

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

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23 **Abstract**

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

42

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

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47 **1. Introduction**

48 Fish immunology research has attracted much interest in recent years for theoretical and practical
49 reasons. Fish immune systems provide important comparative outgroups for understanding the
50 evolution of disease resistance. As a large vertebrate group, fish may have evolved novel mechanisms
51 to tackle infections, and research into their responses should eventually lead to an increased
52 understanding of the general principles of immune system adaptability in vertebrates (Feng et al., 2015;
53 Flajnik, 2018). At the same time, the expanding aquaculture industry and associated disease risks
54 requires fish immunology research to identify ways to manipulate the immune response and allow
55 development of novel/ efficacious vaccines (Secombes, 2008; Van Muiswinkel, 2008; Lafferty et al.,
56 2015; Little et al., 2016). From a functional perspective, this research needs methods to rapidly prepare
57 leucocytes from immune tissues such as head kidney, spleen and blood that are rich in erythrocytes.
58 Peripheral blood leucocytes (PBL) are particularly relevant to vaccine development work, since samples
59 can be obtained multiple times from the same individual during an immune response without killing the
60 fish.

61

62 Classically, mammalian PBL have been purified by lysis of the non-nucleated erythrocytes that they
63 possess with hypotonic ammonium chloride solutions that are commercially available. However, teleost
64 erythrocytes are nucleated and resistant to ammonium chloride lysis (Rowley, 1990). Fish PBL have
65 been routinely prepared by continuous or discontinuous density gradient centrifugation through
66 separation media such as Percoll and Histopaque (Reitan and Thuvander, 1991; Korytar et al, 2013;
67 Maisey et al., 2016; Takizawa et al., 2016, Zhang et al., 2017). Density gradient preparation of
68 leucocytes is time consuming, expensive, and prone to erythrocyte contamination. Crippen et al (2001)
69 reported a simple, rapid and inexpensive leucocyte purification method by hypotonic lysis of
70 erythrocytes. In their method, blood was diluted (1:2) and erythrocytes lysed in a hypotonic solution by
71 addition of distilled water for 20-40 seconds (s). The osmotic pressure was then brought back to
72 isotonicity by addition of 10x phosphate-buffered saline (PBS). The cell suspension was centrifuged
73 (750 g, 10 min) leaving a viscous mass containing cell debris and nuclear material on top of the cells,
74 that could be removed and discarded. Whilst the resultant PBL were comparable to PBL prepared by
75 gradient methods (Crippen et al., 2001), this method has not gained popularity in fish immunology
76 research. This is partly due to the difficulty in separating the PBL from a viscous mass of cell debris
77 and nuclear material in their method, and partly the lack of demonstrated functionality of the PBL
78 prepared.

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

89

90 **2. Methods and Materials**

91 **2.1 Fish**

92 Juvenile rainbow trout were purchased from College Mill Trout Farm (Perthshire, U.K.) and maintained
93 in aerated fibreglass tanks supplied with a continuous flow of recirculating freshwater at 14°C. Fish
94 were fed twice daily on a commercial pellet diet (EWOS), and were reared to 200 - 500 g prior to use.
95 All the experiments described comply with the Guidelines of the European Union Council
96 (2010/63/EU) for the use of laboratory animals, and were carried out under UK Home Office project
97 licence PPL 60/4013, approved by the ethics committee at the University of Aberdeen.

98

99 **2.2. Peripheral blood leucocyte (PBL) preparation by hypotonic lysis of erythrocytes**

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

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

116

117 **2.3. PBL preparation by gradient centrifugation using Histopaque-1077.**

118 1 ml of blood obtained as above was diluted to 8 ml using 1x PBS and carefully layered onto 7 ml of
119 Histopaque-1077 (Sigma, UK) in a 15 mL conical centrifuge tube, and centrifuged (without brake) at
120 500 g for 40 min. The PBL were collected from the Histopaque interface, washed twice and counted as
121 above.

122

123 **2.4. FACS analysis**

124 Peripheral blood leucocytes were isolated as above and processed for flow cytometric analysis as
125 follows. Prior to incubation with primary antibody a total of 5×10^5 cells per sample were blocked with
126 PBS + 2% FCS (FACS buffer, FB) for 30 min at 4°C. The cells were then pelleted by centrifugation at
127 250 g for 5 min and resuspended in 200 µl FB containing mouse anti-trout IgM (protein G-purified I-
128 14) (Deluca et al., 1983) and mouse anti-CD3ε (protein G-purified) (Maisey et al., 2016). Cells were
129 incubated for 30 min at 4°C and then were washed with 800 µl FB. Cells were resuspended in FB (400
130 µl) containing the secondary antibody (Alexa 647 donkey anti-mouse IgG, Molecular Probes). Cells
131 were incubated for 30 min at 4°C, then washed with 800 µl FB and finally resuspended in 300 µl FB
132 prior to analysis. For autofluorescence measurement, cells were resuspended with FB containing no
133 antibody and for isotype controls, cells were treated only with the corresponding conjugated secondary
134 antibody. Accuri C6 Flow Cytometer was used to analyse the samples, and at least 30,000 events were
135 recorded for each sample. Flow cytometry analyses always included cell viability (propidium iodide)
136 staining for exclusion of dead cells. Leucocytes exhibited a characteristic distribution in forward (FSC)
137 and side scatter (SSC) allowing the distinction between the lymphoid ($FSC^{low}SSC^{low}$) and the myeloid
138 cell population ($FSC^{hi}SSC^{hi}$). Doublets discrimination was performed in FSC-H/FSC-A and SSC-
139 H/SSC-A dot plots. Cells were analysed on a gate set on lymphocyte-sized cells. The gating procedure
140 for cell analysis is shown in Supplementary Fig. S1. CFlow Plus software was used for the analysis.

141

142 **2.5. Marker gene expression analysis**

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

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

154

155 **2.6. Phagocytosis of PBL prepared by hypotonic lysis of erythrocytes**

156 PBL in complete cell culture medium prepared as above (2×10^6 cells/ml) were added to 12-well
157 suspension cell culture plates (Greiner bio-one, UK). Fluorescent latex beads (FluoSpheres Fluorescent
158 Microspheres yellow green fluorescent, 1.0 µm, Life technology) were added at a cell/bead ratio of
159 1:20, and incubated at 20°C for 3 h. Both non-adherent and adherent cells were harvested using 0.5%
160 trypsin-EDTA (GIBCO). Non-ingested beads were removed by centrifuging ($100 \times g$ for 10 min at 4°C)
161 over a 3% BSA and 4.5% D-glucose cushion prepared with FACS buffer (HBSS supplemented with
162 2% FCS, 5 mM EDTA, and 0.1% sodium azide). Cells were washed with FACS buffer and analysed
163 with a C6 Accuri Flow Cytometer, measuring at least 75,000 cells after live cell gating according to the
164 FCS/SSC.

165

166 For the effects of cytokine stimulation on phagocytosis, fresh PBL were stimulated with recombinant
167 trout IL-1β (25 ng/ml, Hong et al., 2001), IL-6 (200 ng/ml, Costa et al., 2011), or medium alone as
168 control. Fluorescent latex beads were added 20 h later at a cell/bead ratio of 1:20, incubated for a further
169 3 h and phagocytosis analysed as above.

170

171 **2.7. Proliferation of PBL prepared by hypotonic lysis of erythrocytes**

172 PBL proliferation was quantified by measuring BrdU incorporation during DNA synthesis in replicating
173 cells using a Cell Proliferation ELISA, BrdU (colorimetric) kit (Sigma, UK) as per the manufacturer's
174 instructions. Briefly, PBL from each fish in complete cell culture medium, at 4×10^5 cells/well, were
175 cultured in 96-well cell culture plates in the presence of 2.5, 10 and 25 µg/ml of PHA. A control without
176 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 2×10^6 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 2001b) 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.

206

207

208 **3. Results and Discussion**

209 **3.1. The improved hypotonic method**

210 PBL prepared by the hypotonic lysis of erythrocytes described above were free of red blood cell
211 contamination, as determined by microscopy, and had >99.9% viability as assessed by trypan blue
212 exclusion. The yield from healthy fish was ~30 x10⁶ PBL/ml blood. The yield and viability of PBL
213 prepared by the gradient method were similar but the preparations were typically contaminated with
214 some red blood cells.

