immunological and vaccine development research in fish.

Key words: Rainbow trout *Oncorhynchus mykiss*, peripheral blood leucocytes (PBL), hypotonic lysis of erythrocytes, phagocytosis, proliferation, immune response

1. Introduction

Fish immunology research has attracted much interest in recent years for theoretical and practical reasons. Fish immune systems provide important comparative outgroups for understanding the evolution of disease resistance. As a large vertebrate group, fish may have evolved novel mechanisms to tackle infections, and research into their responses should eventually lead to an increased understanding of the general principles of immune system adaptability in vertebrates (Feng et al., 2015; Flajnik, 2018). At the same time, the expanding aquaculture industry and associated disease risks requires fish immunology research to identify ways to manipulate the immune response and allow development of novel/ efficacious vaccines (Secombes, 2008; Van Muiswinkel, 2008; Lafferty et al., 2015; Little et al., 2016). From a functional perspective, this research needs methods to rapidly prepare leucocytes from immune tissues such as head kidney, spleen and blood that are rich in erythrocytes. Peripheral blood leucocytes (PBL) are particularly relevant to vaccine development work, since samples can be obtained multiple times from the same individual during an immune response without killing the fish.

Classically, mammalian PBL have been purified by lysis of the non-nucleated erythrocytes that they possess with hypotonic ammonium chloride solutions that are commercially available. However, teleost erythrocytes are nucleated and resistant to ammonium chloride lysis (Rowley, 1990). Fish PBL have been routinely prepared by continuous or discontinuous density gradient centrifugation through separation media such as Percoll and Histopaque (Reitan and Thuvander, 1991; Korytar et al, 2013; Maisey et al., 2016; Takizawa et al., 2016, Zhang et al., 2017). Density gradient preparation of leucocytes is time consuming, expensive, and prone to erythrocyte contamination. Crippen et al (2001) reported a simple, rapid and inexpensive leucocyte purification method by hypotonic lysis of erythrocytes. In their method, blood was diluted (1:2) and erythrocytes lysed in a hypotonic solution by addition of distilled water for 20-40 seconds (s). The osmotic pressure was then brought back to isotonicity by addition of 10x phosphate-buffered saline (PBS). The cell suspension was centrifuged (750 g, 10 min) leaving a viscous mass containing cell debris and nuclear material on top of the cells, that could be removed and discarded. Whilst the resultant PBL were comparable to PBL prepared by gradient methods (Crippen et al., 2001), this method has not gained popularity in fish immunology research. This is partly due to the difficulty in separating the PBL from a viscous mass of cell debris and nuclear material in their method, and partly the lack of demonstrated functionality of the PBL prepared.

We report here an improved hypotonic method to prepare leucocytes from fish blood using rainbow trout as a model. The blood was collected from the caudal vein and erythrocytes lysed by direct addition of cold water for 20 s (i.e. without dilution). 10x PBS was then added and the resultant PBL preparation kept on ice for 5-10 min to allow cell debris and nuclear material to clump and settle. The PBL are then easily separated from cell debris by passing through a cell strainer. The method is simple, rapid and inexpensive. The cell composition of the PBL isolated in this way is comparable to PBL prepared by use of density gradients and is free from erythrocyte contamination. Furthermore, we demonstrate that these PBL can proliferate, phagocytose and respond to pathogen associated molecular patterns (PAMPs) and cytokine stimulation.

2. Methods and Materials

2.1 Fish

Juvenile rainbow trout were purchased from College Mill Trout Farm (Perthshire, U.K.) and maintained in aerated fibreglass tanks supplied with a continuous flow of recirculating freshwater at 14℃. Fish were fed twice daily on a commercial pellet diet (EWOS), and were reared to 200 - 500 g prior to use. All the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals, and were carried out under UK Home Office project licence PPL 60/4013, approved by the ethics committee at the University of Aberdeen.

2.2. Peripheral blood leucocyte (PBL) preparation by hypotonic lysis of erythrocytes

The method for hypotonic lysis of erythrocytes was modified from that of Crippen et al. (2001). Dilution of blood was found to be unnecessary and so was omitted from the procedure, and a cell strainer was used to aid the separation of PBL from cell debris. The blood was drawn from the caudal vein using a BD Vacutainer Plus blood collection tube (with Lithium heparin, BD, UK). Premeasured HyPure cell culture grade water (36 ml, GE Healthcare Life Sciences, UK) and 10x PBS (4 ml, Sigma, UK) were cooled on ice. 4 ml of blood was transferred to a Falcon 50 mL conical centrifuge tube (or 15 mL tube for up to 1 ml blood). The erythrocytes were disrupted by combining the blood and ice-cold water and mixing by inversion for 20 s. The 10x PBS was then added to return the solution to isotonicity. The resultant PBL preparation was immediately put on ice for 5-10 min to allow the cell debris and nuclear materials to clump and settle to the bottom. The PBL were then separated from cell debris by passing through an EASYstrainer (70 µm, Greiner Bio One, UK), pelleted by centrifugation (200 g, 5 min), and washed once with incomplete cell culture medium (Leibovitz medium L-15, Life Technologies) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (P/S), and 1% foetal calf serum (FCS,

Sigma, UK). The PBL were then resuspended in complete cell culture medium (as above except 10% FCS), and live cells counted using Trypan blue exclusion. A typical PBL preparation using 4 ml caudal vein blood resulted in 120-150 million PBL.

2.3. PBL preparation by gradient centrifugation using Histopaque-1077.

1 ml of blood obtained as above was diluted to 8 ml using 1x PBS and carefully layered onto 7 ml of Histopaque-1077 (Sigma, UK) in a 15 mL conical centrifuge tube, and centrifuged (without brake) at 500 g for 40 min. The PBL were collected from the Histopaque interface, washed twice and counted as above.

2.4. FACS analysis

Peripheral blood leucocytes were isolated as above and processed for flow cytometric analysis as follows. Prior to incubation with primary antibody a total of $5x10^5$ cells per sample were blocked with PBS + 2% FCS (FACS buffer, FB) for 30 min at 4°C. The cells were then pelleted by centrifugation at 250 g for 5 min and resuspended in 200 µl FB containing mouse anti-trout IgM (protein G-purified I-14) (Deluca et al., 1983) and mouse anti-CD3ε (protein G-purified) (Maisey et al., 2016). Cells were incubated for 30 min at 4°C and then were washed with 800 µl FB. Cells were resuspended in FB (400 µl) containing the secondary antibody (Alexa 647 donkey anti-mouse IgG, Molecular Probes). Cells were incubated for 30 min at 4°C, then washed with 800 µl FB and finally resuspended in 300 µl FB prior to analysis. For autofluorescence measurement, cells were resuspended with FB containing no antibody and for isotype controls, cells were treated only with the corresponding conjugated secondary antibody. Accuri C6 Flow Cytometer was used to analyse the samples, and at least 30,000 events were recorded for each sample. Flow cytometry analyses always included cell viability (propidium iodide) staining for exclusion of dead cells. Leucocytes exhibited a characteristic distribution in forward (FSC) and side scatter (SSC) allowing the distinction between the lymphoid ($FSC^{low}SSC^{low}$) and the myeloid cell population ($FSC^{hi}SSC^{hi}$). Doublets discrimination was performed in FSC-H/FSC-A and SSC-H/SSC-A dot plots. Cells were analysed on a gate set on lymphocyte-sized cells. The gating procedure for cell analysis is shown in Supplementary Fig. S1. CFlow Plus software was used for the analysis.

2.5. Marker gene expression analysis

Freshly prepared PBL (10^7 cells) obtained by hypotonic lysis and Histopaque-1077, as described above, were used directly for total RNA preparation using TRI reagent (Sigma, UK). The cDNA synthesis and

gene expression analysis by real-time PCR were as described previously (Wang et al., 2011a, 2016). Samples from whole blood were included for comparison. To prepare total RNA, 100 µl of whole blood were washed with 1.5 ml of PBS and centrifuged (400g, 5 min). The resultant cell pellet was dissolved in 1.5 ml TRI reagent. A variety of marker genes for T cells, B cells, neutrophils, monocytes/ macrophages, thrombocytes and erythrocytes were selected (**Table 1**) for expression analysis in PBL and whole blood. The primers (**Table 2**) were designed with at least one primer of each pair crossing an intron and tested to ensure that no genomic DNA could be amplified. The expression level was normalised to that of EF-1α and expressed as arbitrary units where the expression in whole blood was defined as 1 or 100.

