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Advances in salmonid fish immunology: A review of methods and techniques for lymphoid tissue and peripheral blood leucocyte isolation and application

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ABSTRACT

Evaluating studies over the past almost 40 years, this review outlines the current knowledge and research gaps in the use of isolated leucocytes in salmonid immunology understanding. This contribution focuses on the techniques used to isolate salmonid immune cells and popular immunological assays. The paper also analyses the use of leucocytes to demonstrate immunomodulation following dietary manipulation, exposure to physical and chemical stressors, effects of pathogens and parasites, vaccine design and application strategies assessment. We also present findings on development of fish immune cell lines and their potential uses in aquaculture immunology. The review recovered 114 studies, where discontinuous density gradient centrifugation (DDGC) with Percoll density gradient was the most popular leucocyte isolation method. Fish head kidney (HK) and peripheral blood (PB) were the main sources of leucocytes, from rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Phagocytosis and respiratory burst were the most popular immunological assays. Studies used isolated leucocytes to demonstrate that dietary manipulations enhance fish immunity, while chemical and physical stressors suppress immunity. In addition, parasites, and microbial pathogens depress fish innate immunity and induce pro-inflammatory cytokine gene transcripts production, while vaccines enhance immunity. This review found 10 developed salmonid cell lines, mainly from *S. salar* and *O. mykiss* HK tissue, which require fish euthanisation to isolate. In the face of high costs involved with density gradient reagents, the application of hypotonic lysis in conjunction with mico-volume blood methods can potentially reduce research costs, time, and using nonlethal and ethically flexible approaches. Since the targeted literature review for this study retrieved no metabolomics study of leucocytes, indicates that this approach, together with traditional technics and novel flow cytometry could help open new opportunities for *in vitro* studies in aquaculture immunology and vaccinology.

1. Introduction

For almost four decades, global aquaculture has been responsible for the increased amount of fish consumed, surpassing population growth to become the world's fastest growing food production primary industry [1,2]. In the past five years, over 50% of food fish has come from aquaculture [1], and is tipped to contribute 60% of all food fish by 2030 [2]. The sector is thus poised to be the main protein source for the world's estimated 8.6 billion people by 2030 [3]. To meet the growing global demand for seafood, aquaculture farms aim to produce high quantity of healthy and fast-growing fish, under optimal husbandry and management practices in limited water volumes [4]. Husbandry practices include, but are not limited to maintaining good water quality, proper feeding, and biosecurity protocols with the aim to safeguard fish health.

Several intrinsic and extrinsic factors, including physical and chemical stressors have the ability to alter the fish innate immunity [5]. One of the sustainable practices recommended to enhance fish immunity and promote growth involves the administration of dietary supplements and immunostimulants [6,7]. Application of

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Abbreviations

PBL	peripheral blood leucocytes
TKL	total kidney leucocyte
HKL	head kidney leucocytes
MKL	middle kidney leucocyte
PKL	posterior kidney leucocyte
ATK	anterior part of trunk kidney
SPL	spleen leucocytes
THL	thymus leucocytes
F	frequency
HK	head kidney
PB	peripheral blood
SP	spleen
TH	thymus
DDGC	discontinuous density gradient centrifugation
CDGC	continuous density gradient centrifugation
HL	hypotonic lysis
ICSC	ice-cold sedimentation and centrifugation
SC	suspension centrifugation
TS	tissue suspension
CL	calorimetry
FC	flow cytometry
PCM	phase contrast microscopy
LM	light microscopy
EFM	epifluorescence microscopy
FM	fluorescence microscopy
SEM	scanning electron microscopy
SP	spectrophotometer
MMR	multimode microplate reader
LU	Luminometer
CC	colony count
MPF	Microplate fluorometer
ER	ELISA reader

immunostimulants is preferred over the use of antibiotics, which have been associated with food safety concerns [8] and occupational health hazards [9]. Antibiotic use in aquaculture has also been associated with antimicrobial resistance [10], residue accumulation [11], aquatic toxicity, antibiotic resistant microbial community selection [12,13], and the emergence of multi-antibacterial resistant strains [14]. Farmed fish often co-exist with a multitude of bacteria, viruses, fungi, and parasites, which exploit lapses in optimal culture conditions to proliferate and compromise the innate immune system. Indeed, disease outbreaks routinely lead to heavy industry losses [reviewed in Ref. [15]]. These scenarios make fish immunology understanding an indispensable asset to allow routine detection of health problems [reviewed in Ref. [5]].

Salmonid production is dominated by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), with a contribution of 6% of the world's finfish aquaculture production in 2016 [2]. *S. salar* is the ninth most farmed finfish species globally [16]. Major farmed salmonid producers include Norway, Chile, Scotland, and Canada [17]. In the salmon farming industry, major losses have been caused by the caligid copepod sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus)* [18]. In