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

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

233

234 **3.2. Characterisation of PBL prepared using the hypotonic and gradient method**

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

240 We further evaluated the B cell and T cell populations in the PBL using monoclonal antibodies against
241 trout IgM and CD3 ϵ respectively. No staining was observed in secondary antibody controls (**Fig. 1C**).
242 The IgM antibody stained 31.4 \pm 7.7 % (Mean \pm SD) and 34.7 \pm 4.5 % leucocytes in the lymphocyte gate
243 of PBL prepared by the hypotonic lysis and gradient method, respectively (**Fig. 1D**). Meanwhile the
244 CD3 ϵ antibody (as a T cell marker) stained 31.8 \pm 7.6 % and 32.7 \pm 7.6 % leucocytes in the lymphocyte
245 gate (**Fig. 1E**). Paired samples T-test of the six fish analysed showed no significant difference in the
246 percentages of IgM $^{+}$ and CD3 ϵ^{+} in the lymphocyte populations of the PBL prepared by both methods
247 (**Fig. 1F**). IgM $^{+}$ B cells and T cells with similar percentages have been recorded in rainbow trout PBL
248 by Korytar et al. (2013).

249

250 **3.3. Characterisation of cell marker gene expression in PBL prepared using the hypotonic lysis** 251 **and gradient method**

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

255

256 **3.3.1 Expression of marker genes for erythrocytes and proliferation**

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

263 The head kidney and spleen are the main erythropoietic organs in fish, however peripheral blood can
264 contain a high proportion of immature erythrocytes, mostly erythroblasts that divide and undergo their
265 final maturation within the circulation (Witeska, 2013). In rainbow trout, 10.6% erythrocytes are
266 immature (Keen, et al., 1989). In contrast, most peripheral blood leucocytes are mature and do not
267 proliferate without stimulation. To examine whether signs of proliferation could be detected the
268 expression of several proliferation markers, proliferation cell nuclear antigen (PCNA, Leung et al,
269 2005), Ki63 and mini-chromosome maintenance protein (MCM)2 (Jurikova et al., 2016), were
270 investigated. All of these proliferation marker genes had higher expression in whole blood compared to
271 PBL prepared by both the hypotonic and gradient method, which were not different (**Fig. S2D-F**). These
272 results confirm the removal of erythrocytes and the low proliferation level in PBL.

273

274 **3.3.2 Expression of marker genes for T cells and B cells**

275 The expression of lymphocyte markers for T cells (TCR α , TCR β , CD3 ϵ , CD3 ζ , CD4-1, CD4-2, CD8 α
276 and CD8 β) and B cells (CD79a, CD79b, secreted (s) IgM, membrane (m) IgM, sIgD, mIgD, sIgT and
277 mIgT) were next examined (**Fig. 2**). The lymphocyte marker gene expression was consistently higher
278 in PBL prepared by the hypotonic method compared to whole blood. Their expression in PBL prepared
279 by the gradient method was also higher (CD3 ζ , CD4-1, CD79a and mIgD), or showed intermediate
280 levels that were not significantly different to the hypotonic prepared PBL or whole blood (TCR α , TCR β ,
281 CD3 ϵ , CD4-2, CD8 α , CD8 β , CD79b, sIgM, sIgT and mIgT). The exception was the expression of
282 mIgM and sIgD where levels were not different to whole blood but lower than in hypotonic prepared
283 PBL (**Fig. 2**). These expression patterns suggest that PBL prepared by the hypotonic method are
284 enriched for both T cells and B cells due to complete removal of erythrocytes whilst PBL prepared by
285 the gradient method are more prone to erythrocyte contamination and may lose some high-density B
286 cells (Ramirez-Gomez, et al., 2012).

287

288 **3.3.3 Expression of marker genes for other cell types**

289 Other leucocyte markers examined were the pan leucocyte marker L plastin, pan myeloid marker Spi-
290 1a and Spi-1b, thrombocyte markers CD41 and G6F, neutrophil marker myeloperoxidase (MPO),
291 macrophage markers lysozyme C and MCSFR and dendritic cell markers CD83 and CD80/86 (**Table**
292 **1**). Expression of all the leucocyte marker genes was higher in PBL prepared by the hypotonic method
293 compared to whole blood. Their expression in PBL prepared by the gradient method was also higher (L
294 plastin and Spi-1b), or at intermediate levels that were not significantly different to PBL prepared by
295 the hypotonic method or to whole blood (Spi-1a, CD41, MCSFR, lysozyme C, CD83 and CD86). The
296 exception was the expression of G6F and the neutrophil marker MPO where levels were no different to
297 whole blood but lower than in hypotonic prepared PBL (**Fig. 3**). These expression patterns suggest that
298 PBL prepared by the hypotonic method are enriched for thrombocytes, neutrophils, macrophages and
299 dendritic cells due to complete removal of erythrocytes and are potentially superior to PBL prepared by
300 the gradient method that may lose some higher density cells such as neutrophils.

301

302 **3.4. Phagocytosis**

303 Fish PBL contain B cells and other myeloid cells that are phagocytic (Takizawa et al., 2016; Zhang et
304 al., 2017). The phagocytic potential of PBL prepared by the hypotonic method was analysed by flow
305 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-1 β 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-1 β 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 $\mu\text{g/ml}$ (**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-1 β and
330 IFN γ). The expression of all the pro-inflammatory cytokines (IL-1 β 1, IL-1 β 2, TNF α 1, TNF α 2, TNF α 3,
331 IL-6, and IL-8) was up regulated by all the PAMPs and rIL-1 β at least at one time point, except TNF α 1
332 that was refractory to PHA (**Fig. 6**). IFN γ had no effect on the expression of IL-1 β 2, TNF α 1, TNF α 2,
333 TNF α 3, IL-6 and IL-8, but down-regulated IL-1 β 1 expression. IFN γ expression itself was up regulated
334 by Poly I:C and PHA but was refractory to LPS, IL-1 β and IFN γ (**Fig. 6F**). Lastly, CXCL11_L1
335 expression was up regulated by Poly I:C, PHA and IFN γ from 4 h to 24 h, and by LPS at 8 h, but was
336 refractory to IL-1 β 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.

339

340 **3.7. General discussion and prospective**

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

353

354 **4. Acknowledgements**

355 This work was funded by the Biotechnology and Biological Sciences Research Council (BBSRC,
356 BB/N024052/1) under the Newton Fund RCUK-CONICYT Research Partnership. YH was supported
357 by a PhD Studentship from the Ministry of Education, Republic of China (Taiwan). PAS was supported
358 by the Newton-Bhabha PhD placement programme (268692473) and FL was supported by a Newton
359 International Fellowship funded by the Academy of Medical Sciences (AMS, NIF004\1036). MI was
360 funded by Fondecyt 1161015 and KM was funded by Fondecyt 11171057.

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364 **6. References**

- 365 1. Aerts-Toegaert C, Heirman C, Tuybaerts S, Corthals J, Aerts JL, Bonehill A, Thielemans K,
366 Breckpot K. CD83 expression on dendritic cells and T cells: correlation with effective immune
367 responses. *Eur J Immunol.* 2007; 37:686-695.
- 368 2. Boardman T, Warner C, Ramirez-Gomez F, Matrisciano J, Bromage E. Characterization of an
369 anti-rainbow trout (*Oncorhynchus mykiss*) CD3ε monoclonal antibody. *Vet Immunol*
370 *Immunopathol.* 2012; 145:511-515.
- 371 3. Chen J, Xu Q, Wang T, Collet B, Corripio-Miyar Y, Bird S, Xie P, Nie P, Secombes CJ, Zou
372 J. Phylogenetic analysis of vertebrate CXC chemokines reveals novel lineage specific groups
373 in teleost fish. *Dev Comp Immunol.* 2013; 41:137-152.
- 374 4. Costa MM, Maehr T, Diaz-Rosales P, Secombes CJ, Wang T. Bioactivity studies of rainbow
375 trout (*Oncorhynchus mykiss*) interleukin-6: effects on macrophage growth and antimicrobial
376 peptide gene expression. *Mol Immunol.* 2011; 48:1903-1916.
- 377 5. Crippen TL, Bootland LM, Leong JC, Fitzpatrick MS, Schreck CB, Vella AT. Analysis of
378 salmonid leukocytes purified by hypotonic lysis of erythrocytes. *Journal of Aquatic Animal*
379 *Health* 2001; 13: 234-245.
- 380 6. DeLuca D, Wilson M, Warr GW. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*,
381 defined with monoclonal antibodies to IgM. *Eur J Immunol.* 1983;13:546-551.
- 382 7. Feng Y, Martin P. Imaging innate immune responses at tumour initiation: new insights from
383 fish and flies. *Nat Rev Cancer.* 2015; 15:556-562.
- 384 8. Fillatreau S, Six A, Magadan S, Castro R, Sunyer JO, Boudinot P. The astonishing diversity of
385 Ig classes and B cell repertoires in teleost fish. *Front Immunol.* 2013; 4:28.
- 386 9. Flajnik MF. A cold-blooded view of adaptive immunity. *Nat Rev Immunol.* 2018; doi:
387 10.1038/s41577-018-0003-9.
- 388 10. Hall C, Flores MV, Storm T, Crosier K, Crosier P. The zebrafish lysozyme C promoter drives
389 myeloid-specific expression in transgenic fish. *BMC Dev Biol.* 2007; 7:42.
- 390 11. Hong S, Zou J, Crampe M, Peddie S, Scapigliati G, Bols N, Cunningham C, Secombes CJ. The
391 production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1 beta. *Vet*
392 *Immunol Immunopathol.* 2001; 81:1-14.