2.6. Phagocytosis of PBL prepared by hypotonic lysis of erythrocytes

PBL in complete cell culture medium prepared as above (2×10^6 cells/ml) were added to 12-well suspension cell culture plates (Greiner bio-one, UK). Fluorescent latex beads (FluoSpheres Fluorescent Microspheres yellow green fluorescent, 1.0 um, Life technology) were added at a cell/bead ratio of 1:20, and incubated at 20°C for 3 h. Both non-adherent and adherent cells were harvested using 0.5% trypsin-EDTA (GIBCO). Non-ingested beads were removed by centrifuging (100×g for 10 min at 4°C) over a 3% BSA and 4.5% D-glucose cushion prepared with FACS buffer (HBSS supplemented with 2% FCS, 5 mM EDTA, and 0.1% sodium azide). Cells were washed with FACS buffer and analysed with a C6 Accuri Flow Cytometer, measuring at least 75,000 cells after live cell gating according to the FCS/SSC.

For the effects of cytokine stimulation on phagocytosis, fresh PBL were stimulated with recombinant trout IL-1β (25 ng/ml, Hong et al., 2001), IL-6 (200 ng/ml, Costa et al., 2011), or medium alone as control. Fluorescent latex beads were added 20 h later at a cell/bead ratio of 1:20, incubated for a further 3 h and phagocytosis analysed as above.

2.7. Proliferation of PBL prepared by hypotonic lysis of erythrocytes

PBL proliferation was quantified by measuring BrdU incorporation during DNA synthesis in replicating cells using a Cell Proliferation ELISA, BrdU (colorimetric) kit (Sigma, UK) as per the manufacturer's instructions. Briefly, PBL from each fish in complete cell culture medium, at $4x10^5$ cells/well, were cultured in 96-well cell culture plates in the presence of 2.5, 10 and 25 µg/ml of PHA. A control without PHA and a blank control without cells were also included. Three replicate wells were used for each

177 treatment. The plates were then sealed and incubated at 20°C for 3 days. BrdU was added 20 h before
178 fixation. The cell culture medium was removed after centrifugation (400 g, 5 min) and the cells fixed
179 and DNA denatured by adding FixDenat solution. Anti-BrdU-peroxidase was then added and detected
180 using tetramethylbenzidine. The colour reaction was read at 450 nm using an ELISA plate reader
181 (SoftMax Pr0 5.3). To calculate a stimulation index, the average OD450 of triplicate wells from each
182 fish was first subtracted from the background value (without cell blank control). A stimulation index
183 was then calculated as the average of the resulting OD450 of PHA stimulated samples divided by that
184 of untreated samples.

185

186 **2.8. Immune stimulation of PBL**

187 Freshly prepared PBL obtained by hypotonic lysis of erythrocytes as above were seeded into 12-well
188 cell culture plates (Greiner bio-one, UK) at $2x10^6$ cells/ml, 2 ml/well, and stimulated with PAMPS and
189 cytokines. These included polyinosinic: polycytidylic acid (Poly I:C, 50 μg/ml, Sigma, UK),
190 lipopolysaccharide (LPS, 25 μg/ml, Sigma, UK), phytohemagglutinin from red kidney beans (PHA, 5
191 μg/ml, Sigma, UK), recombinant IL-1β (25 ng/ml, Hong et al., 2001) and IFN-γ (20 ng/ml, Wang et al.,
192 20011b) for 4 h, 8 h and 24 h. The concentration chosen for each stimulant was deemed optimal for
193 immune gene expression experiments based on our previous studies (Hong et al., 2001, 2013; Wang et
194 al., 2011a, b). Real-time PCR quantification of the expression of a variety of cytokines (**Table 2**) was
195 then undertaken as described above. The genes analysed included IL-1β1, IL-1β2 (Husain et al, 2012),
196 TNFα1 (Laing et al., 2001), TNFα2 (Zou et al., 2003), TNFα3 (Hong et al., 2013), IL-6 (Costa et al.,
197 2011), IL-8 (Laing et al., 2002), IFNγ (Zou et al., 2005) and CXCL11_L1 (Chen et al., 2013). The
198 results were expressed as a fold change relative to the time-matched unstimulated controls after
199 normalising to EF-1α.

200

201 **2.9. Statistical analysis**

202 The data were analysed statistically using the SPSS Statistics package 24.0 (SPSS Inc., Chicago,
203 Illinois). Real-time PCR data were scaled, log2 transformed and used for statistical analysis as described
204 previously (Wang et al., 2011a). The OD450 and percentage of cells were directly used for statistical
205 analysis using a paired-sample T-test, with $P < 0.05$ between groups considered significant.

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207

3. Results and Discussion

3.1. The improved hypotonic method

PBL prepared by the hypotonic lysis of erythrocytes described above were free of red blood cell contamination, as determined by microscopy, and had >99.9% viability as assessed by trypan blue exclusion. The yield from healthy fish was ~30 x10^6 PBL/ml blood. The yield and viability of PBL prepared by the gradient method were similar but the preparations were typically contaminated with some red blood cells.

Compared to the method introduced by Crippen et al. (2001), our method firstly eliminated the dilution of blood, and this allows large volumes of blood to be processed easily. Secondly, our method used premeasured cold water and PBS (kept on ice on the day of use) that streamlined the procedure. We found that the removal of the viscous mass formed in Crippen's method is difficult to perform reliably and leads to low yield or contamination with cell debris. Putting the erythrocyte lysed cell suspension on ice and letting the cell debris and nuclear materials clump and settle to the bottom of the tube overcomes this, and the PBL can be easily separated using a cell strainer. A 70 μm cell strainer has been routinely used for this purpose, although a 40 μm or 100 μm cell strainer can also be used. Clumping of cell debris and nuclear material may be affected by the low temperature in our procedure, and the use of undiluted blood that provides a larger amount of erythrocytes and hence more cell debris/ nuclear material. Therefore, there is no need for high force centrifugation (i.e. 750 g, 10 min) to pellet the viscous mass, as used in the original method, that may damage the cells. We found that 200g for 5 min was suffice to pellet the PBL in our procedure.

The lysis time is important as the PBL may be irreversibly damaged if they spend too long in a hypotonic solution. It takes 2-3 s to close the lid after combining the blood and water, and 2 s to open the lid to add the 10xPBS. Thus if the total time of exposure to the hypotonic solution is 20 s this leaves 15 s for mixing by gentle inversion to completely lyse the erythrocytes. Longer time (eg. 40 s) for lysis is unnecessary. The PBL prepared using a 20 s lysis are of high quality as described later.

3.2. Characterisation of PBL prepared using the hypotonic and gradient method

For flow cytometric analysis, PBL were prepared by the hypotonic lysis and gradient method simultaneously and compared side by side using each individual blood sample from six rainbow trout. **Fig. S1** shows the gating analysis after PBL isolation. PBL prepared by both methods showed similar size and granularity characteristics of lymphocytes and granulocytes (**Fig. 1A**), as seen by Crippen et al. (2001). The cells had >97.5% viability as assessed by propidium iodide staining (**Fig. 1B**).

We further evaluated the B cell and T cell populations in the PBL using monoclonal antibodies against trout IgM and CD3ε respectively. No staining was observed in secondary antibody controls (**Fig. 1C**). The IgM antibody stained 31.4±7.7 % (Mean±SD) and 34.7±4.5 % leucocytes in the lymphocyte gate of PBL prepared by the hypotonic lysis and gradient method, respectively (**Fig. 1D**). Meanwhile the CD3ε antibody (as a T cell marker) stained 31.8±7.6 % and 32.7±7.6 % leucocytes in the lymphocyte gate (**Fig. 1E**). Paired samples T-test of the six fish analysed showed no significant difference in the percentages of IgM+ and CD3ε+ in the lymphocyte populations of the PBL prepared by both methods (**Fig. 1F**). IgM+ B cells and T cells with similar percentages have been recorded in rainbow trout PBL by Korytar et al. (2013).

3.3. Characterisation of cell marker gene expression in PBL prepared using the hypotonic lysis and gradient method

To complement the limited FACS analysis using antibodies, we analysed the expression of a large number of marker genes for different cell types present in PBL in whole blood and PBL prepared by the hypotonic lysis and gradient method (**Table 1**).

3.3.1 Expression of marker genes for erythrocytes and proliferation

We first examined the expression of three genes, haemoglobin-a, haemoglobin-b and 5-aminolevulinate synthase erythroid (ALAS), specifically expressed in erythrocytes (Krasnov, et al., 2013). All these genes are highly expressed in whole blood samples but significantly reduce to less than 0.01% in PBL prepared by the hypotonic lysis method (**Fig. S2A-C**), suggesting efficient removal of erythrocytes. The expression of these genes was significantly higher in PBL prepared by the gradient method relative to the hypotonic method.