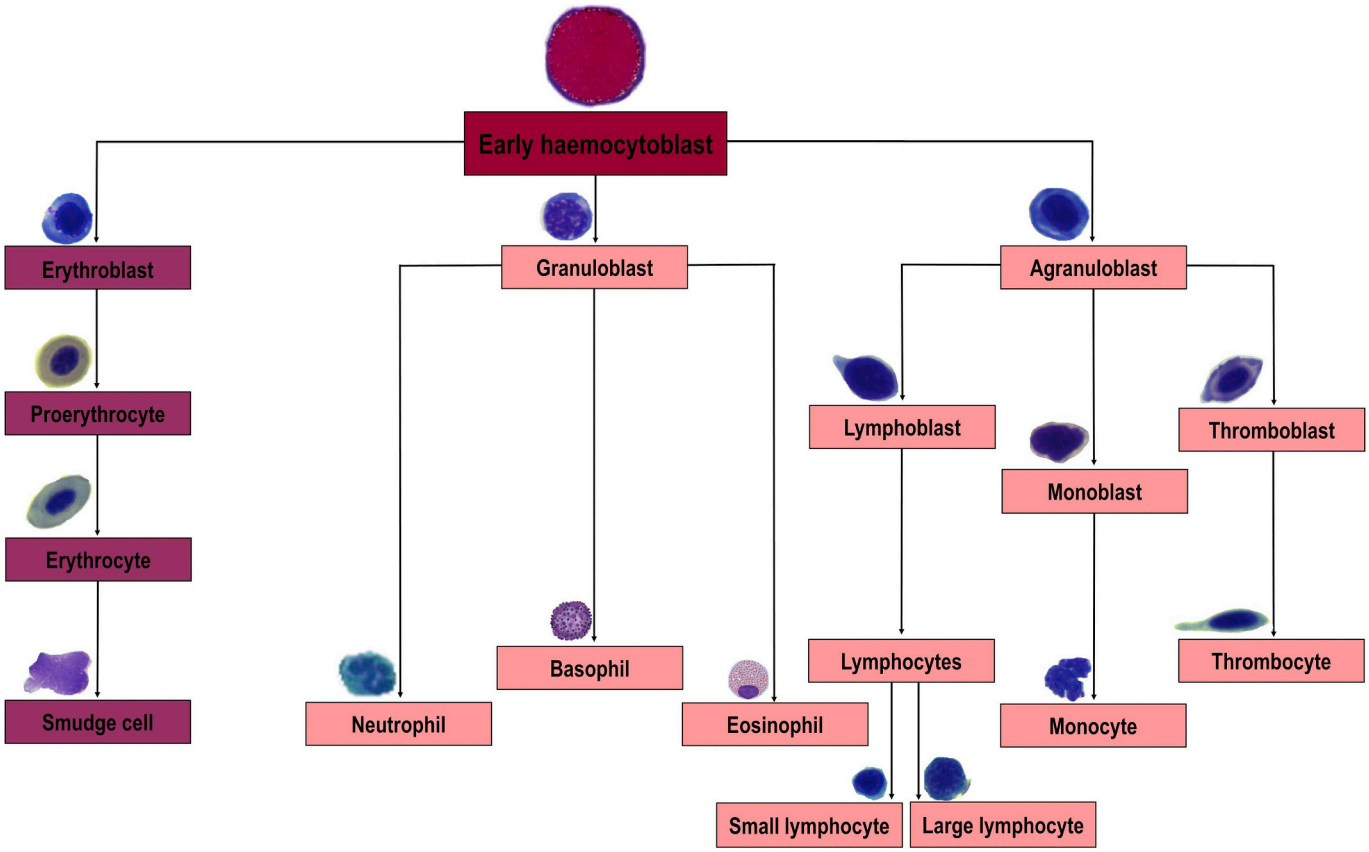

Fig. 1. Schematic representation of salmonid red blood and white blood cells haematopoiesis. Most of the cells come from slide smears of Chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood. Only basophil, eosinophils are adapted from Claver and Quaglia [31] and haemocytoblast from Ref. [32]. NB: as monocytes age, they differentiate into macrophages [33]. Other cells not shown include dendritic cells (DC) and mast cells (MC). [1.5]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Commonly studied salmonid species and popular lymphoid tissues for isolation of fish leucocytes in fish immunology. PBL: Peripheral blood leucocytes, HKL: Head kidney leucocytes, SPL: Spleen leucocytes, and THL: Thymus leucocytes. F: Frequency. Data comes from 114 reviewed salmonid studies.

Studied salmonid species	Studies reviewed	PBL	HKL	SPL	THL
	F	F	F	F	F
O. kisutch	4	1	3	2	1
O. mykiss	62	27	41	9	1
O. tshawytscha	11	3	10	6	–
O. nerka	1	–	1	–	–
S. trutta	2	–	2	1	–
S. fontinalis	1	–	1	–	–
S. salar	33	17	27	4	1
Total	**114**	**48**	**85**	**22**	**3**

Chile, bacterial kidney disease (BKD), *Piscirickettsia salmonis*, and infectious salmon anaemia virus (ISAV) have devastated those industries [19] and *P. salmonis* remains the biggest industry health challenge [20]. In Norway, *L. salmonis* remains a major salmon health challenge [21], and pancreases disease (PD) caused by *Salmonid alphavirus* (SAV) and ISAV [22]. In Scotland, *L. salmonis*, is the number one challenge [23,24]; while furunculosis caused by *Aeromonas salmonicida*, and Piscine Reovirus (PRV) poses the biggest problem in British Colombia, Canada [25].

The role of immunity in fish is to act as a barrier that ensures protection against pathogens [26]. The immune system consists of the innate components, which form the first line of defence [reviewed in Refs. [27,28]]. The adaptive immune components complement the fish immune machinery and rely on humoral and cellular responses, characterized by specific antigen recognitions, which invoke a quick secondary pathogen-specific response [28]. Thus, fish mostly rely on the innate immune system for defence [29]. For literature on the cellular specifics and functions of the innate and adaptive immune components of fish, see literature in Lulijwa et al. [30] and Fig. 1. For details on specific molecules produced by the physical and humoral components, see Refs. [34,35]. The primary lymphoid organs of fish include the head kidney (HK), spleen (SP) and thymus (TH) a paired organ, located in the dorsolateral area of the opercular cavity in teleosts [36]. Teleost's T cell development takes place in the thymus, and B cells in the bursa [36]. Thus, to conduct immunological studies, fish leucocytes must be purified from lymphoid tissues and peripheral blood by hypotonic lysis and density gradient centrifugation [37–39]. Possession of the adaptive and innate immune capacity makes fish an important immunological model to investigate vaccine design, efficacy and vaccination strategies, dietary manipulations, including effects induced by pathogens and stressors.

In studying salmonid fish immunology, several approaches are commonly used, including characterisation of physical barriers [28], peripheral blood (PB) haematology [30,40–42], HK, PB and SP isolated cellular functional characterisation [43–46], and humoral parameters [47–49]. Consequently, integrated haematology, and the study of humoral and cellular parameters have been employed in salmonids to investigate immunomodulatory effects of: dietary manipulations [46,50,51]; pathogen challenges [45,52]; physical and chemical stressors [43,53–55].

In fish immunology, previous reviews have focussed on: developmental aspects of the fish immune system [26,56,57], innate immunity [5,7,58], humoral immunity [7], cellular granulocytes [59,60], macrophage biology [61] and macrophage antimicrobial mechanisms [62], immune related genes [63], cytokines [64], plus mucosal immunology [34,65–67]. However, no review has previously focussed on the use of isolated salmonid lymphoid tissue or PB leucocytes (PBL) to illustrate immunological effects induced by dietary manipulations, vaccines and vaccination strategies, pathogen challenges, and effects induced by stressors. Literature on the use of isolated lymphoid tissue and PBL in immunological investigations has been accumulating across salmonid species since the 1980s, forming the basis for the current review. To achieve our objective, we searched using the terms “fish leucocytes*immunology/immunological assays” in the Web of Science and “fish AND leucocytes AND salmonid AND immunology” in Scopus. Only papers on salmonids were considered by title and quick abstract scan for the key words isolated leucocytes, immune or immunological response, and/or characterisation. Subsequently, we highlight the major findings of this review with respect to salmon immune understanding. We also mention developed and characterised salmonid immunological cell lines, identify research gaps and make recommendations for affordable, nonlethal and ethically appropriate techniques for leucocyte isolation.