- 393 12. Hong S, Li R, Xu Q, Secombes CJ, Wang T. Two types of TNF- α exist in teleost fish:
394 Phylogenetic, expressional and bioactivity analysis of Type II TNF-a3 in rainbow trout
395 *Oncorhynchus mykiss*. J Immunol. 2013; 191:5959-5972.
- 396 13. Hsu K, Traver D, Kutok JL, Hagen A, Liu TX, Paw BH, Rhodes J, Berman JN, Zon LI, Kanki
397 JP, Look AT. The pu.1 promoter drives myeloid gene expression in zebrafish. Blood. 2004;
398 104:1291-1297.
- 399 14. Husain M, Bird S, van Zwieten R, Secombes CJ, Wang T. Cloning of the IL-1 β 3 gene and IL-
400 1 β 4 pseudogene in salmonids uncovers a second type of IL-1 β gene in teleost fish. Dev Comp
401 Immunol. 2012; 38:431-446.
- 402 15. Juríková M, Danihel L, Polák Š, Varga I. Ki67, PCNA, and MCM proteins: Markers of
403 proliferation in the diagnosis of breast cancer. Acta Histochem. 2016; 118:544-552.
- 404 16. Kang SJ. The bloodline of CD8 α (+) dendritic cells. Mol Cells. 2012; 34:219-229.
- 405 17. Keen, J. E., A. M. Calarco Steele, and A. H. Houston. "The circulating erythrocytes of rainbow
406 trout (*Salmo gairdneri*)." Comparative Biochemistry and Physiology A. 1989; 94: 699–711.
- 407 18. Korytář T, Dang Thi H, Takizawa F, Köllner B. A multicolour flow cytometry identifying
408 defined leukocyte subsets of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol.
409 2013;35:2017-2019.
- 410 19. Krasnov A, Timmerhaus G, Afanasyev S, Takle H, Jørgensen SM. Induced erythropoiesis
411 during acute anemia in Atlantic salmon: a transcriptomic survey. Gen Comp Endocrinol. 2013;
412 192:181-190.
- 413 20. Lafferty KD, Harvell CD, Conrad JM, Friedman CS, Kent ML, Kuris AM, Powell EN, Rondeau
414 D, Saksida SM. Infectious diseases affect marine fisheries and aquaculture economics. Ann
415 Rev Mar Sci. 2015; 7:471-496.
- 416 21. Laing KJ, Wang T, Zou J, Holland J, Hong S, Bols N, Hirono I, Aoki T, Secombes CJ. Cloning
417 and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-alpha.
418 Eur J Biochem. 2001; 268:1315-1322.
- 419 22. Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T, Secombes CJ. Identification and analysis
420 of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss*. Dev Comp Immunol.
421 2002; 26:433-444.

- 422 23. Leung AY, Leung JC, Chan LY, Ma ES, Kwan TT, Lai KN, Meng A, Liang R. Proliferating
423 cell nuclear antigen (PCNA) as a proliferative marker during embryonic and adult zebrafish
424 hematopoiesis. *Histochem Cell Biol.* 2005; 124:105-111.
- 425 24. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional
426 characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood.*
427 2001; 98:3087-3096.
- 428 25. Lin HF, Traver D, Zhu H, Dooley K, Paw BH, Zon LI, Handin RI. Analysis of thrombocyte
429 development in CD41-GFP transgenic zebrafish. *Blood.* 2005; 106:3803-3810.
- 430 26. Little DC, Newton RW, Beveridge MC. Aquaculture: a rapidly growing and significant source
431 of sustainable food? Status, transitions and potential. *Proc Nutr Soc.* 2016; 75:274-286.
- 432 27. Liu X, Li YS, Shinton SA, Rhodes J, Tang L, Feng H, Jette CA, Look AT, Hayakawa K, Hardy
433 RR. Zebrafish B cell development without a pre-B cell stage, revealed by CD79 fluorescence
434 reporter transgenes. *J Immunol.* 2017; 199:1706-1715.
- 435 28. Liu Y, Moore L, Koppang EO, Hordvik I. Characterization of the CD3zeta, CD3gammadelta
436 and CD3epsilon subunits of the T cell receptor complex in Atlantic salmon. *Dev Comp*
437 *Immunol.* 2008; 32:26-35.
- 438 29. Maisey K, Montero R, Corripio-Miyar Y, Toro-Ascuy D, Valenzuela B, Reyes-Cerpa S,
439 Sandino AM, Zou J, Wang T, Secombes CJ, Imarai M. Isolation and characterization of
440 salmonid CD4+ T cells. *J Immunol.* 2016; 196:4150-4163.
- 441 30. Witeska M. Erythrocytes in teleost fishes: a review, *Zoology and Ecology.* 2013; 23:275-281.
- 442 31. Moore LJ, Somamoto T, Lie KK, Dijkstra JM, Hordvik I. Characterisation of salmon and trout
443 CD8alpha and CD8beta. *Mol Immunol.* 2005; 42:1225-1234.
- 444 32. Ohashi K, Takizawa F, Tokumaru N, Nakayasu C, Toda H, Fischer U, Moritomo T, Hashimoto
445 K, Nakanishi T, Dijkstra JM. A molecule in teleost fish, related with human MHC-encoded
446 G6F, has a cytoplasmic tail with ITAM and marks the surface of thrombocytes and in some
447 fishes also of erythrocytes. *Immunogenetics.* 2010; 62:543-559.
- 448 33. Ramirez-Gomez F, Greene W, Rego K, Hansen JD, Costa G, Kataria P, Bromage ES. Discovery
449 and characterization of secretory IgD in rainbow trout: secretory IgD is produced through a
450 novel splicing mechanism. *J Immunol.* 2012; 188:1341-1349.

- 451 34. Reitan LJ, Thuvander A. In vitro stimulation of salmonid leucocytes with mitogens and with
452 *Aeromonas salmonicida*. Fish & Shellfish Immunol. 1991; 1: 297-307
- 453 35. Rowley A. Collection, separation and identification of fish leucocytes. In Techniques in Fish
454 Immunology (Stolen JS, Fletcher TC, Anderson DP, Roberson BS and van Muiswinkel WB,
455 editors). SS Publications, Fair Haven, New Jersey, p113-135.
- 456 36. Secombes C. Will advances in fish immunology change vaccination strategies? Fish Shellfish
457 Immunol. 2008; 25:409-16
- 458 37. Takizawa F, Dijkstra JM, Kotterba P, Korytář T, Kock H, Köllner B, Jaureguiberry B,
459 Nakanishi T, Fischer U. The expression of CD8 α discriminates distinct T cell subsets in teleost
460 fish. Dev Comp Immunol. 2011; 35:752-763.
- 461 38. Takizawa F, Magadan S, Parra D, Xu Z, Korytář T, Boudinot P, Sunyer JO. Novel teleost CD4-
462 bearing cell populations provide insights into the evolutionary origins and primordial roles of
463 CD4+ lymphocytes and CD4+ macrophages. J Immunol. 2016; 196:4522-4535.
- 464 39. Van Muiswinkel WB. A history of fish immunology and vaccination I. The early days. Fish
465 Shellfish Immunol. 2008; 25:397-408.
- 466 40. Wang T, Diaz-Rosales P, Costa MM, Campbell S, Snow M, Collet B, et al. Functional
467 characterization of a nonmammalian IL-21: rainbow trout *Oncorhynchus mykiss* IL-21
468 upregulates the expression of the Th cell signature cytokines IFN-gamma, IL-10, and IL-22. J
469 Immunol. 2011a; 186:708-821.
- 470 41. Wang T, Huang W, Costa MM, Martin SAM, Secombes CJ. Two copies of the genes encoding
471 the subunits of putative interleukin (IL)-4/IL-13 receptors, IL-4Ra, IL-13Ra1 and IL-13Ra2,
472 have been identified in rainbow trout (*Oncorhynchus mykiss*) and have complex patterns of
473 expression and modulation. Immunogenetics. 2011b; 63:235–253.
- 474 42. Wang T, Johansson P, Abós B, Holt A, Tafalla C, Jiang Y, et al., First in-depth analysis of the
475 novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and
476 modulation but overlapping bioactivities. Oncotarget 2016; 7:10917-10946.
- 477 43. Wang T, Hu Y, Wangkahart E, Liu F, Wang A, Zahran E, Maisey KR, Liu M, Xu Q, Imarai
478 M, Secombes C. Interleukin (IL)-2 is a key regulator of Th1 and Th2 cytokine expression in
479 fish: functional characterisation of two divergent IL2 paralogues in salmonids. Frontiers in
480 Immunology. 2018. doi: 10.3389/fimmu.2018.01683