The head kidney and spleen are the main erythropoietic organs in fish, however peripheral blood can contain a high proportion of immature erythrocytes, mostly erythroblasts that divide and undergo their final maturation within the circulation (Witeska, 2013). In rainbow trout, 10.6% erythrocytes are immature (Keen, et al., 1989). In contrast, most peripheral blood leucocytes are mature and do not proliferate without stimulation. To examine whether signs of proliferation could be detected the expression of several proliferation markers, proliferation cell nuclear antigen (PCNA, Leung et al, 2005), Ki63 and mini-chromosome maintenance protein (MCM)2 (Jurikova et al., 2016), were investigated. All of these proliferation marker genes had higher expression in whole blood compared to PBL prepared by both the hypotonic and gradient method, which were not different (**Fig. S2D-F**). These results confirm the removal of erythrocytes and the low proliferation level in PBL.

3.3.2 Expression of marker genes for T cells and B cells

The expression of lymphocyte markers for T cells (TCRα, TCRβ, CD3ε, CD3ζ, CD4-1, CD4-2, CD8α and CD8β) and B cells (CD79a, CD79b, secreted (s) IgM, membrane (m) IgM, sIgD, mIgD, sIgT and mIgT) were next examined (**Fig. 2**). The lymphocyte marker gene expression was consistently higher in PBL prepared by the hypotonic method compared to whole blood. Their expression in PBL prepared by the gradient method was also higher (CD3ζ, CD4-1, CD79a and mIgD), or showed intermediate levels that were not significantly different to the hypotonic prepared PBL or whole blood (TCRα, TCRβ, CD3ε, CD4-2, CD8α, CD8β, CD79b, sIgM, sIgT and mIgT). The exception was the expression of mIgM and sIgD where levels were not different to whole blood but lower than in hypotonic prepared PBL (**Fig. 2**). These expression patterns suggest that PBL prepared by the hypotonic method are enriched for both T cells and B cells due to complete removal of erythrocytes whilst PBL prepared by the gradient method are more prone to erythrocyte contamination and may lose some high-density B cells (Ramirez-Gomez, et al., 2012).

3.3.3 Expression of marker genes for other cell types

Other leucocyte markers examined were the pan leucocyte marker L plastin, pan myeloid marker Spi-1a and Spi-1b, thrombocyte markers CD41 and G6F, neutrophil marker myeloperoxidase (MPO), macrophage markers lysozyme C and MCSFR and dendritic cell markers CD83 and CD80/86 (**Table 1**). Expression of all the leucocyte marker genes was higher in PBL prepared by the hypotonic method compared to whole blood. Their expression in PBL prepared by the gradient method was also higher (L plastin and Spi-1b), or at intermediate levels that were not significantly different to PBL prepared by the hypotonic method or to whole blood (Spi-1a, CD41, MCSFR, lysozyme C, CD83 and CD86). The exception was the expression of G6F and the neutrophil marker MPO where levels were no different to whole blood but lower than in hypotonic prepared PBL (**Fig. 3**). These expression patterns suggest that PBL prepared by the hypotonic method are enriched for thrombocytes, neutrophils, macrophages and dendritic cells due to complete removal of erythrocytes and are potentially superior to PBL prepared by the gradient method that may lose some higher density cells such as neutrophils.

3.4. Phagocytosis

Fish PBL contain B cells and other myeloid cells that are phagocytic (Takizawa et al., 2016; Zhang et al., 2017). The phagocytic potential of PBL prepared by the hypotonic method was analysed by flow cytometry using fluorescent beads (**Fig. 4**). 5.4% of the lymphoid cells were phagocytic in control PBL.

306 This percentage was decreased to 4.5 by IL-1β treatment but not affected by IL-6 (**Fig. 4D**). The
307 phagocytic lymphoid cells are presumably B cells (Zhang et al., 2017). Myeloid cells are more
308 phagocytic, with 24.9% of these cells phagocytic in control PBL. This percentage was increased
309 significantly to 45.3% and 29.2% by IL-1β and IL-6, respectively (**Fig. 4D**). The phagocytic myeloid
310 cells showed a variety of granularities (**Fig. 4C**), suggesting different myeloid cells (eg neutrophils,
311 macrophages and dendritic cells) might all contribute to the phagocytosis observed. The PBL prepared
312 by the hypotonic method can also phagocytose bacteria, such as GFP expressing *Aeromonas*
313 *salmonicida* and *E. coli* (data not shown). These results indicate that the PBL prepared by hypotonic
314 lysis of erythrocytes are fully functional in terms of phagocytic capacity.

315

316 **3.5. Proliferation of PBL prepared by the hypotonic lysis of erythrocytes**

317 Most circulating PBL are non-proliferating mature cells as demonstrated by the low-level expression of
318 proliferation marker genes (**Fig. S2**). They can be activated to proliferate by mitogens or vaccines, the
319 latter a marker of antigen-mediated adaptive immune responses (Reitan and Thuvander, 1991). The
320 proliferation potential of PBL prepared by the hypotonic method was demonstrated using PHA as a
321 stimulant. BrdU incorporation was significantly increased in PBL treated with PHA from 2.5 to 25
322 μg/ml (**Fig. 5**). Enhanced BrdU incorporation was also observed in PBL stimulated by a bacterin and
323 recombinant trout cytokines (Wang et al., 2018). This result confirms the proliferation potential of PBL
324 prepared by the hypotonic method.

325

326 **3.6. Immune response to PAMPs and pro-inflammatory cytokines of PBL prepared using the**
327 **hypotonic lysis of erythrocytes**

328 The functionality of PBL prepared using the hypotonic method was further assessed by stimulating
329 them with PAMPs (LPS, Poly I:C and PHA) and recombinant proinflammatory cytokines (IL-1β and
330 IFNγ). The expression of all the pro-inflammatory cytokines (IL-1β1, IL-1β2, TNFα1, TNFα2, TNFα3,
331 IL-6, and IL-8) was up regulated by all the PAMPs and rIL-1β at least at one time point, except TNFα1
332 that was refractory to PHA (**Fig. 6**). IFNγ had no effect on the expression of IL-1β2, TNFα1, TNFα2,
333 TNFα3, IL-6 and IL-8, but down-regulated IL-1β1 expression. IFNγ expression itself was up regulated
334 by Poly I:C and PHA but was refractory to LPS, IL-1β and IFNγ (**Fig. 6F**). Lastly, CXCL11_L1
335 expression was up regulated by Poly I:C, PHA and IFNγ from 4 h to 24 h, and by LPS at 8 h, but was
336 refractory to IL-1β stimulation (**Fig. 6I**). Gene-specific and stimulant-specific responses have also been
337 seen previously using these stimulants and genes, suggesting that the PBL prepared by the hypotonic
338 method are fully responsive and show typical responses.

3.7. General discussion and prospective

Rapid preparation of high quality PBL is highly desirable in fish immunology research and fish vaccine development. The density gradient method currently used is time consuming, costly and prone to erythrocyte contamination. Our modified PBL preparation method includes a 20 s hypotonic lysis of erythrocytes and a subsequent separation of PBL from cell debris by a cell strainer. This method is simple to perform, rapid and cost effective. The PBL obtained are free from erythrocyte contamination but have similar leucocyte composition to those prepared by density separation. The cell marker gene analysis suggested that PBL prepared by hypotonic lysis are superior in that certain cell markers were higher in these cells, suggesting some high-density cells (particularly B cells and neutrophils) might be lost by the gradient method. The PBL prepared using our hypotonic method can phagocytose, proliferate and respond to immune stimulants and cytokines. These data suggest that hypotonic lysis of erythrocytes is a rapid way to prepare high quality PBL that will enable more studies on PBL in disease and vaccine development research in fish.

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Table 1. Marker genes analysed in blood cell populations.