2. Commonly studied salmonids and target tissues

The current review found 114 studies that discussed the isolation and use of lymphoid tissue and PBL (Table 1). These studies spead

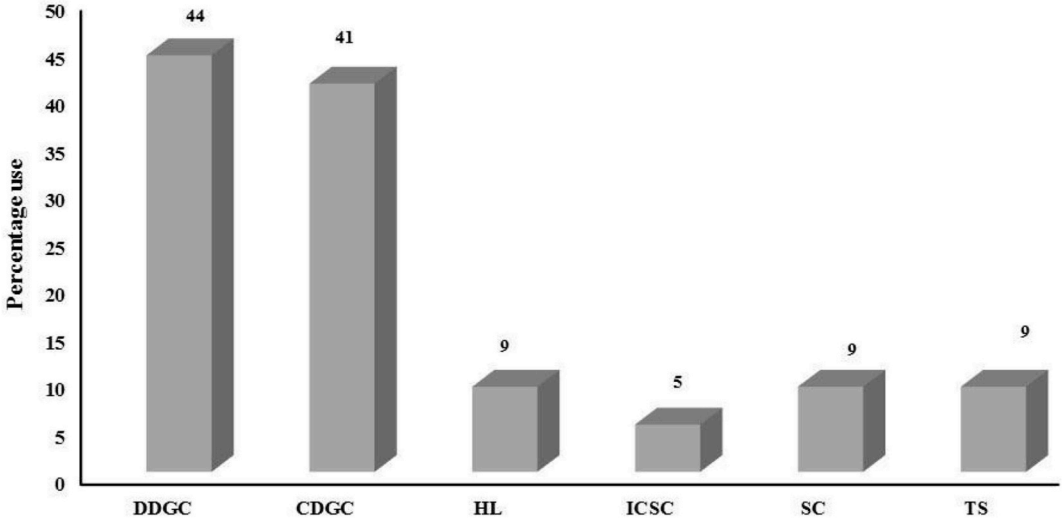

Fig. 2. Techniques commonly used to isolate salmonid fish leucocytes for immunological assays. DDGC: Discontinuous density gradient centrifugation, CDGC: Continuous density gradient centrifugation, HL: Hypotonic lysis, ICSC: Ice-cold sedimentation and centrifugation, SC: Suspension centrifugation, and TS: Tissue suspension. Data comes from 114 reviewed salmonid studies. [1.5].

Table 2
Techniques commonly used in salmonid leucocyte isolation and intended assays. PBL: Peripheral blood leucocyte, TKL: Total kidney leucocyte, HKL: Head kidney leucocyte, MKL: Middle kidney leucocyte, PKL: Posterior kidney leucocyte, SPL: Spleen leucocyte, and THL: Thymus leucocytes. Unless otherwise stated, the antibiotic solution used consisted of a combination of penicillin/streptomycin (P/S) at ratios e.g. 1% P/S, P 100 UmL^{-1}/S 100 $\mu g\,L^{-1}$, etc., while singly used antibiotics included oxytetracycline, gentamycin sulphate and streptomycin.

Species	Cells	Technique	Intended assays	Reference
O. kisutch	PBL	Heparinised PB 1 mL of 4, 5 or 6% Ficoll-Sodium Metrizoate Solution (SMS Sigma) were purified by Ficoll-SMS DCDGC for 20 min at 100 g and 15 °C.	Microscopic characterisation	[70]
	HKL	Unfiltered HK tissue suspension in holding medium (HM) on ice was sediment to remove debris, the supernatant, was removed and washed once in HM for 10 min at 500 g and 4 °C. The resulting cell pellet was resuspended in TCM and cells counted on a haemocytometer.	Plaque forming cells Antibody production Total IgM	[71]
	HK-L, SPL	Unfiltered HK and SP tissue suspensions in tissue culture medium, and leucocyte suspensions were kept on ice to allow debris sedimentation tissue leucocytes were purified according to Yui and Kaattari [72]. The resulting cell pellet was resuspended in TCM and adjusted to $10^7\,mL^{-1}$ by haemocytometer.	Lymphocyte mitogen stimulation Plaque forming cell assay	[72]
	HK-L, SPL, THL	Unfiltered tissue single cell suspensions were obtained by repeated aspiration of tissue fragments with a 1 mL syringe on ice in L-15 complete medium with 10% FCS, L-glutamine at pH 7.4. The suspension was washed twice at 1000 g at 4 °C for 10 min and the pellet resuspended in L-15 complete medium for assaying.	Plaque forming cell assay	[73]
O. mykiss	PBL	Heparinised PB diluted 1:2 in heparinised MEM with Eagle's salt with 0.2% sodium heparin, buffered with 7.5% sodium hydrogen carbonate (pH 7.6) and leucocyte suspension purified by Percoll (54%) CDGC at 400 *g* for 25 min. The leucocytes were washed with PBS (pH 7.6) at 500 g for 5 min. Leucocytes were counted by haemocytometer fixed with RNA later® and stored at −20 °C.	Cell proliferation and apoptosis Immune gene expression	[74]
	PBL	PBL were purified according to Hu et al. [38].	Immune gene expression Cell proliferation Phagocytosis	[75,76]
	PBL	PBL were purified according to Hu et al. [38].	Immune gene expression Flow cytometry	[77,78]
	PBL	Heparinised PB diluted 1: 8 in 1x phosphate buffered saline (PBS) and leucocytes purified by Histopaque ($1.077\,g\,mL^{-1}$) CDGC at 500 g for 40 min. PBL were	Marker gene expression Phagocytosis Cell proliferation Immune gene expression	[38]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		collected from the interface, washed twice and counted. Also, PBL were prepared by modified hypotonic lysis via erythrocytes lysis in ice-cold water mixing for 20 s, then immediate return to isotonic condition with 10x PBS. The PBL preparation was left on ice for 5–10 min to allow debris and nuclear materials to settle. The PBL were separated by passing through an EASYstrainer (70 μm) pelleted at 200 g, for 5 min, washed once and resuspended in complete culture medium.		
	HKL	Filtered (70 μm) HK tissue suspension in L-15 medium with 2% FBS, were centrifuged into single cells for 10 min at 600 g and 4 °C. Cell pellet was resuspended in L-15 medium and leucocytes purified by Percoll (60%) CDGC for 60 min at 700 g and 20 °C. Leucocyte were posteriorly centrifuged for 10 min at 400 *g* and 4 °C; collected and resuspended in a supplemented L-15 medium. This procedure was repeated twice and cells counted on a haemocytometer.	Immune gene expression	[50]
	HK-L, SPL	Nylon mesh filtered tissue suspensions were purified according to Chung and Secombes [79]. Leucocytes were adjusted to 10^6 mL^{-1} in PBS with 2% FCS, and 0.1% sodium azide.	Flow cytometry characterisation of trout polyclonal antibody to rainbow C-type lectin (CLEC4T1 +).	[44]
	HKL	Nylon mesh (100 μm) filtered HK tissue in incomplete heparinised L-15 medium with antibiotic solution, 0.5% FBS, was purified by centrifugation at 200 g for 5 min, the pellet washed once with complete medium with 10% FBS and adjusted to 10^6 mL^{-1}.	Immune gene expression	[80]
	PBL	Heparinised PB was purified to obtain PBL by Percoll (1.075 g mL^{-1}) CDGC at unspecified settings.	Monoclonal antibody (MAb) detection	[81]
	PBL, SPL, HKL & PKL	Cell strainer (70 μm) filtered tissue cell suspension in L-15 medium with 10% bovine growth serum (BGS), gentamicin (50 μg mL^{-1}), and fungizone (0.25 μg mL^{-1}) on ice and adjusted to the right density used for direct assay. PB diluted 1:5 in heparinised PBS was allowed to settle for at least 20 min at room temperature. Leucocyte rich plasma was removed from settled erythrocyte and purified by Nycoprep (1.077 g mL^{-1}) CDGC. Cells were adjusted to 10^6 mL^{-1}	Phagocytosis Antibody detection Transmission electron microscopy	[82]