- 481 44. Yazawa R, Cooper GA, Hunt P, Beetz-Sargent M, Robb A, Conrad M, McKinnel L, So S,
482 Jantzen S, Phillips RB, Davidson WS, Koop BF. Striking antigen recognition diversity in the
483 Atlantic salmon T-cell receptor alpha/delta locus. *Dev Comp Immunol.* 2008; 32:204-212.
- 484 45. Zakrzewska A, Cui C, Stockhammer OW, Benard EL, Spaink HP, Meijer AH. Macrophage-
485 specific gene functions in Spi1-directed innate immunity. *Blood.* 2010; 116:e1-11.
- 486 46. Zhang XJ, Wang P, Zhang N, Chen DD, Nie P, Li JL, Zhang YA. B Cell functions can be
487 modulated by antimicrobial peptides in rainbow trout *Oncorhynchus mykiss*: Novel insights
488 into the innate nature of B cells in fish. *Front Immunol.* 2017; 8:388.
- 489 47. Zhang YA, Hikima J, Li J, LaPatra SE, Luo YP, Sunyer JO. Conservation of structural and
490 functional features in a primordial CD80/86 molecule from rainbow trout (*Oncorhynchus*
491 *mykiss*), a primitive teleost fish. *J Immunol.* 2009; 183:83-96.
- 492 48. Zou J, Carrington A, Collet B, Dijkstra JM, Yoshiura Y, Bols N, Secombes C. Identification
493 and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type
494 cytokine characterized functionally in fish. *J Immunol.* 2005;175:2484-2494.
- 495 49. Zou J, Wang T, Hirono I, Aoki T, Inagawa H, Honda T, Soma GI, Ototake M, Nakanishi T,
496 Ellis AE, Secombes CJ. Differential expression of two tumor necrosis factor genes in rainbow
497 trout, *Oncorhynchus mykiss*. *Dev Comp Immunol.* 2002; 26:161-172.

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501 **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

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

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Gene	Sequence (5'-3')	Sequence (5'-3')	Size (bp)	Acc. No.
Housekeeping gene				
EF-1 α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	AF498320
Cytokine genes				
IL-1 β 1	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGTGTGGAAGAACATATAG	179	AJ278242
IL-1 β 2	GAGCGCAGTGGAGTGTGG	AGACAGGTTCAAATGCACTTTATGGT	204	AJ245925
TNF α 1	TGTGTGGGGTCTCTTAATAGCAGGTC	CCTCAATTCATCCTGCATCGTTGA	102	AJ277604
TNF α 2	CTGTGTGGCGTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	98	AJ401377
TNF α 3	GCTGCACTCTTCTTACCAAGAAACAAG	CCACTGAGGACTTGTAAATCACCATAGGT	148	HE798544
IL-6	GGGAGAAAATGATCAAGATGCTCGT	GCAGACATGCCTCCTTGTGG	180	DQ866150
IL-8	TCCTGACCATTACTGAGGGGATGA	AGCGCTGACATCCAGACAAATCTC	200	AJ279069
IFN γ	CAAACGTAAAGTCCACTATAAGATCTCCA	TCCTGAATTTCCCTTGACATATTT	210	AJ616215
CXCL11_L1	TCATCAGCTTCTGGCCTGTC	TTCTCCGTTCTTCAAGTGACAATGAT	191	AF396869
Marker genes				
TCR α	CAAACGTGGTATTTTGACACAGATGCAA	TTCTTGTGTGTCTTTGAGGGACTGA	165/162	BT073987
TCR β	CAAAGTGGGGGAGAGATAACGACAGA	TGTTTCTCTGAGGAGCCCTGGAA	149	AF329700
CD3 ϵ	AAGGAAAGGTGTGAAGGACTGCTATG	GAGCGGGAAGTGGGTTTTTG	160	NM_001195174
CD3 ζ	CGTCTACTGCACTACCCTATGTCGAAG	AAGTGTGTCATTATTCTGCGATTATC	273	BT073940
CD4-1	GTGTGGAGGTGCTACAGGTTTTTTC	ATCGTCACCCGCTGTCTGTG	396	AY973028
CD4-2	CGACATTGTACAGTCAAGTCC	CCTCATTTGGCAACAACTTCTCAC	284	AY973029
CD8 α	CAAGTCGTGCAAGTGGGAAA	TCTGTTGTTGGCTATAGGATGTTGTTG	214	AF178053
CD8 β	GAACATCAAACCCAGAGGCTGTG	GACACTTTTTGGGTAGTCGGCTGAA	125	AY563420
slgM H	TACAAGAGGGAGACCGGAGGAGT	CTTCTGATTGAATCTGGCTAGTGGT	221	X65261
mIgM H	CCTACAAGAGGGAGACCGATTGTC	GTCTTCATTTACCTTGATGGCAGT	168	OMU04616
slgD H	TGAACATATCCAAACCAGGTGTCTG	GTCCTGAAGTCATCATTTTGTCTTGA	357	JQ003979
mIgD H	TGAACATATCCAAACCAGACTCC	GTCTGAAGTCATCATTTTGTCTTGA	191	AY870260
slgT H	CATCAGCTTCCAAAGGAAGTGA	TCACTTGTCTTACATGAGTTACCCGT	361	AY870268
mIgT H	TCGAAGTCCACGGCGAACA	GTGTTCTTACCCGCTTCACTTGA	187	AY870264
CD79a	CGAGGGAATGTTACTGATGGTGG	GCATTCTCCAGATTTAGACCCTCATA	142	CA362887
CD79b	CCTTTGTGAACCTCTCAGTGGC	GGCCTTGACCATTCCACCGT	238	XM_021565350
Hemoglobin-a	GAGGCTTTGGGAAGGATGCTGAC	CACAAGGATGTTGTTGGACAGAATCTT	170	EZ765953
Hemoglobin-b	CACTGGCTCTGGGAAGAGTCTCTGA	TGAGGACGTCAGCCAAACCCCT	186	FX112568
ALAS	CAGCCACATCATCCCAATAAAGGTTG	CACCTCCACGAGCCTCTCCAC	202	XM_021615925
PCNA	GGGCACTGGCAACGTCAAAC	TGTCAGCTATCTTGACTCCACCACTAGGG	206	XM_021621842
Ki67	GATGGCGGAGAGATTAATCAAATGTCA	TGGCACTTGGGAAGCAAATCC	250	BT073583
MCM2	CGCAAAGAGTCCATGGCGAC	TGAACAGCAGCAACTCATTGTTGTCTTT	253	XM_021610724
L plastin	CACAGATCAGCAGCTTCAAGGATAAGC	GCTCCAATCTTCTGGATACCGTGATA	178	XM_021571076
Spi-1a	AGTATGGCTCGTTACAGCGATGTTGA	GGATCTTCTCTTGTGGCCCAATTCTC	259	NM_001124513
Spi-1b	CAGGCCCTGTGTCCCTAGTTA	CGGATCTTCTCTTGTACCAGGAGTC	149	XM_021578477
CD41	AAGTGGACAGGCTGAGACCCAGA	GCGATTAAGAACCCACCTTCCA	68.6	XM_021624569
G6F	ACTGGGGACTTCTCTGCTGTTCA	GGCTGAAGGAAAGATAGAGACTGTGAGGAC	141	GU393010
MPO	TTTCGGTGACATGGCCAACAG	TCCACACGAACATCACCTGCAAC	282	XM_021616849
Lysozyme C	AACAGCCTGCCCAACTGGGT	ACACGCTTGGCACACCGGAT	227	XM_021601582
MCSFR	GGACTTTGCCCTCCAGAGATATACAC	GATCACAATCCTCACTAATCTTAGCTTGGC	212	AB091826
CD83	GTGAGGTGGTACAAGCTGGGTG	GCTGCCAGGAGACACTGTACCT	203	AY263797
CD80/86	CAGGAACACACTGTCTGCAGGC	CTGCTCCCTTCTCCTTGATTACTTC	163	EU927451

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511 **Figure legend:**

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

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

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

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

540 **Figure 5. Rainbow trout PBL proliferation.** Freshly prepared PBL from 4 fish were incubated with
541 2.5, 10 and 25 μg/ml PHA, or with medium alone as control, in triplicate wells per fish, for 3 days.
542 BrdU was added 20 h before incorporation of BrdU was detected by ELISA. The data are presented as
543 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-1 β and IFN γ for 4 h,
548 8 h and 24 h. The expression of IL-1 β 1 (A), IL-1 β 2 (B), TNF α 1 (C), TNF α 2 (D), TNF α 3 (E), IFN γ (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 *** $p \leq 0.001$.