Gene	Main population	Other cell populations	Reference
CD3ζ	T cells		Liu et al., 2008
CD3ε	T cells		Boardman et al., 2012
CD4-1	T cells	Monocytes/macrophages	Takizawa et al., 2016
CD4-2	T cells	Monocytes/macrophages	Takizawa et al., 2016
CD8α	T cells	Dendritic cells	Takizawa et al., 2011, Kang 2012
CD8β	T cells		Moore et al., 2005
TCRα	T cells		Yazawa et al., 2008
TCRβ	T cells		Yazawa et al., 2008
sIgM H	B cells		Fillatreau et al., 2013
mIgM H	B cells		Fillatreau et al., 2013
sIgD H	B cells		Fillatreau et al., 2013
mIgD H	B cells		Fillatreau et al., 2013
sIgT H	B cells		Fillatreau et al., 2013
mIgT H	B cells		Fillatreau et al., 2013
CD79a	B cells		Liu et al., 2017
CD79b	B cells		Liu et al., 2017
Hemoglobin-a	Erythrocytes		Krasnov et al., 2013
Hemoglobin-b	Erythrocytes		Krasnov et al., 2013
ALAS	Erythrocytes		Krasnov et al., 2013
PCNA	Proliferating cells		Leung et al., 2005; Juríková et al., 2016
Ki67	Proliferating cells		Juríková et al., 2016
MCM2	Proliferating cells		Juríková et al., 2016
L plastin	Leucocytes		Hsu et al., 2004
Spi-1a	Myeloid cells	Immature lymphoid cells	Hsu et al., 2004
Spi-1b	Myeloid cells	Immature lymphoid cells	Hsu et al., 2004
CD41	Thrombocytes		Lin et al., 2005
G6F	Thrombocytes		Ohashi et al., 2010
Myeloperoxidase (MPO)	Neutrophils	Other myeloid cells	Lieschke et al., 2001
Lysozyme C	Macrophages	Other myeloid cells/granulocytes	Hall et al., 2007
MCSFR2	Macrophages	Neural crest cells	Zakrzewska et al., 2010
CD83	Dendritic cells	T cells, B cells	Aerts-Toegaert et al., 2007
CD80/86	Dendritic cells	B cells, macrophages	Zhang et al., 2009

507 **Table 2. Primers used for real-time PCR analysis.**

508

Gene	Sequence (5'-3')	Sequence (5'-3')	Size (bp)	Acc. No.
Housekeeping gene				
EF-1α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	AF498320
Cytokine genes				
IL-1β1	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGCTGTGGAAGAACATATAG	179	AJ278242
IL-1β2	GAGCGCAGTGGAAGTGTTGG	AGACAGGTTCAAATGCACTTTATGGT	204	AJ245925
TNFα1	TGTGTGGGGTCCTCTTAATAGCAGGTC	CCTCAATTTCATCCTGCATCGTTGA	102	AJ277604
TNFα2	CTGTGTGGCGTTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	98	AJ401377
TNFα3	GCTGCACTCTTCTTTACCAAGAAACAAG	CCACTGAGGACTTGTAATCACCATAGGT	148	HE798544
IL-6	GGGAGAAAATGATCAAGATGCTCGT	GCAGACATGCCTCCTTGTTGG	180	DQ866150
IL-8	TCCTGACCATTACTGAGGGGATGA	AGCGCTGACATCCAGACAAATCTC	200	AJ279069
IFNγ	CAAACTGAAAGTCCACTATAAGATCTCCA	TCCTGAATTTTCCCCTTGACATATTT	210	AJ616215
CXCL11_L1	TCATCAGCTTCCTGGCCTGTC	TTCTCCGTTCTTCAGAGTGACAATGAT	191	AF396869
Marker genes				
TCRα	CAAACTGGTATTTTGACACAGATGCAA	TTCTTGTGTTGTCTTTGAGGGACTGA	165/162	BT073987
TCRβ	CAAAGTGGGGGAGAGATAACGACAGA	TGTTTCTCTGAGGAGCCCTGGAA	149	AF329700
CD3ε	AAGGAAAGGTGTGTAAGGACTGCTATG	GAGCGGGAAGTGGGTTTTTG	160	NM_001195174
CD3ζ	CGTCTACTGCACTACCCTATGTCGAAG	AAGTGTCGTCATTATTCCTGCGATTATC	273	BT073940
CD4-1	GTGTGGAGGTGCTACAGGTTTTTTC	ATCGTCACCCGCTGTCTGTG	396	AY973028
CD4-2	CGACATTGTCACAGTCAAGGTCC	CCTCATTTGGCAACAAACTTCTCAC	284	AY973029
CD8α	CAAGTCGTGCAAAGTGGGAAA	TCTGTTGTTGGCTATAGGATGTTGTTG	214	AF178053
CD8β	GAACTATCAAACCCCAGAAGGCTGTG	GACACTTTTTGGGTAGTCGGCTGAA	125	AY563420
sIgM H	TACAAGAGGGAGACCGGAGGAGT	CTTCCTGATTGAATCTGGCTAGTGGT	221	X65261
mIgM H	CCTACAAGAGGGAGACCGATTGTC	GTCTTCATTTCACCTTGATGGCAGT	168	OMU04616
sIgD H	TGAACATATCCAAACCAGGTGTCTG	GTCCTGAAGTCATCATTTTGTCTTGA	357	JQ003979
mIgD H	TGAACATATCCAAACCAGAGCTCC	GTCCTGAAGTCATCATTTTGTCTTGA	191	AY870260
sIgT H	CATCAGCTTCACCAAAGGAAGTGA	TCACTTGTCTTCACATGAGTTACCCGT	361	AY870268
mIgT H	TCGAAGTCCACGGCGAACA	GTGTTCTTCACCGCTTCATCTTGAA	187	AY870264
CD79a	CGAGGGAATGTTACTGATGGTGG	GCATTCCTCCAGATTTAGACCCTCATA	142	CA362887
CD79b	CCTTTGTGAACCTCTCAGTGGC	GGCCTTGTACCATTCCACCGT	238	XM_021565350
Hemoglobin-a	GAGGCTTTGGGAAGGATGCTGAC	CACAAGGATGTTGTGGGACAGAATCTT	170	EZ765953
Hemoglobin-b	CACTGGCTCTGGGAAGAGTCCTGA	TGAGGACGTCAGCCAACACCCT	186	FX112568
ALAS	CAGCCACATCATCCCAATAAAGGTTG	CACCTCCACGAGCCTCTCCAC	202	XM_021615925
PCNA	GGGCACTGGCAACGTCAAAC	TGTCAGCTATCTTGTACTCCACCACTAGGG	206	XM_021621842
Ki67	GATGGGCGAGAGATTAATCAAATGTCA	TGGCACTTGGGAAGCAAATCC	250	BT073583
MCM2	CGCAAAGAGTCCATGGCGAC	TGAACAGCAGCAACTCATTGTTGTCTTT	253	XM_021610724
L plastin	CACAGATCAGCAGCTTCAAGGATAAGC	GCTCCAATCTTCCTGGATACCGTGATA	178	XM_021571076
Spi-1a	AGTATGGCTCGTTACAGCGATGTTGA	GGATCTTCTTCTTGTTGCCCAATTCTC	259	NM_001124513
Spi-1b	CAGGCCCCTGTGTCCCCTAGTTA	CGGATCTTCCTCTTGTTACCGGAGTC	149	XM_021578477
CD41	AAGTGGACAGGCTGAGACCCAGA	GCGATTAAAGAACCCCACCTTCCA 68.6	157	XM_021624569
G6F	ACTGGGGACTTCTCTCTGCTGTTCA	GGCTGAAGGAAAGATAGAGACTGTGAGGAC	141	GU393010
MPO	TTTCGGTGACATGGCCAACAG	TCCACACGAACATCACCTGCAAC	282	XM_021616849
Lysozyme C	AACAGCCTGCCCAACTGGGT	ACACGCTTGGCACAACGGAT	227	XM_021601582
MCSFR	GGACTTTGCCCCTCCAGAGATATACAC	GATCACAATCCTCACTAATCTTAGCTTGGC	212	AB091826
CD83	GTGAGGTGGTACAAGCTGGGTG	GCTGCCAGGAGACACTTGTACCT	203	AY263797
CD80/86	CAGGAACACACTGTCTGCAGGC	CTGCTCCCTTTCCTCCTTGATTACTTC	163	EU927451

509

510

Figure legend:

Figure 1. Flow cytometry detection of IgM^+ and $CD3\varepsilon^+$ lymphocytes in trout PBL prepared by the hypotonic lysis and gradient method. (A) Gate selection of isolated PBL. (B) Live cell gating by propidium iodide exclusion. (C) Secondary antibody control. (D) Percentages of IgM^+ lymphocytes. (E) Percentages of $CD3\varepsilon^+$ lymphocytes. (F) Mean + standard deviation of the percentages of IgM and CD3ε positive cells in PBL from six fish. The flow cytometry data (A-E) are for a representative fish of the 6 analysed.

Figure 2. The expression of T cell and B cell marker genes in whole blood, and PBL prepared by the hypotonic and gradient method. cDNA samples were prepared from whole blood (W), or PBL prepared by the hypotonic (H) and gradient (G) method from four fish. The expression of TCRα (A), TCRβ (B), CD3ε (C), CD3ζ (D), CD4-1 (E), CD4-2a (F), CD8α (G), CD8β (H), CD79a (I), CD79b (J), secreted (s) IgM (K), membrane (m) IgM (L), sIgD (M), mIgD (N), sIgT (O) and mIgT (P) was quantified by qPCR. The data are presented as mean (+SEM) arbitrary units where one unit equals the average expression level in whole blood. Different letters over the bars indicate significant differences ($p<0.05$, Paired samples T test).