(*continued on next page*)

Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		by Trypan Blue in supplemented media.		
	HKL	HKL isolated aseptically according to Secombes [83]. Nylon mesh (100 μm) filtered tissue cell suspension in unheparinised medium and leucocytes purified by Percoll (51%) CDGC for 30 min at 400 g and 4 °C. Cells were washed twice with L-15 medium by centrifugation at 800*g* for 10 min, and adjusted to 10^6 mL^{-1} by haemocytometer in L-15 medium with 0.1% FBS.	Flow cytometry of PB Respiratory burst Bactericidal activity	[49]
	HKL	Nylon mesh (100 μm) filtered HK tissue suspension in heparinised RPMI 1640 with antibiotic solution and 0.1% FCS were purified by Percoll (34/51%) DCDGC for 25 min at 400 g and 4 °C according to M. Sakai, Kobayashi, and Yoshida [84]. Cells were washed twice with RPMI 1640, adjusted to 10^5 mL^{-1} by haemocytometer.	Phagocytosis Phagocytic index	[85]
	HKL	Nylon mesh (100 μm) filtered HK tissue cell suspension in heparinised RPMI 1640 with antibiotic solution and 0·1% FCS were purified by Percoll (34/51%) DCDGC for 25 min at 2000 g and 4 °C, according to M. Sakai et al. [84] washed twice in RPMI 1640 and adjusted to 10^5 mL^{-1} with a haemocytometer.	Phagocytosis Phagocytic index	[86]
	PBL, HKL	PB and HK leucocytes were isolated according to Markkula, Salo, Rikalainen, and Jokinen [87]. Briefly, nylon net (80 μm) filtered HK tissue cell suspension in heparinised HBSS were purified by Percoll (1.040/1.090 g mL^{-1}) DDGC. For PBL, the blood pellet was resuspended in heparinised HBSS medium with Ultroser G serum substitute, sodium pyruvate and Hepes, pH 7.4 and the suspension purified by Percoll (1.075 g mL^{-1}) CDGC at 400 g for 30 min. Leucocytes were washed twice and resuspended in RPMI-1640 with Ultroser G Serum substitute, sodium pyruvate, L-glutamine, antibiotic solution and Hepes, and adjusted accordingly with a haemocytometer.	Respiratory burst Lymphocyte proliferation	[40]
	HKL	Nylon cell strainer (37 μm) filtered HKL tissue cell suspension in MEM were purified by Percoll (34/55%) DCDGC at 400 *g* for 50 min. The leucocyte were	Superoxide anion production Phagocytosis Immune gene expression	[88]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		harvested, washed with PBS (pH 7.6), and suspended in MEM containing 0.5% trout serum. Isolated leucocytes were adjusted to 10^7 mL^{-1} MEM with 0.5% trout serum.		
	HKL	Filtered HK tissue cell suspension in heparinised L-15 medium were purified by Percoll (1.070/1.080 g mL^{-1}) DCDGC for 40 min at 400 g as described by Braun-Nesje, Bertheussen, Kaplan, and Seljelid [89].	Respiratory burst	[90]
	HKL	Nylon mesh (100 μm) filtered HK tissues suspension diluted 1: 10 in heparinised sterile filtered L-15 medium with antibiotic solution and 2% FCS were purified by Percoll (34/51%) DCDGC for 25 min at 400 g and 4 °C according to Secombes [83]. Leucocytes at the interface were collected and washed thrice in L-15 medium with 0.1% FCS and antibiotic solution, and adjusted to 10^7 mL^{-1}.	Phagocytosis Respiratory burst	[91]
	HKL	Nylon mesh (100 μm) filtered HK tissue suspension in heparinised RPMI 1640 medium with 2% FCS, and antibiotic solution were purified by Percoll (34/51%) DCDGC for 25 min at 400 g and 4 °C according to Secombes [83]. Leucocytes at the interface were collected and washed twice in HBSS, adjusted to 10^6 mL^{-1} with a haemocytometer in RPMI 1640 with 0.1% FCS and antibiotic solution.	Phagocytosis Respiratory burst	[92]
	HKL	Nylon mesh (100 μm) filtered HK tissue suspension in heparinised L-15 medium were directly used for assay	Respiratory burst	[55]
	HKL	Nylon net (80 μm) filtered HK tissue cell suspension in heparinised RPMI 1640 with Ultroser G serum substitute, sodium pyruvate, mercaptoethanol, sodium chloride, Hepes, pH 7.4 were purified by Percoll (1.040/1.080 g mL^{-1}) DCDGC, at unspecified centrifuge parameters. Cells were washed twice and resuspended in culture medium: incubation medium supplemented with L-glutamine, antibiotic solution and sodium bicarbonate and adjusted to desired density by a haemocytometer.	Respiratory burst Natural cytotoxicity	[93]
	HKL	Nylon net (80 μm) filtered HK tissue cell suspension in heparinised RPMI 1640 with Ultroser G serum substitute, sodium pyruvate and Hepes	Respiratory burst Natural cytotoxicity	[87]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		were purified by Percoll (1.040/1.090 g mL^{-1}) DCDGC at 400 g for 30 min. Cells were washed twice and resuspended in supplemented RPMI-1640 culture media, and adjusted accordingly with a haemocytometer.		
	HKL	Sterile nylon mesh (200 μm) bags filtered HK tissue suspension in Dulbecco's Modified Eagle Medium with high glucose, 10% heat inactivated FCS and antibiotic solution were adjusted to 10^7 mL^{-1} on a haemocytometer, for direct assay use.	Immune gene expression Microarray analyses	[94]
	PBL	PBL were isolated by Histopaque (1.077 g mL^{-1}) CDGC at unspecified settings and stored at −80 °C until needed.	Gene expression Indirect epifluorescent detection of surface antigens	[95]
	PBL	Leucocyte buffy coat was isolated from heparinised PB by centrifuging at 1000 rpm for 10 min at room temperature, collected and resuspended in heparinised RPMI 1640 with antibiotic solution. Leucocytes were purified by Histopaque 1077 CDGC at 1500 rpm for 30 min. Leucocytes were resuspended in Trizol for RNA extraction and in PBS for protein extraction.	Northern blot analysis Gene expression	[96]
	HKL	Nylon mesh (70 μm) filtered HK tissue cell suspension in heparinised L-15 medium with 2% FCS, L-glutamine, and antibiotic solution were purified by Percoll (34/51%) DCDGC at 400 *g* for 25 min at 4 °C. Cells were collected and washed twice in heparinised L-15 medium with 0·1% FCS, L-glutamine, antibiotic solution and adjusted by haemocytometer to 10^7 mL^{-1} according to Secombes [83].	Phagocytosis Pinocytosis Antibody synthesis	[97]