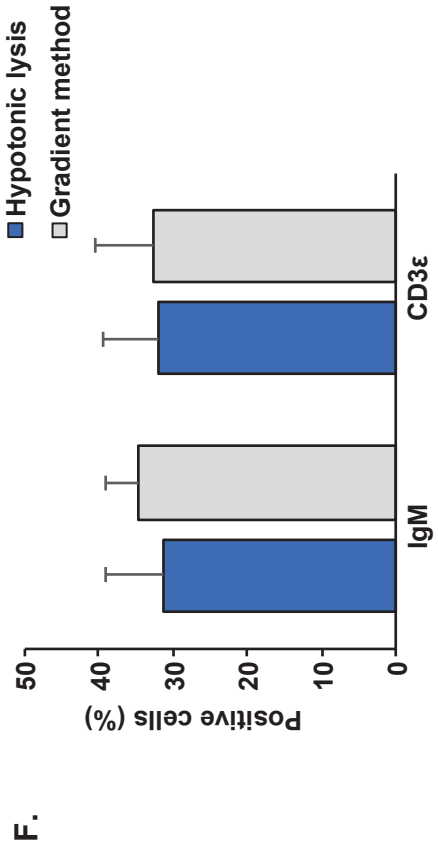
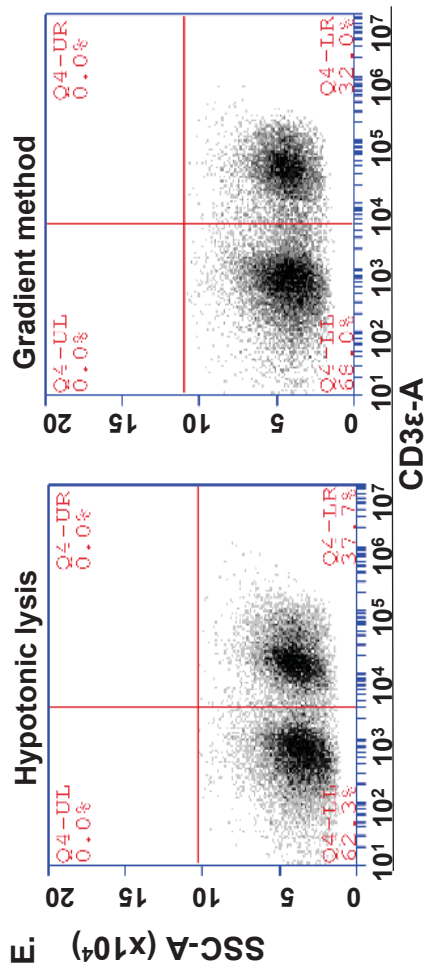
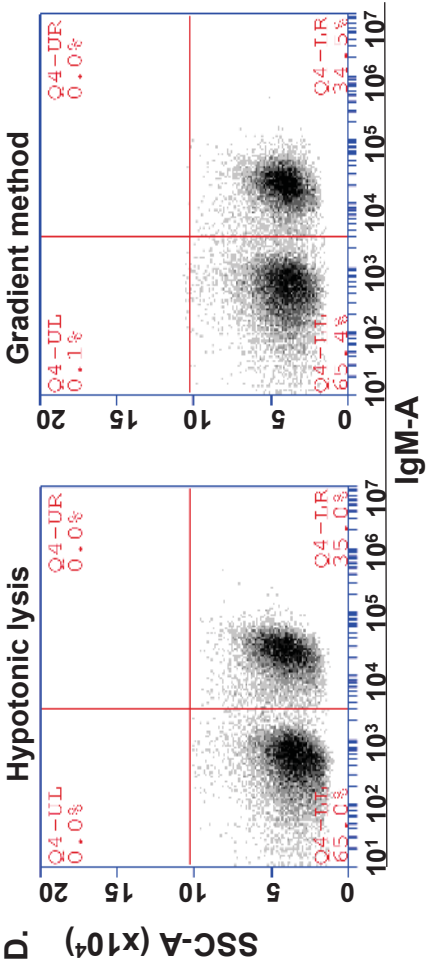
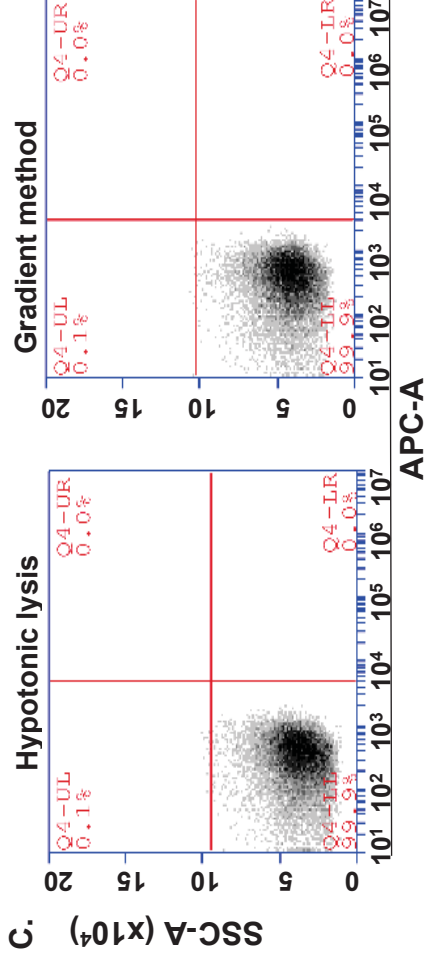
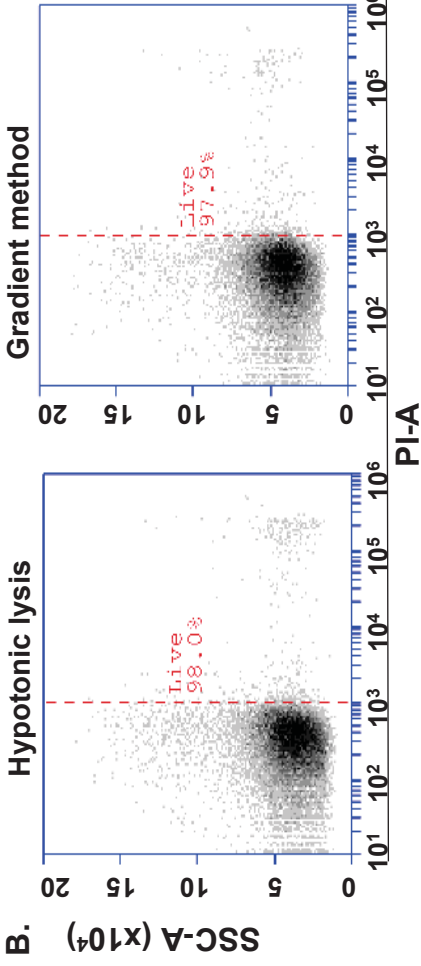
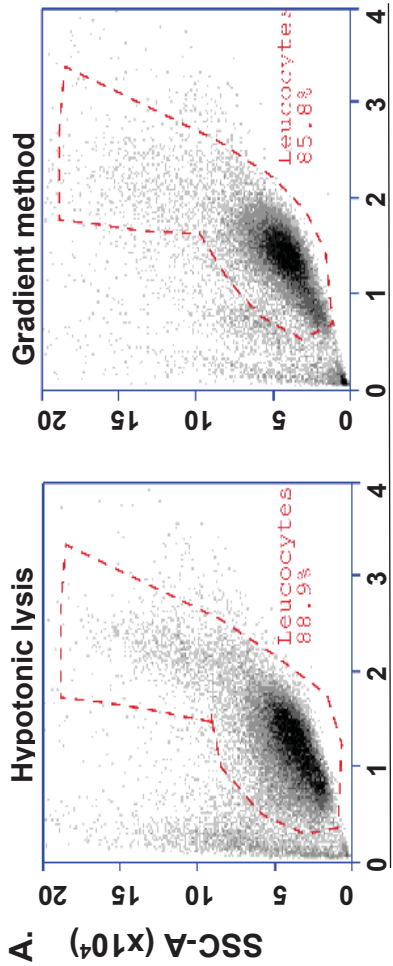
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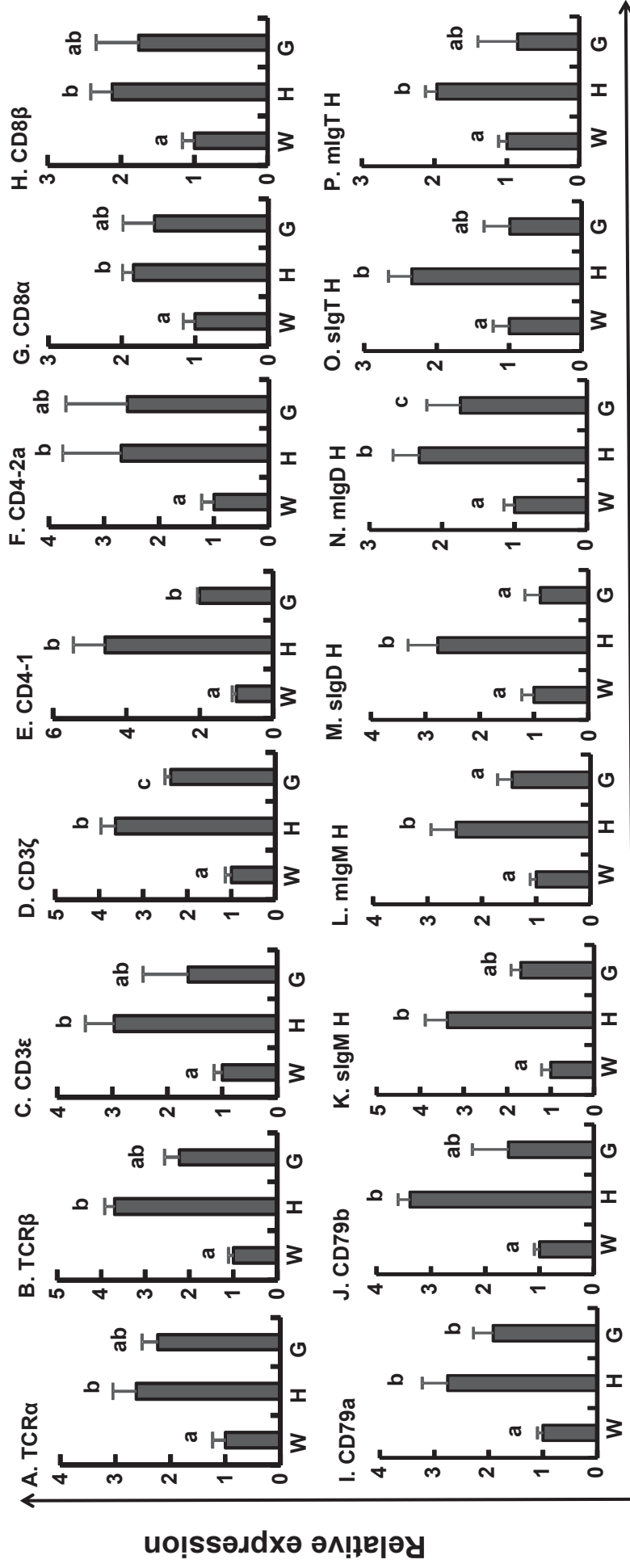
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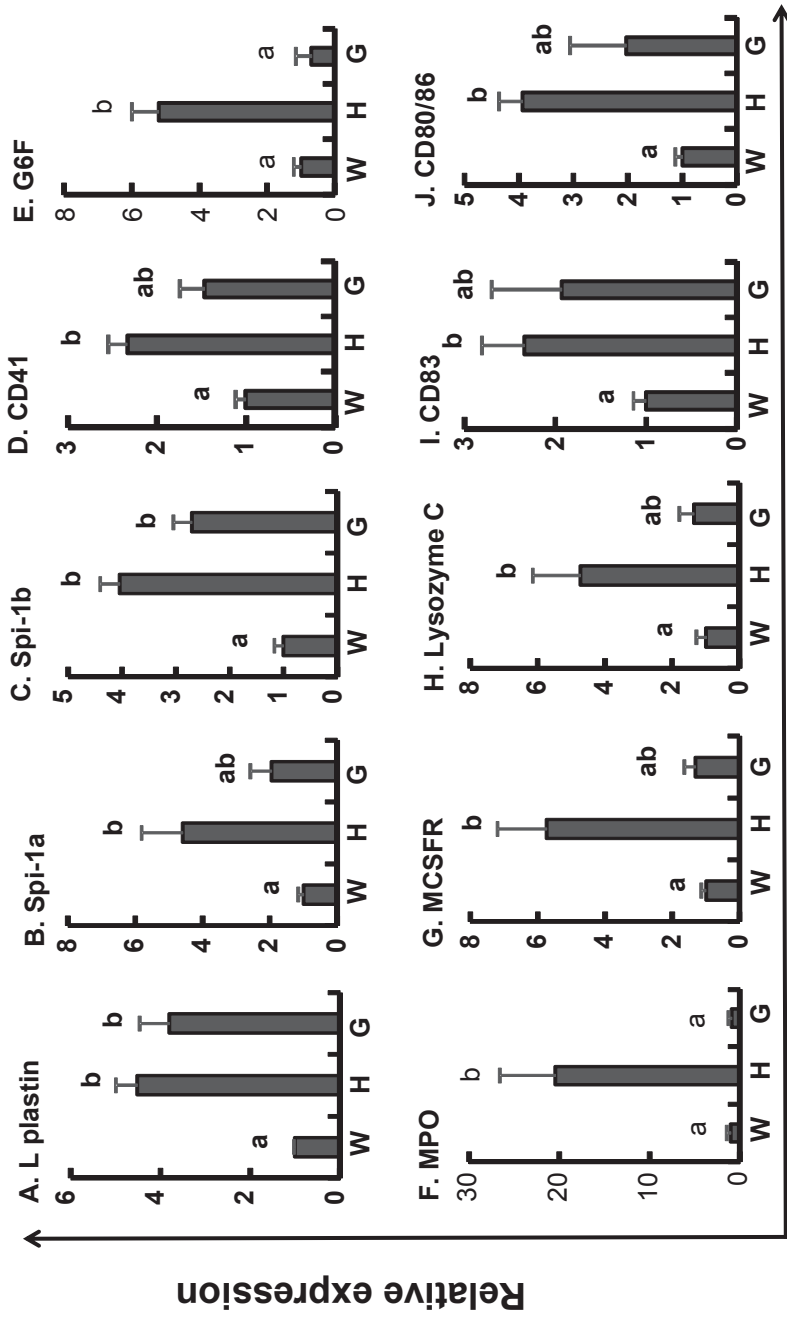
556 **Figure S1. Gating strategy for flow cytometric analysis.** Examples for PBL purified by hypotonic
557 lysis or the density gradient method. P1 = Leucocyte gate (A). P2 = Gate of excluded doublets in FSC-
558 H/FSC-A (B). P3 = Gate of excluded doublets in SSC-H/SSC-A (C). P4 = Live cells (as in 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).

