Developmental and Comparative Immunology 88 (2018) 104–113

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

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

Yehfang Hu[a], Kevin Maisey[b], Parasuraman Aiya Subramani[a,1], Fuguo Liu[a], Camila Flores-Kossack[b], Mónica Imarai[c], Christopher J. Secombes[a,**], Tiehui Wang[a,*]

[a] Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, United Kingdom
[b] Laboratorio de Immunología Comparativa, Centro de Biotecnología Acuícola, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Santiago, Chile
[c] Laboratorio de Immunología, Centro de Biotecnología Acuícola, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Santiago, Chile

ARTICLE INFO

Keywords:
Rainbow trout *Oncorhynchus mykiss*
Peripheral blood leucocytes (PBL)
Hypotonic lysis of erythrocytes
Phagocytosis
Proliferation
Immune response

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 s 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.

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 and Martin, 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.

* Corresponding author.
** Corresponding author.
E-mail addresses: c.secombes@abdn.ac.uk (C.J. Secombes), t.h.wang@abdn.ac.uk (T. Wang).
[1] Current address: Centre for Fish Immunology, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai, Tamil Nadu, 600117, India.

https://doi.org/10.1016/j.dci.2018.07.010
Received 13 June 2018; Received in revised form 10 July 2018; Accepted 10 July 2018

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 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 °C. 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 5×10^5 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 (400 g, 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.