	HKL	Nylon mesh (100 μm) filtered HK tissue cell suspension in heparinised RPMI 1640 with 0.1% FCS and oxytetracycline were purified by Percoll CDGC at 2500 rpm for 25 min at 4 °C, washed thrice in RPMI 1640 and adjusted to 10^5 mL^{-1} according to M. Sakai et al. [84].	Phagocytosis Respiratory burst	[98]
	HKL	Nylon mesh filtered HK tissue cell suspension in heparinised L-15 medium with 0.1% FCS and antibiotic solution were purified by Percoll (34%/51%) DCDGC for 25 min at 400 g and 4 °C according to Chung and Secombes [79]. Cells were adjusted to 10^6 and 10^7 mL^{-1} in L-15 medium	Phagocytosis Superoxide anion production	[99]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		with antibiotic solution for further use.		
	HKL	HKL tissue in heparinised L-15 medium with sodium salt and antibiotic solution were purified by Percoll (34%/51%) DCDGC for 20 min at 400 g and 4 °C according to Secombes [83]. Cells were adjusted to 10^6 mL^{-1} in L-15 medium, with antibiotic solution.	Phagocytosis Oxidative burst Lymphocyte proliferation	[100]
	PBL	PBL were isolated according to Espelid, Løkken, Steiro, and Bøgwald [101]. Briefly, PB diluted 1:2 in heparinised MEM with Eagle's salt, and leucocyte suspension purified by Percoll (54%) CDGC at 400 *g* for 25 min. The leucocytes were washed with PBS (pH 7.6) and suspended in MEM with 0.5% trout serum, and adjusted to 10^7 mL^{-1}.	Cell proliferation PBL lysozyme activity Immune gene expression	[102]
	HKL	Sterile nylon mesh (200 μm) bags filtered HKL tissue suspension in Dulbecco's Modified Eagle Medium with high glucose, 10% heat inactivated FCS and antibiotic solution was adjusted to 10^7 mL^{-1} on a haemocytometer, for direct assay use.	Immune gene expression	[103]
	HKL	Sterile filtered HK tissue suspension in ice-cold incomplete L-15 medium with L-glutamine, and Hepes, pH 7.1, were partially purified for 10 min at 500 *g* and 4 °C, and washed thrice. Cells were resuspended in complete L-15 medium with ciprofloxacin, antibiotic solution, fungizone, M β-mercaptoethanol and 10% FCS. Cells were adjusted to 10^6 mL^{-1} using a haemocytometer. Due to the toxicity by Ficoll-Paque, no density gradient was used further in the study.	Lymphocyte mitogen stimulation	[104]
	PBL	Heparinised PB dilute 1: 2 with isolation medium Histopaque (1.077 g mL^{-1}) with bacto haemagglutination buffer. The suspension was purified by Histopaque (1.077 g mL^{-1}) with bacto haemagglutination buffer DCDGC for 15 min at 500 *g* and 4 °C. Leucocytes were gently collected and dispensed into a siliconized tube, washed twice in phenol red-free HBSS and adjusted to 10^6 mL^{-1}.	Respiratory burst Phagocytosis	[105]
	HK-L, PBL	Nylon mesh filtered HK tissue cell suspensions in heparinised RPMI 1640 with antibiotic solution were centrifuged at 500 g for 5 min and washed thrice	Phagocytosis assay Cellular killing capacity Binding capacity	[106]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		with medium. Purified macrophages were obtained by Percoll (34/51%) DCDGC for 30 min at 300 g and 4 °C, cells were collected, washed twice at 300 g for 10 min with HBSS and adjusted to 10^6 mL^{-1}. For PBL, heparinised PB diluted 1:3 in HBSS medium was washed thrice for 5 min at 14 °C and 500 g, the cloudy layers removed following each centrifugation, and the free erythrocyte pellet (95%) was gently resuspended in RPMI 1640 to 10^6 mL^{-1}.		
	HKL	HKL were isolated according to M. Sakai et al. [84]. Briefly, unfiltered HK tissue suspension diluted 1:10 in filter sterilized heparinised RPMI 1640 with oxytetracycline, and 0.1% FCS were centrifuged for 5 min at 500 g and 4 °C, washed thrice in RPMI and adjusted to 10^7 mL^{-1} in heparinised RPMI 1640 with oxytetracycline and 0.1% FCS.	Phagocytosis	[107]
	PBL	PBL were isolated according to Espelid et al. [101]. Briefly, PB was diluted 1:2 in heparinised MEM with Eagle's salt, sodium biphosphate (pH 7.6). The leucocyte suspension was purified by Percoll (54%) CDGC at 400 g for 20 min. Leucocytes were harvested, washed with PBS (pH 7.6), and suspended in MEM with 10% *O. mykiss* serum.	Respiratory burst	[108]
	HKL	Nylon mesh (100 μm) filtered HK tissue cell suspension in heparinised RPMI-1640 with antibiotic solution were purified by Percoll (34%/51%) DCDGC for 20 min at 400 g and 4 °C according to Secombes [83]. Leucocytes were washed and resuspended in HBSS medium to desired concentration by Trypan Blue exclusion.	Phagocytosis Pinocytosis Chemiluminescence	[109]
	HKL	Sterile mesh screen filtered HK tissue cell suspension in heparinised medium with antibiotic solution, and gentamycin were purified by Percoll (51%) CDGC at 400 *g* for 25 min. Cells were washed twice in serum-free medium and centrifuged at 200 *g* for 10 min. Leucocytes were adjusted to appropriate concentration with haemocytometer.	Nitric oxide production Respiratory burst HKL flow cytometry characterisation	[110]
	HK-L, PBL	PB diluted 1:2 in cold isolation medium, erythrocytes lysed with cold sterile distilled water for 20 s and immediately returned	Viability Leucocyte differential counts Flow cytometry characterisation	[37]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		isotonic with 10x PBS and the suspension centrifuged for 10 min at 750 g and 4 °C. The cell pellet was resuspended in MEM and kept on ice to sediment out debris. Leucocyte supernatant was collected and further centrifuged as above, the pellet resuspended in MEM with sodium bicarbonate, 5% FCS antibiotic solution, streptomycin and L-glutamine. After a 1:12 dilution of PB in isolation medium, PBL were purified by Histopaque ($1.077\ g\ mL^{-1}$) CDGC for 40 min at 400 g and 4 °C. Unfiltered HK tissue leucocyte suspension were prepared by hypotonic lysis in MEM and were purified by Percoll (34%/51%) DCDGC for 40 min at 2000 g and 4 °C.	Sudan Black B Neutral Red Staining	