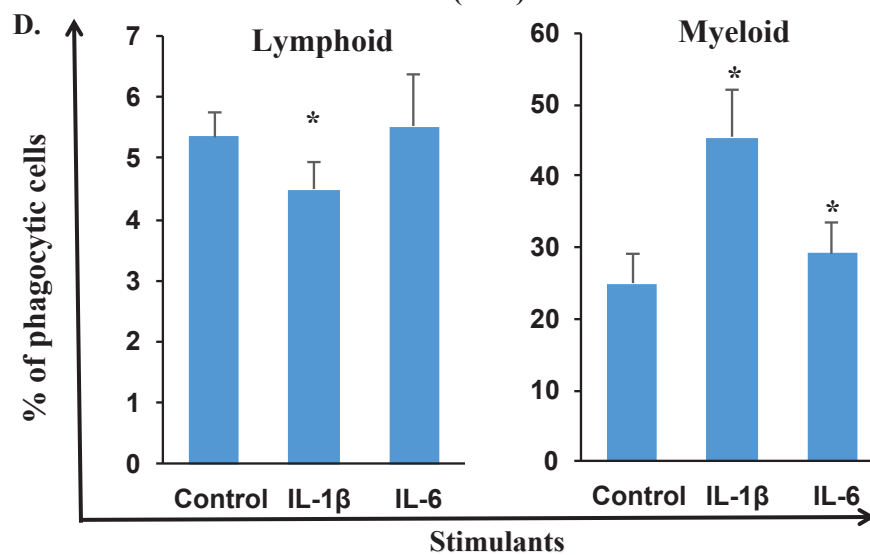
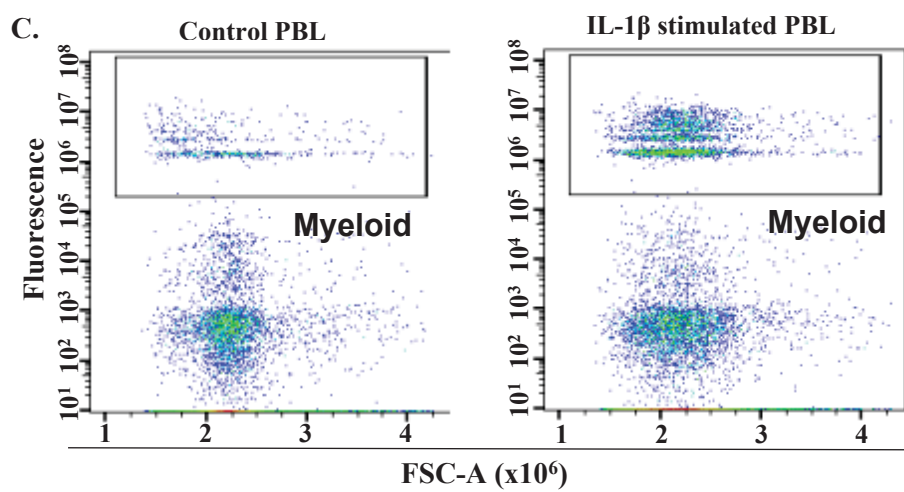
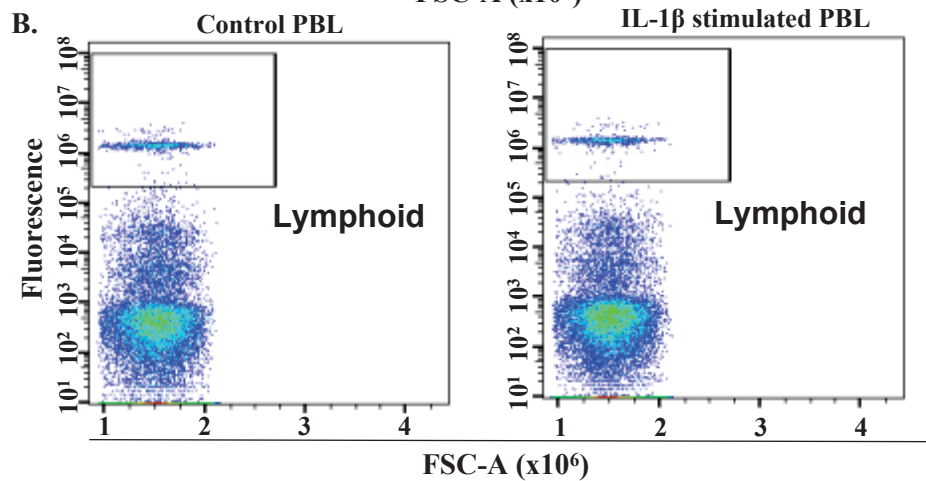
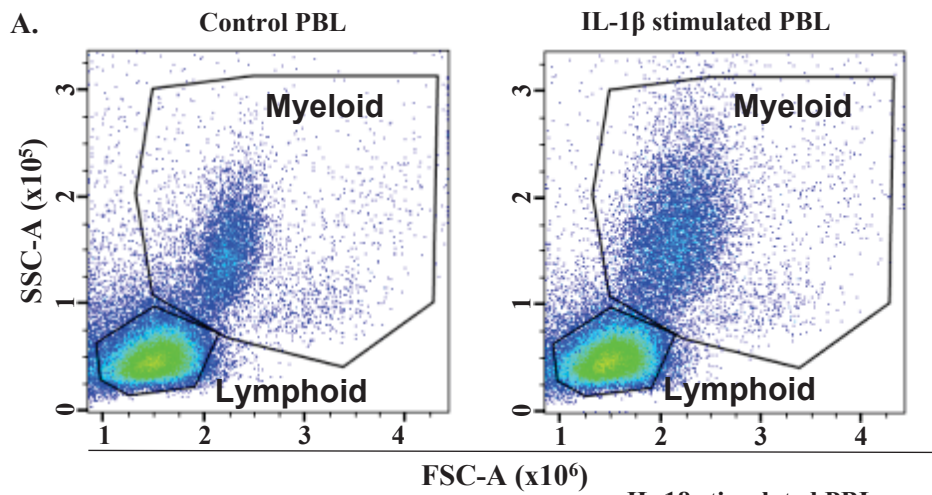
561 **Figure S2. The expression of erythrocyte and proliferation marker genes in whole blood, and PBL**
562 **prepared by the hypotonic and gradient method.** cDNA samples were prepared from whole blood
563 (W), or PBL prepared by the hypotonic (H) and gradient (G) method from four fish. The expression 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).