Figure 3. The expression of other leucocyte marker genes in whole blood, and PBL prepared by the hypotonic and gradient method. cDNA samples were prepared from whole blood (W), or PBL prepared by the hypotonic (H) and gradient (G) method from four fish. The expression of L plastin (A), Spi-1 (B), Spi-2 (C), CD41 (D), G6F (E), MCSFR (F), lysozyme C (G), MPO (H), CD83 (I) and CD80/86 (J) was quantified by qPCR. The data are presented as mean (+SEM) arbitrary units, where one unit equals the average expression level in whole blood. Different letters over the bars indicate significant differences ($p<0.05$, Paired samples T test).

Figure 4. Flow cytometry analysis of phagocytosis. Trout PBL were incubated with IL-1β, IL-6 or medium alone as control for 20 h. PBL were then incubated with 1 μm fluorescent beads for 3 h and analysed by flow cytometry. Typical results from a single fish are shown in panels A-C. **(A)** Gating of lymphoid and myeloid cell populations. **(B)** Phagocytosis by cells in the lymphoid gate. **(C)** Phagocytosis by cells in the myeloid gate. **(D)** The percentage of phagocytic leucocytes in the lymphoid and myeloid gates. The results are presented as the mean (+ SEM) of four fish. Significant differences of paired sample T tests between stimulated and control samples is shown above the bars as * $p \leq 0.05$.

Figure 5. Rainbow trout PBL proliferation. Freshly prepared PBL from 4 fish were incubated with 2.5, 10 and 25 μg/ml PHA, or with medium alone as control, in triplicate wells per fish, for 3 days. BrdU was added 20 h before incorporation of BrdU was detected by ELISA. The data are presented as the mean (+SEM) stimulation index, calculated as the OD450 of PHA treated cells divided by that of

544 untreated samples. Significant differences of paired sample T tests between PHA-stimulated and control
545 samples are shown above the bars as: * $p \leq 0.05$.

546 **Figure 6. Modulation of PBL cytokine gene expression by PAMPs and recombinant cytokines.**
547 Freshly prepared PBL were stimulated with LPS, Poly I:C, PHA, recombinant IL-1β and IFNγ for 4 h,
548 8 h and 24 h. The expression of IL-1β1 (A), IL-1β2 (B), TNFα1 (C), TNFα2 (D), TNFα3 (E), IFNγ (F),
549 IL-6 (G), IL-8 (H) and CXCL11_L1 (I) was quantified by real-time RT-PCR. Modulated expression
550 was expressed as a fold change calculated as the mean expression level in stimulated cells normalized
551 to that of time-matched controls. The means (+ SEM) of cells from four fish are shown. The relative
552 significance of paired sample T tests between stimulated and time-matched control samples is shown
553 above the bars as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