	HK-L, PBL	Heparinised PB was diluted 1:2.5 in heparinised HBSS with 2% FCS and purified by Percoll (51%) CDGC. Unfiltered HK cell suspension in HBSS with 2% FCS were purified by Percoll (34%/51%) DDGC. PBL and HKL suspensions were centrifuged for 20 min at 800 g and 4 °C. PB and HK leucocytes were harvested, diluted 10x in HBSS, washed for 10 min at 800 g and 4 °C. PB and HK leucocyte pellets were resuspended in medium with Hepes, sodium chloride, pH 7·4 and homogenised on ice for 1 min. PBL were homogenised with a glass piston tissue grinder and the homogenate diluted with an equal volume of Hepes, sodium, pH 7·4 and centrifuged for 10 min at 800 g and 4 °C to remove cellular debris. Similarly, HKL homogenate was diluted with an equal volume as used for PBL and centrifuged for 10 min at 13,000 g and 4 °C.	Antibacterial activity HKL lysozyme content	[111]
	HK-L, PBL	Stainless steel mesh (100 μm) filtered tissue cell suspension in heparinised HBSS and leucocyte buffy coat preparation diluted in heparinised HBSS were purified by Histopaque ($1.077\ g\ mL^{-1}$) CDGC for 30 min at 400 *g* and 9 °C. Cells were collected at the interface, washed thrice with HBSS, and resuspended in RPMI 1640 medium with Hepes, L-glutamine, 10% FCS and antibiotic solution.	HKL respiratory burst HKL phagocytosis HKL lymphocyte proliferation PBL Surface IgM marking	[112]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
	PBL & HKL	Heparinised PB diluted 1:20 in MEM and stainless steel filtered HK tissue cell suspensions were prepared in cold serum-free MEM. Leucocytes were purified by Ficoll (1·077 g mL^{-1}) CDGC for 20 min at 400 g, cells were collected, washed in PBS and resuspended in MEM 10.	Flow cytometry characterisation Phagocytosis Oxidative burst Lymphoproliferation Flow cytometry cell population analysis	[113]
	PBL & HKL	HK and PB leucocyte suspensions were purified by Lymphoprep CDGC at unspecified settings. Cells were collected at the interface, washed twice and diluted in heparinised L-15medium with antibiotic solution, glutamine and 5% FCS.	Leucocyte proliferation	[114]
	PBL & HKL	Heparinised PB diluted 1:5 in L-15 medium with 5% FCS were purified by Percoll (51%) CDGC for 20 min at 400 g and 4 °C. Leucocytes were washed twice in RPMI-1640 with sodium hydrogen carbonate (pH 7·2), antibiotic solution and 2-Mercaptoethanol, and adjusted to 10^6 mL^{-1}. Nylon mesh (100 μm) filtered HK tissue cell suspensions in L-15 medium, were purified by Percoll (51%) CDGC for 35 min at 400 g and 4 °C. Leucocytes were collected, washed and adjusted to 10^6 mL^{-1} in L-15 medium with antibiotic solution and 2-Mercaptoethanol.	Respiratory burst	[115]
	HKL	Nylon mesh (100 μm) filtered HK tissue cell suspensions in heparinised L-15 medium, with antibiotic solution and 2% FCS were purified by Percoll (34%/51%) DCDGC for 30 min at 400 g and 4 °C following Chung and Secombes [79]. Leucocytes were collected, washed twice for 5 min at 400 g and 4 °C in L-15 with 0.1% FCS.	Respiratory burst assays	[116]
	HKL	Nylon mesh filtered HK tissue suspension in heparinised RPMI 1640 medium with antibiotic solution were purified by centrifugation at 500g for 5 min and washed thrice RPMI 1640, cells counted by Trypan Blue exclusion.	Phagocytosis Chemiluminescence assay Superoxide anion production	[84]
	PBL	PBL were purified according to Marsden, Hamdani, and Secombes [117] below.	Leucocyte proliferation Antibody production	[118]
	PBL	Heparinised PB was diluted 1:5 with L-15 medium with 5% FCS, and leucocytes purified by Percoll (51%) CDGC for 20 min at 400 g and 4 °C. Leucocytes were washed for 5 min at 400 g	Leucocyte proliferation Lymphocyte separation	[117]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		and 4 °C in L-15 medium with 5% FCS. Leucocytes were counted by Trypan Blue and adjusted to 10^6 mL^{-1} in L-15 with 5% FCS.		
	HKL	Nylon mesh (100 μm) filtered HK tissue cell suspension in in L-15 medium were purified by Percoll (51%) CDGC for 35 min at 400 g and 4 °C. Leucocytes were collected at the interface, washed and adjusted to 10^6 mL^{-1} in L-15 medium with antibiotic solution and 2-mercaptoethanol.	Respiratory burst Leucocyte proliferation	[119]
	SPL	Metallic nets pressed SP tissue suspensions in RPMI 1640 with Hepes, glutamine, gentamycin, and 5% FCS were adjusted to 10^6 mL^{-1} with a haemocytometer for direct assay use.	Respiratory burst	[120]
	HKL	Unfiltered HKL cell suspension in heparinised RPMI 1640 with antibiotic solution, sodium carbonate were purified by Percol (51%) CDGC at 400 g for 25 min at 4 °C. The cell pellet was adjusted to 10^6 mL^{-1} in RPMI for further analysis.	Lymphocyte proliferation Respiratory burst	[121]
	PBL	Heparinised PBL were separated on a Percoll (54%) CDGC according to Thuvander, Norrgren, and Fossum [122].	Lymphocyte membrane total IgM	[123]
	PBL, SPL	Unfiltered SP tissue suspensions were obtained according to Kaattari and Irwin. [73]. PB diluted 1 in RPMI-1640 were purified by centrifugation at 500*g* for 10 min at 17 °C, and the resulting pellet suspended in RPMI-1640. The suspension was purified by Histopaque 1077 CDGC for 20 min at 800 g and 17 °C. Cells at the interface were washed once in TCM and adjusted to 10^7 mL^{-1} in TCM.	Plaque forming cells assay	[124]
	HK-L, SPL, TH-L, PBL	PB diluted 1:5 in RPMI 1640, and metal nets pressed HK, SP and TH tissue suspensions in medium were purified according to Thuvander et al. [122]. Cells were purified by Percoll (54%) CDGC at 400 g. Cells at the interface were washed, counted and resuspended in RPMI 1640 with Hepes, glutamine, antibiotic solution, gentamicin and 5% FCS until used.	Cell proliferation Antibody production	[125]