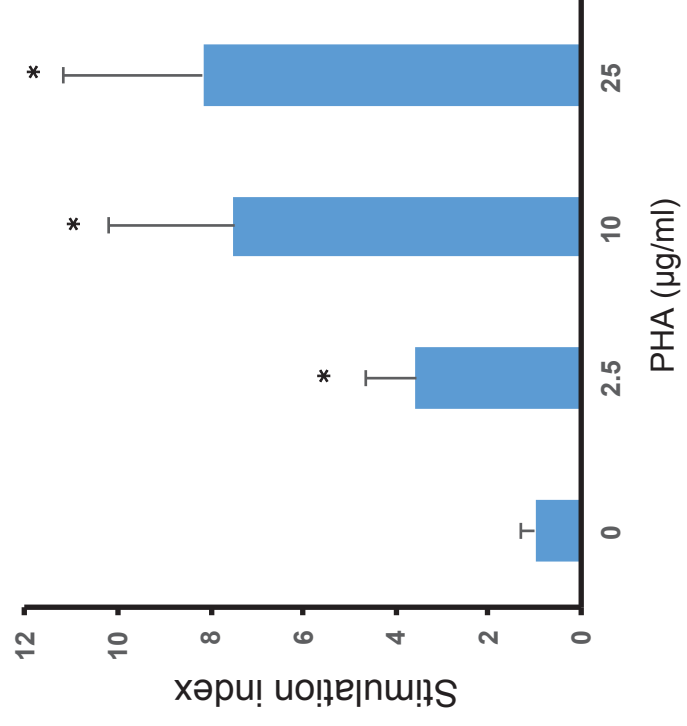
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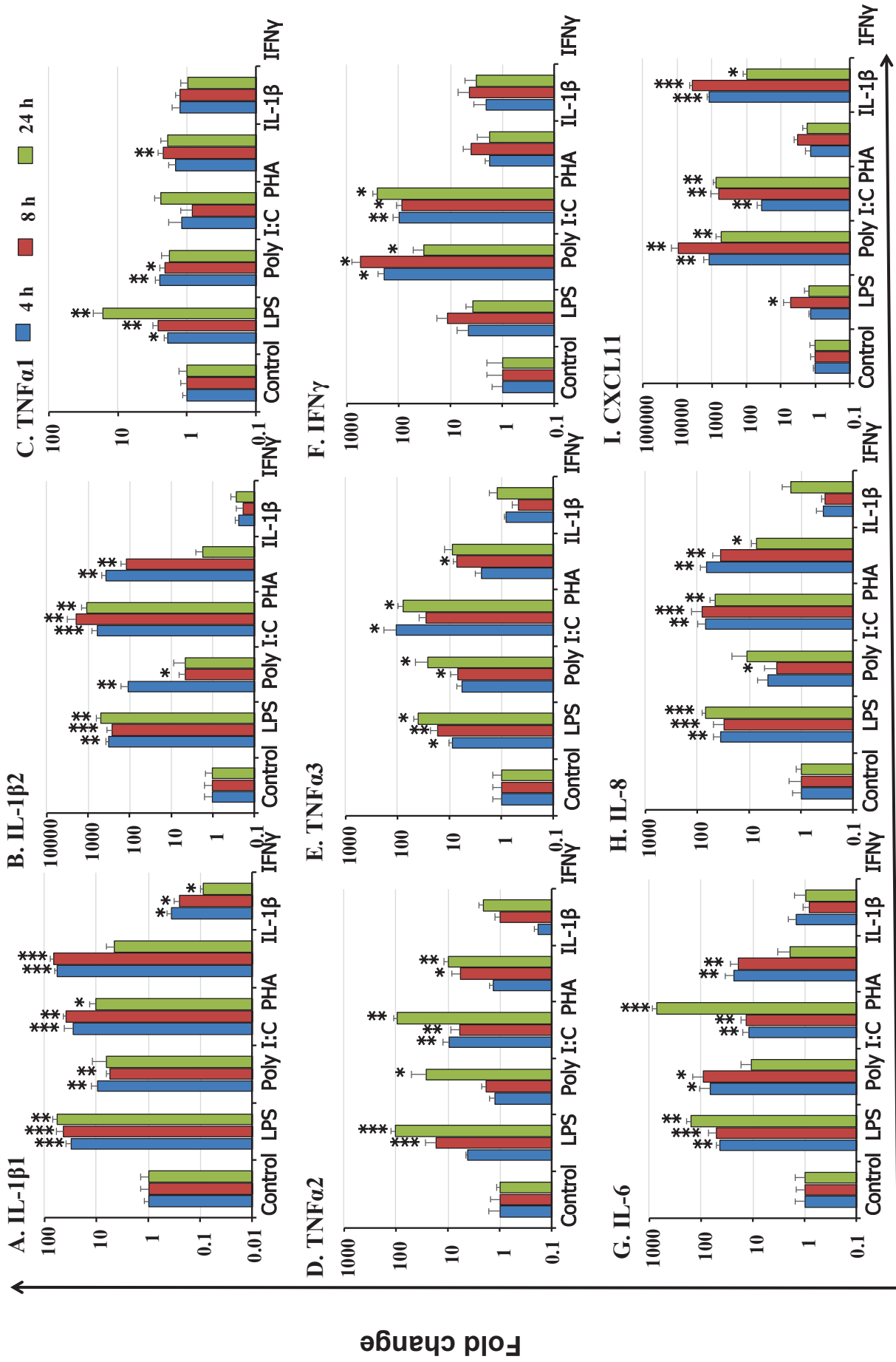