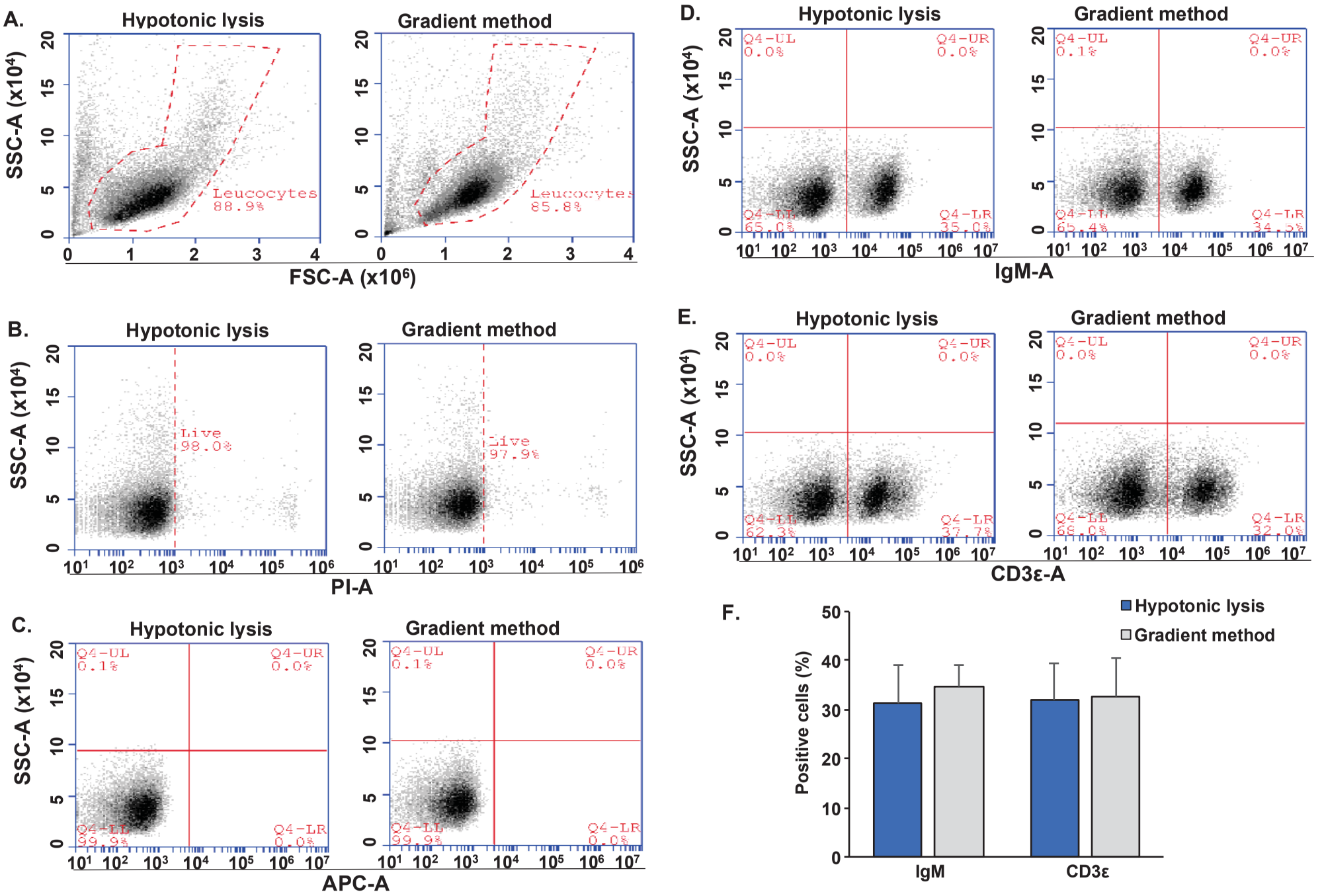
554

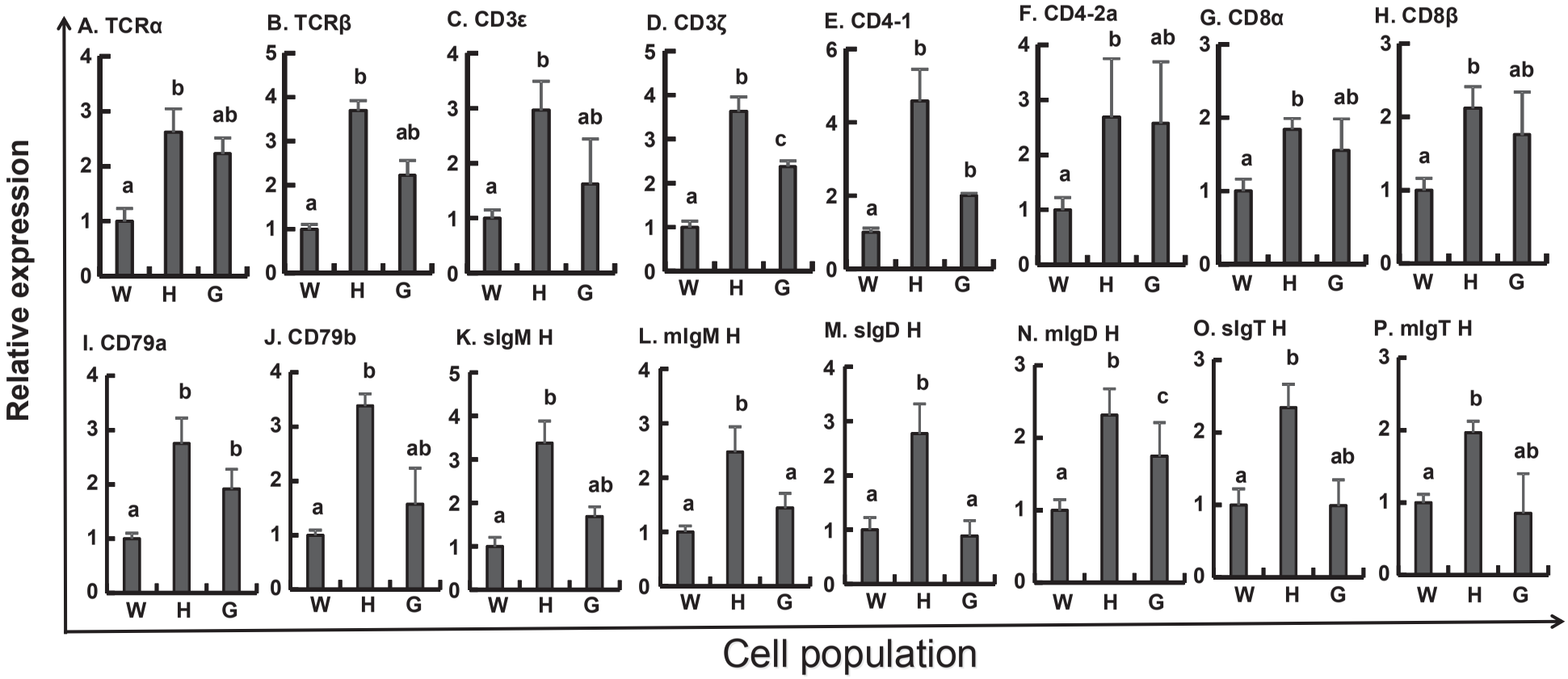
555

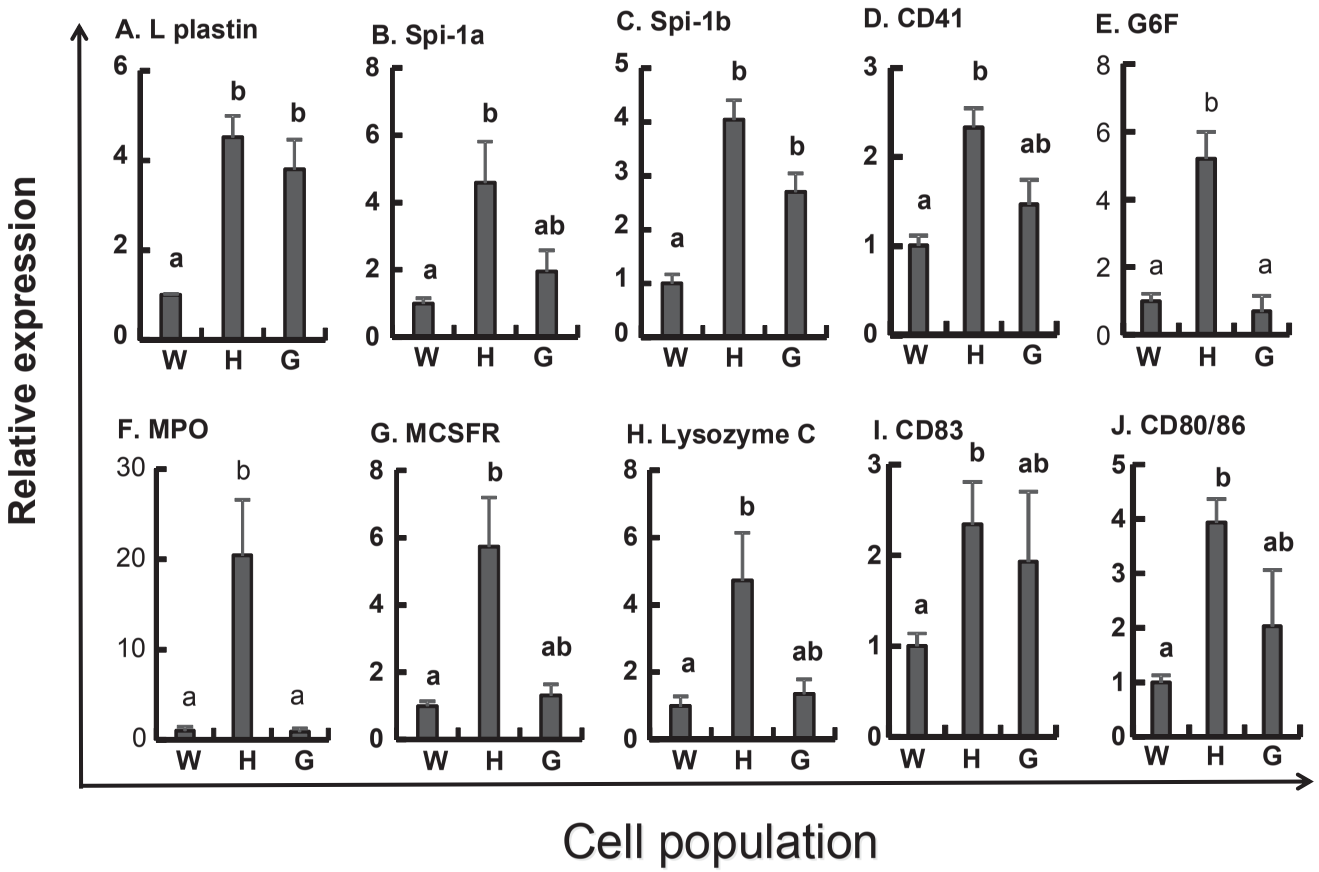
556 **Figure S1. Gating strategy for flow cytometric analysis.** Examples for PBL purified by hypotonic
557 lysis or the density gradient method. P1 = Leucocyte gate (A). P2 = Gate of excluded doublets in FSC-
558 H/FSC-A (B). P3 = Gate of excluded doublets in SSC-H/SSC-A (C). P4 = Live cells (as in Fig. 1B) and
559 live lymphocytes gate (D). Leucocytes were recorded in P1 gate with P2, P3 and P4 gates excluded
560 from counts (total of 30,000 events in P1).

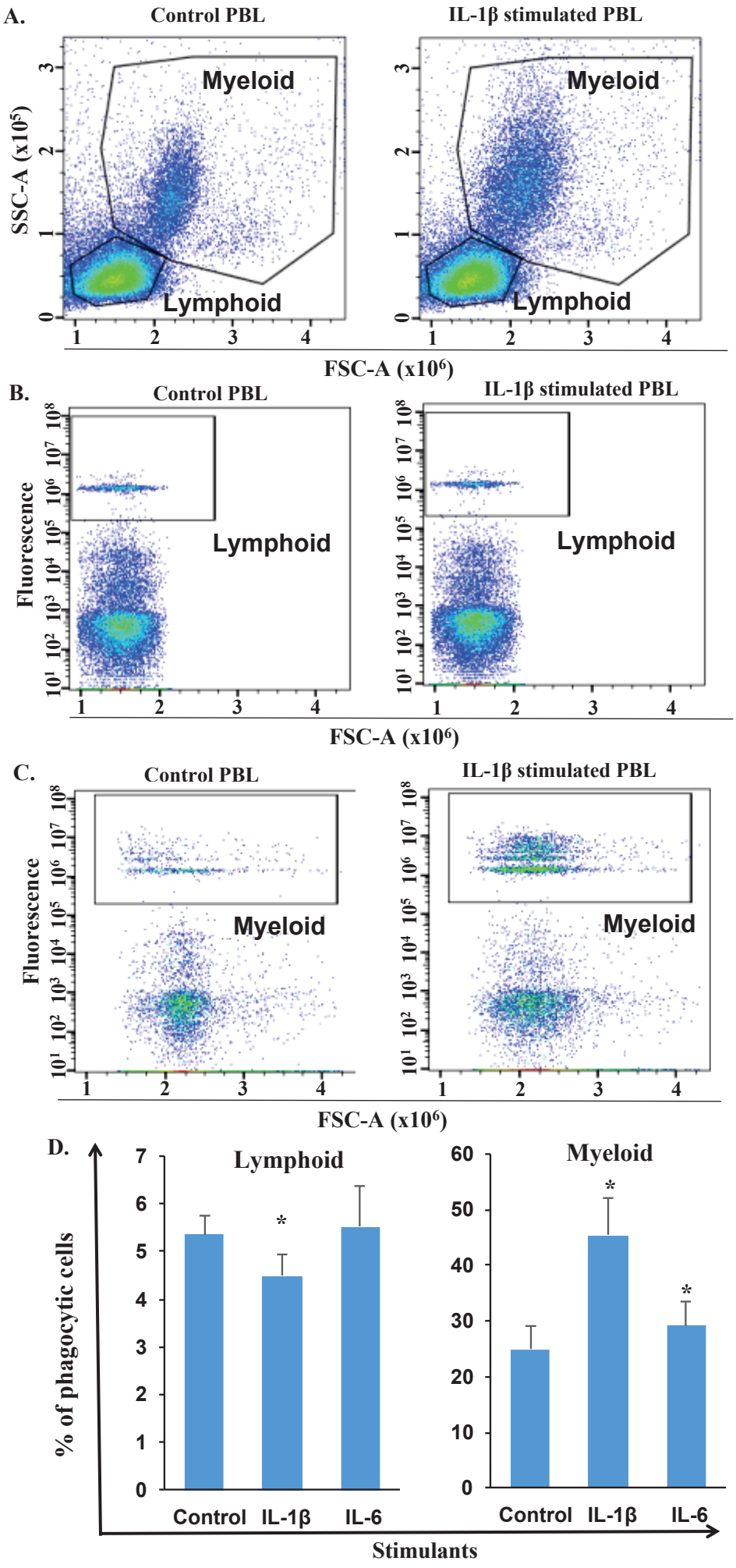
561 **Figure S2. The expression of erythrocyte and proliferation marker genes in whole blood, and PBL**
562 **prepared by the hypotonic and gradient method.** cDNA samples were prepared from whole blood
563 (W), or PBL prepared by the hypotonic (H) and gradient (G) method from four fish. The expression of
564 Hemoglobin-a (A), Hemoglobin-b (B), ALAS (C), PCNA (D), Ki67 (E) and MCM2 (F) was quantified
565 by qPCR. The data are presented as mean (+SEM) arbitrary units, where 100 units equal the average
566 expression level in whole blood. Different letters over the bars indicate significant differences ($p<0.05$,
567 Paired samples T test).