	HKL	Fine wire mesh filtered HK tissue suspensions in cold L-15 medium were drawn into sterile Strumia capillary tubes and centrifuged at 13600 g for 3 min. Tubes were cut at the cell fluid	Cell migration Migration inhibition	[126]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		interface, tubes with cells fixed to the bottom of tissue culture wells using Vaseline. The medium was added to each well through a Millipore Filter Unit and incubated at 12 °C for 12-I6h.		
	SPL	Metallic nets pressed SP tissue suspensions in RPMI 1640 with Hepes, glutamine, mercaptoethanol, gentamicin, sodium pyruvate and 5% heat inactivated *S. trutta* serum were adjusted to 10^6 mL^{-1} with a haemocytometer for direct assay use.	Phagocytosis Lymphocyte stimulation	[127]
	PBL	PB diluted 1:3 in cold complete medium following the procedure by Boyum [128]. Briefly, leucocytes were purified by Ficoll-Paque CDGC for 30 min at 500 g and 10 °C. Lymphocytes at the interface were collected, washed twice in cold, complete medium by centrifugation for 10 min at 100 g and 10 °C.	Lymphocyte proliferation Flow cytometry characterisation	[129]
	HKL	Nylon mesh filtered HK tissue cell suspension in heparinised L-15 medium with 0.1% FCS and antibiotic solution were purified by Percoll (34%/51%) DCDGC for 25 min at 400 g and 4 °C. Leucocytes were adjusted to 10^7 mL^{-1} in L-15 medium with 0.1% FCS and antibiotic solution.	Respiratory burst Hydrogen peroxide production Superoxide dismutase inhibition	[79]
	HK-L, SPL	Unfiltered HK and SP tissue suspensions in tissue culture medium, and leucocyte suspensions were kept on ice to allow debris sedimentation. Single cell suspension was collected and washed once in TCM by centrifugation for 10 min at 500 g and 4 °C. Leucocyte pellets were resuspended in TCM to 10^7 mL^{-1}, using Trypan Blue exclusion assay.	Same assays as for *O. kisutch* above	[72]
	PBL	PB diluted 1:3 in RPMI 1640, was purified by Percoll (54%) CDGC at 400 g for 11 min at room temperature, a leucocyte fraction collected at the interface, washed once in RPMI 1640 at 280 g for 8 min. The resulting pellet was resuspended in RPMI 1640 with Hepes, sodium bicarbonate, glutamine, antibiotic solution and either 5% FCS, 5% *S. trutta* serum (BTS) or 1% Ultroser G serum supplement at 10^6 mL^{-1}.	Phagocytosis	[122]
	SPL	Unfiltered SP tissue suspensions in TCM with FCS and gentamicin, were centrifuged for I0 min at 500 g and 4 °C. The pellet	Plaque forming cells Lymphocyte stimulation	[130]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		was collected, washed and resuspended in the modified TCM, cells were counted on a haemocytometer and adjusted to 10^6 mL^{-1} in TCM.		
	HK-L, SPL, PBL, THL	PB diluted 1:1 with sodium citrate cold anticoagulant buffer, and tissue filtered cell suspension, were purified by Percoll (30%) CDGC at 500 g for 8 min at 5 °C to isolate leucocytes for cell cultures.	Lymphocyte stimulation	[131]
	HKL	Stainless steel mesh (0.3 mm) filtered HK tissue cell suspension in L-15 medium with 10% FCS, glucose and antibiotic solution were purified by Percoll (1.070/1.080 g mL^{-1}) DCDGC for 40 min at 400 g at 5 °C or on ice.	Phagocytosis Microscopic characterisation	[89]
O. nerka	HKL	Stainless steel screen filtered HK tissue suspension in heparinised L-15 medium with 10%FCS, glucose, and antibiotic were passed through a glass wool mat to remove any remaining large aggregates. Leucocytes were purified by passing through Percoll (34%/51%) DCDGC according to Braun-Nesje et al. [89]. Cells were washed once in heparinised L-15 medium at 1000 *g* for 10 min, and resuspended in L-15 with heparin.	Respiratory burst Phagocytosis HKL myeloperoxidase content	[132]
O. tshawytscha	PBL	PBL were isolated according to Lulijwa et al. [30]	Phagocytosis Respiratory burst Immune gene expression	[133]
	PBL	PBL were isolated by a modified method from Pierrard et al. [39]. Briefly, heparinised PB diluted 1:1 in sterile filtered (40 μm) PBS, pH 7.4 were purified from micro blood volume (284 μL) by Lymphoprep (1.077 g mL^{-1}) CDGC at 971 g for 20 min in a 1.5 mL Eppendorf tube at room temperature. Leucocytes at the interface were aspirated with a pipette and washed twice in PBS at 674 g for 7 min. The cell pellet was adjusted to 10^5 - 10^6 mL^{-1} in PBS with 2% FCS and kept at 4 °C.	PBL cell viability PBL cell microscopic characterisation PBL cell flow cytometry characterisation	[30]
	HKL	Unfiltered HK tissue suspension in L-15 medium with 0.1% FBS and antibiotic solution on ice, leucocytes were purified by Histopaque (1.077 g mL^{-1}) CDGC at unspecified settings and macrophages separated by plastic adherence.	Respiratory burst Phagocytosis	[43]
	HKL	Unfiltered HK tissue suspensions in L-15 medium with 0.1% FBS and antibiotic solution were sediment on	Phagocytosis Respiratory burst	[53]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		ice to remove debris for 3–5 min. The leucocyte supernatants were purified by Histopaque ($1.077\ g\ mL^{-1}$) CDGC for 15 min at 500 g and 16 °C. The interface layer with WBCs was washed in L-15 medium with 0.1% FBS and resuspended in L-15 medium with 0.1% FBS at $10^6\ mL^{-1}$ by haemocytometer.		
	HKL & SPL	HKL and SPL were isolated according to Crippen et al. [37]. Nylon cell strainer (40 μm) filtered tissue suspensions in isolation medium (HBSS) and Alsever's solution (AS) and leucocytes partially purified for 7 min at 500 *g* and 4 °C. The cell pellet was resuspended in ice-cold isolation medium, and clumps removed. For hypotonic lysis of erythrocytes, 2 mL of cell suspension were diluted with 9 mL sterile deionized water for 20 s; 1 mL of sterile 10x PBS was immediately added to stop lysis. Cells were washed twice for 7 min at 500 *g* and 4 °C, cells were resuspended in ice-cold TCM, and adjusted to $10^6\ mL^{-1}$ by Trypan Blue assay.	Cell viability Cell apoptosis IgM response	[134]