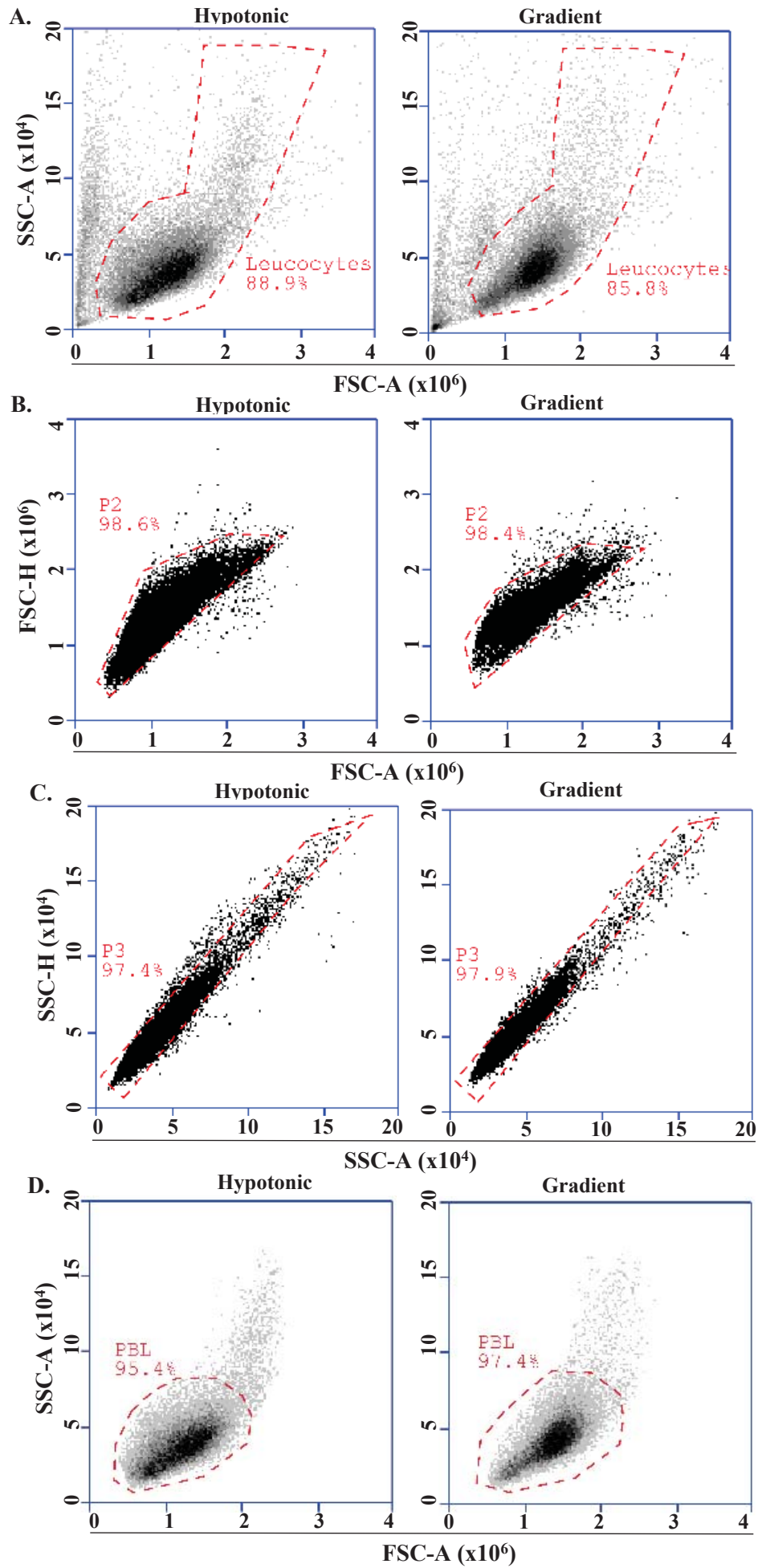


Fig. S1. Gating strategy for flow cytometric analysis. Examples for PBL purified by hypotonic lysis or density gradient method. P1 = Leucocytes gate (A). P2 = Gate of excluded doublets in FSC-H/FSC-A (B). P3 = Gate of excluded doublets in SSC-H/SSC-A (C). P4 = Live cells (as in Fig. 1B) and live lymphocytes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

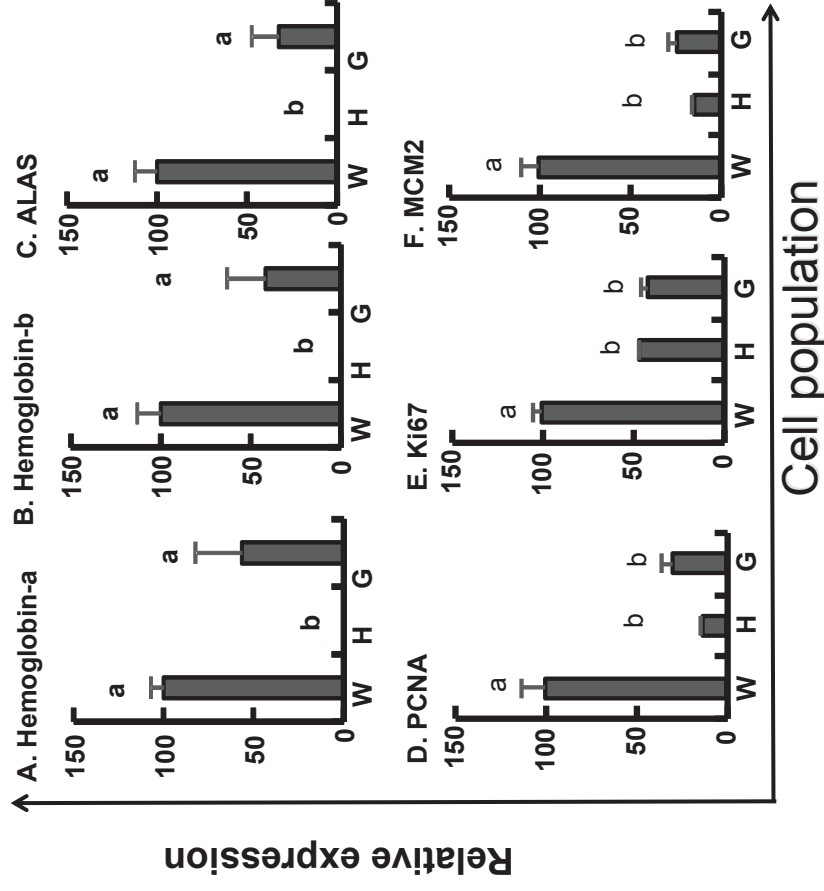


Fig. S2. The expression of erythrocyte and proliferation marker genes in whole blood, and PBL prepared by the hypotonic and gradient method. cDNA samples were prepared from whole blood (W), or PBL prepared by the hypotonic (H) and gradient (G) method from four fish. The expression of Hemoglobin-a (A), 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).