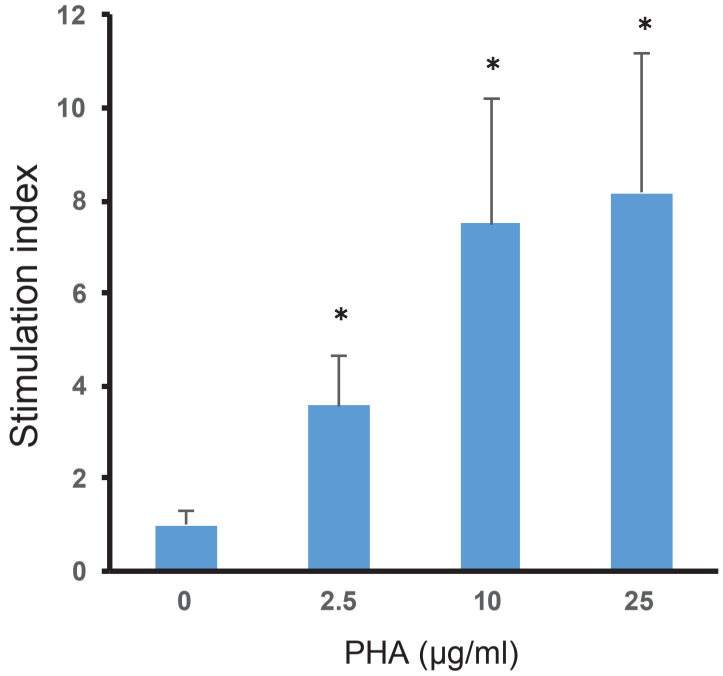
568

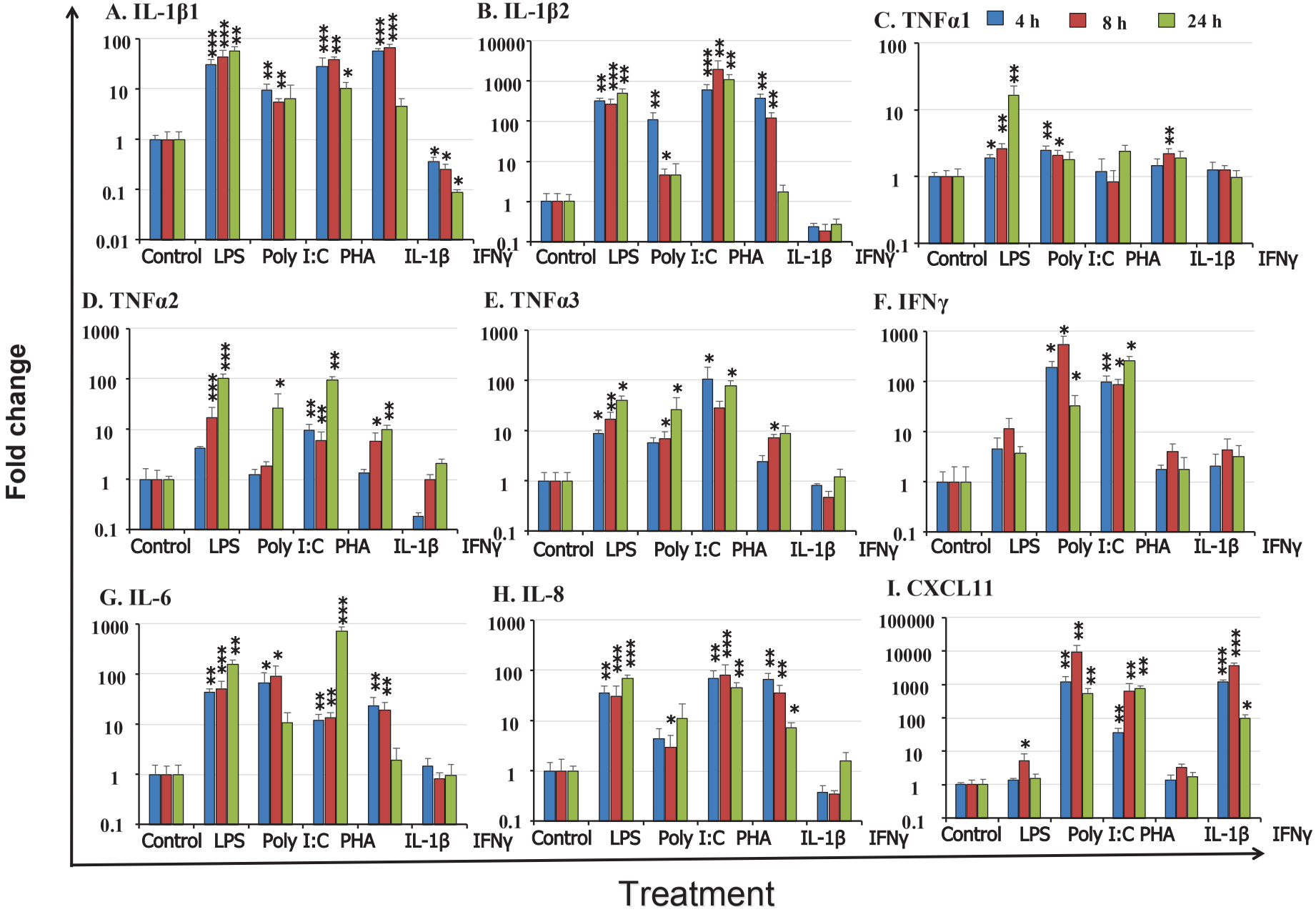

A.
Hypotonic lysis
Gradient method
SSC-A (x10⁴)
FSC-A (x10⁶)
Leucocytes
88.9%
Leucocytes
85.8%
B.
Hypotonic lysis
Gradient method
SSC-A (x10⁴)
PI-A
Live
98.0%
Live
97.9%
C.
Hypotonic lysis
Gradient method
SSC-A (x10⁴)
APC-A
Q4-UL
0.1%
Q4-UR
0.0%
Q4-LL
99.9%
Q4-LR
0.0%
D.
Hypotonic lysis
Gradient method
SSC-A (x10⁴)
IgM-A
Q4-UL
0.0%
Q4-UR
0.0%
Q4-LL
65.0%
Q4-LR
35.0%
Q4-UL
0.1%
Q4-UR
0.0%
Q4-LR
34.5%
E.
Hypotonic lysis
Gradient method
SSC-A (x10⁴)
CD3ε-A
Q4-UL
0.0%
Q4-UR
0.0%
Q4-LL
62.3%
Q4-LR
37.7%
Q4-UL
0.0%
Q4-UR
0.0%
Q4-LL
68.0%
Q4-LR
32.0%
F.
Hypotonic lysis
Gradient method
Positive cells (%)
IgM
CD3ε


A. TCRα
B. TCRβ
C. CD3ε
D. CD3ζ
E. CD4-1
F. CD4-2a
G. CD8α
H. CD8β
I. CD79a
J. CD79b
K. sIgM H
L. mIgM H
M. sIgD H
N. mIgD H
O. sIgT H
P. mIgT H
W H G
Relative expression
Cell population


A. L plastin
B. Spi-1a
C. Spi-1b
D. CD41
E. G6F
F. MPO
G. MCSFR
H. Lysozyme C
I. CD83
J. CD80/86
W
H
G
a
b
ab
Relative expression
Cell population


A.
Control PBL
IL-1β stimulated PBL
Myeloid
Lymphoid
SSC-A (x10⁵)
FSC-A (x10⁶)
B.
Control PBL
IL-1β stimulated PBL
Lymphoid
Fluorescence
FSC-A (x10⁶)
C.
Control PBL
IL-1β stimulated PBL
Myeloid
Fluorescence
FSC-A (x10⁶)
D.
Lymphoid
Myeloid
% of phagocytic cells
Control
IL-1β
IL-6
Stimulants


Stimulation index
12
10
8
6
4
2
0
*
*
*
0
2.5
10
25
PHA (µg/ml)


A. IL-1β1
B. IL-1β2
C. TNFα1
4 h
8 h
24 h
D. TNFα2
E. TNFα3
F. IFNγ
G. IL-6
H. IL-8
I. CXCL11
Control
LPS
Poly I:C
PHA
IL-1β
IFNγ
Fold change
Treatment

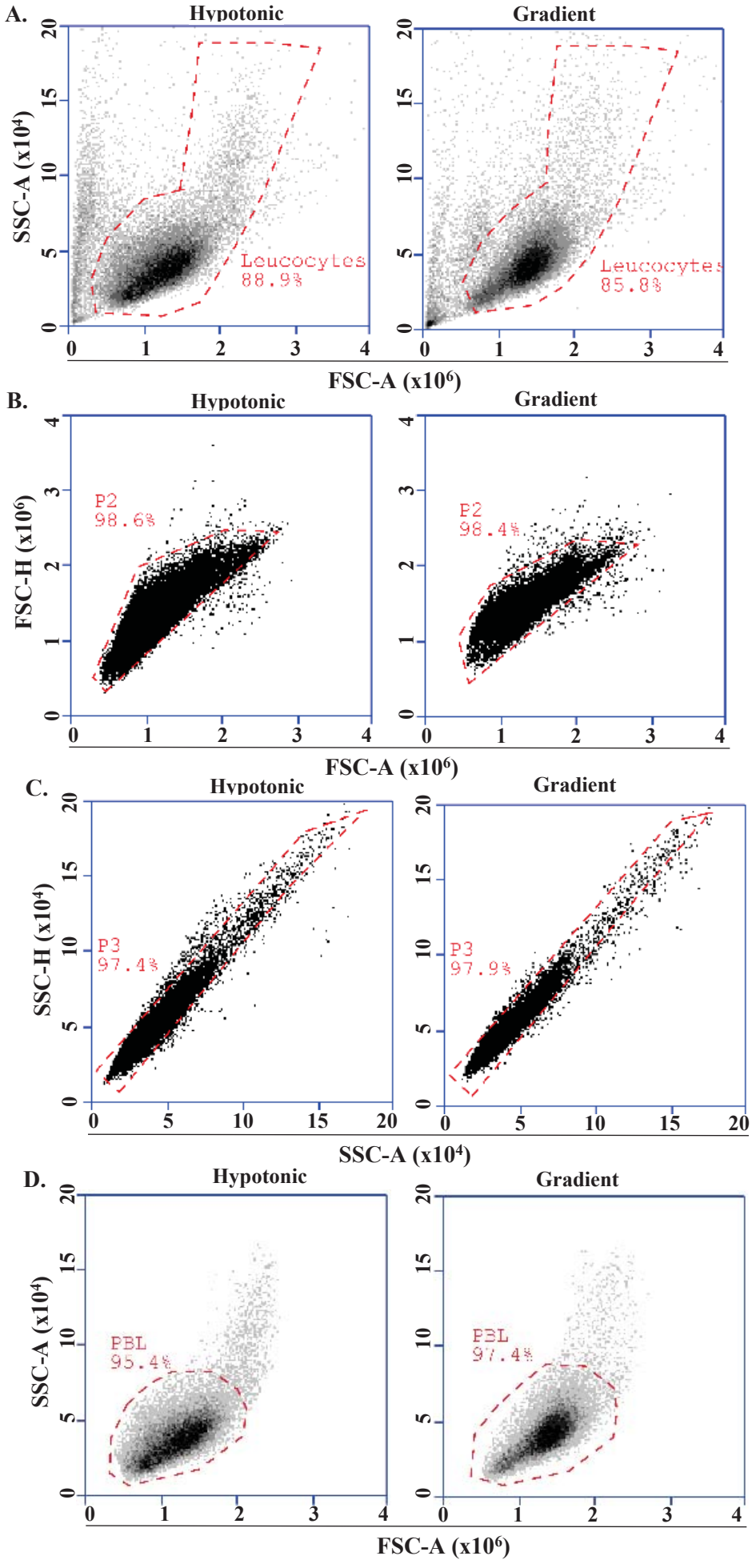

A.
Hypotonic
Gradient
SSC-A (x10⁴)
Leucocytes
88.9%
Leucocytes
85.8%
FSC-A (x10⁶)
B.
Hypotonic
Gradient
FSC-H (x10⁶)
P2
98.6%
P2
98.4%
FSC-A (x10⁶)
C.
Hypotonic
Gradient
SSC-H (x10⁴)
P3
97.4%
P3
97.9%
SSC-A (x10⁴)
D.
Hypotonic
Gradient
SSC-A (x10⁴)
PBL
95.4%
PBL
97.4%
FSC-A (x10⁶)

Fig. S1. Gating strategy for flow cytometric analysis. Examples for PBL purified by hypotonic lysis or density gradient method. P1 = Leucocytes gate (A). P2 = Gate of excluded doublets in FSC-H/FSC-A (B). P3 = Gate of excluded doublets in SSC-H/SSC-A (C). P4 = Live cells (as in Fig. 1B) and live lymphocytes gate (D). Leucocytes were recorded in P1 gate with P2, P3 and P4 gates exclusion from counts (Total of 30,000 events in P1).

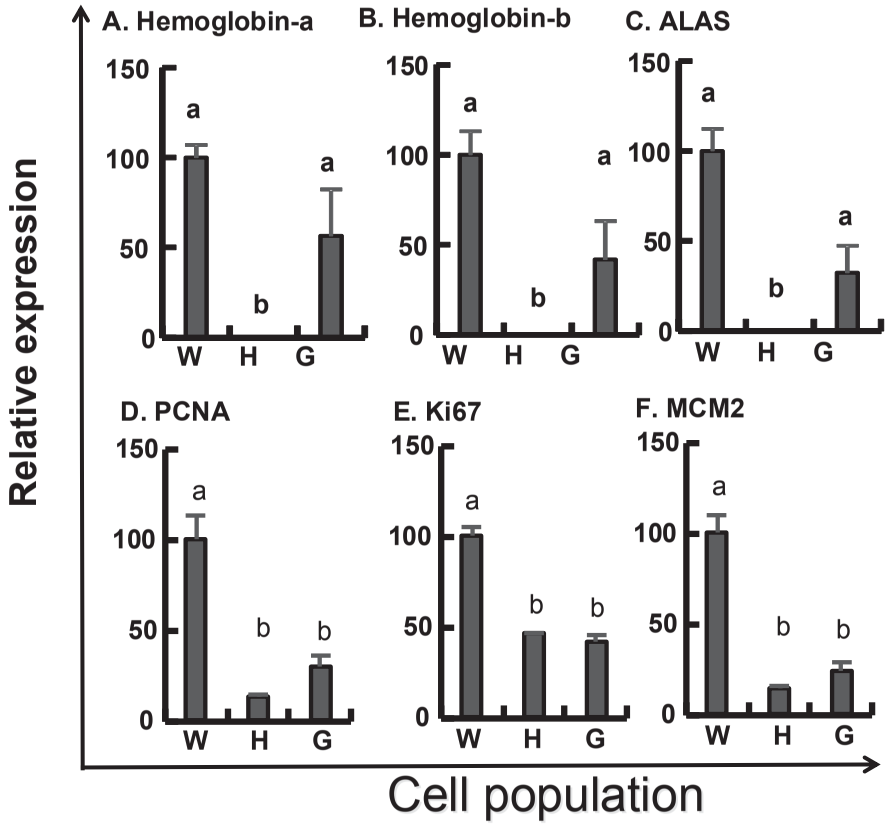


Fig. S2. The expression of erythrocyte and proliferation marker genes in whole blood, and PBL prepared by the hypotonic and gradient method. cDNA samples were prepared from whole blood (W), or PBL prepared by the hypotonic (H) and gradient (G) method from four fish. The expression of Hemoglobin-a (A), Hemoglobin-b (B), ALAS (C), PCNA (D), Ki67 (E) and MCM2 (F) was quantified by qPCR. The data are presented as mean (+SEM) arbitrary units, where 100 units equal the average expression level in whole blood. Different letters over the bars indicate significant differences ($p<0.05$, Paired samples T test).