	HKL & SPL	HKL and SPL were isolated according to Crippen et al. [37]. Nylon cell strainer (40 μm) filtered tissue suspensions in isolation medium (HBSS) and Alsever's solution (AS) and leucocytes partially purified for 7 min at 500 *g* and 4 °C. The cell pellet was resuspended in ice-cold isolation medium, and clumps removed. For hypotonic lysis of erythrocytes, 2 mL of cell suspension were diluted with 9 mL sterile deionized water for 20 s; 1 mL of sterile 10x PBS was immediately added to stop lysis. Cells were washed twice for 7 min at 500 *g* and 4 °C, cells were resuspended in ice-cold TCM, and adjusted to $10^6\ mL^{-1}$ by Trypan Blue assay.	Cell viability Cell apoptosis Cell proliferation IgM response	[135]
	HK-L, SPL, & PBL	PBL were isolated by hypotonic lysis [37]. Briefly, PB erythrocytes were lysed with distilled water for 20 s, immediately returned to isotonic with 10x PBS, erythrocyte debris removed and the PBL washed with isolation media, and finally re-suspended in RPMI for assay use. Unfiltered HK and	MAb Surface IgM Flow cytometry characterisation of isolated cells	[136]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		SP tissue suspensions in ice-cold TCM (MEM) with 5% FCS, antibiotics, sodium bicarbonate, essential and none-essential amino acids and sodium pyruvate. Leucocyte suspensions were allowed to settle, and cell suspensions aspirated off. Cells were adjusted by Trypan Blue to 10^7 mL^{-1} in sterile TCM on ice for direct assay use.		
	HKL	Stainless steel filtered HKL tissue cell suspension in heparinised L-15 medium with 10% FCS, glucose, and antibiotic solution were purified by Percoll (34–51%) DCDGC for 40 min at 400 g and 5 °C. Cells were washed once, resuspended in L-15 medium with 20 IU mL^{-1} heparin, and adjusted to the right concentration using a haemocytometer.	Respiratory burst Phagocytosis	[47]
	HK-L, SPL	Unfiltered tissue suspension in holding medium (HM) on ice was sediment to remove debris, the supernatant, was removed and washed once in HM for 10 min at 500 g and 4 °C according to Yui and Kaattari [72] above. The resulting cell pellet was resuspended in TCM and cells counted on a haemocytometer.	Same assays as for *O. kisutch* above	[72]
	HK-L, SPL	HK and SP leucocytes were isolated according to Arkoosh and Kaattari [124].	Plaque forming cell assay	[137]
	HKL	Unfiltered HKL tissue cell suspension on ice was sedimented to remove debris in RPMI-1640 with 10% FCS, L-glutamine, gentamycin, 2-meracaptoethanol, uracil, cytosine, adenosine and guanine. The suspension was obtained by repeated aspiration of fragments using a 1 mL syringe. Leucocytes were adjusted to 10^7 mL^{-1} with a haemocytometer in supplemented RPMI-1640 for assaying.	Plaque forming cell assay	[138]
	HK-L, SPL	Unfiltered tissue suspension obtained by repeatedly forcing tissue through a 1 cc syringe in TCM was sedimented on ice, supernatant was centrifuged at 1400 g for 10 min, pellet collected and suspended in TCM. Cells were counted on a haemocytometer and adjusted to 10^7 mL^{-1} in TCM until needed for assay.	Plaque forming cell assay	[139]
S. salar	PBL, HK-L, SPL	PB and tissue leucocytes were isolated according to Jørgensen, Johansen, Stenersen, and Sommer [140]. Heparinised PB suspension leucocytes were purified by Percoll (54%)	Flow cytometry sorting Surface protein characterisation Immune gene expression	[141]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		CDGC for 40 min at 400 g and 4 °C. Cell strainer (100 μm) filtered HK and SP tissue suspensions were purified by Percoll (25/54%) DCDGC and centrifuged as above. Leucocytes at the interface were collected and washed twice in L-15 medium with P/S prior to use.		
	HKL	Cell strainer (100 μm) filtered HK tissue cell suspension in heparinised L-15 medium with antibiotic solution, and 2% inactivated FBS were purified by Percoll (25%/54%) DCDGC for 40 min at 400 *g* and 4 °C according to Braun-Nesje et al. [89]. Leucocytes at the interface were collected and washed twice in L-15 medium at 450 *g* for 15 and 10 min respectively, and adjusted to 10^6 mL^{-1} in L-15 with 1% FBS.	Respiratory burst activity Nitric oxide production Immune gene expression	[46]
	HKL	Cell strainer (100 μm) filtered HK tissue suspensions in heparinised L-15 medium with 2% FCS and antibiotic solution were purified by Percoll (25/54%) DCDGC for 45 min at 400 g and 4 °C according to Jørgensen et al. [140]. Leucocytes at the interface were collected and washed twice in L-15 medium and seeded at 10^6 mL^{-1}.	Luciferase reporter gene assay	[52]
	HK-L, PBL	Briefly, heparinised PBL was diluted 1: 2 in heparinised L-15 + medium with sodium carbonate, D-glucose, gentamycin sulphate, and L-gluamin. Homogenised and unfiltered HK tissue suspensions were prepared in heparinised cold L-15 + medium. Leucocytes from PB and HK suspensions were purified by Percoll (1·075/1·060 g mL^{-1}) DCDGC for 30 min at 400 g according to Pettersen, Bjerknes, and Wergeland [142]. Isolated leucocytes were diluted in L-15 + medium and analysed in a CASY-TT, adjusted to 10^6 mL^{-1}.	Surface MAb detection Leucocyte flow cytometry characterisation HK tissue gene expression	[45]
	HK-L, SP	Nylon mesh (80 μm) filtered HK and SP tissue cell suspensions in heparinised RPMI-1640 with 2% healthy *S. salar* serum and antibiotic solution were purified by Percoll (34/51%) DCDGC at 400 *g* for 45 min. Cells were collected at the interface and centrifuged again at 400 *g* for 10 min. Leucocytes were washed thrice in medium and suspended in the same medium to 10^7 mL^{-1}.	Phagocytosis Respiratory burst HKL lysozyme activity	[143]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
	PBL	Heparinised PB was diluted 1:2 in L-15 + medium with gentamicin sulphate, L-glutamine and HEPES. Leucocyte were then purified by Percoll (1·075/1·060 g mL^{-1}) DCDGC for 40 min at 400 g as previously described by Pettersen et al. [144]	Phagocytosis Flow cytometry characterisation Respiratory burst PBL myeloperoxidase Acid phosphatase Mitogen stimulation Cell surface marker gene expression	[145]
	HKL	Nylon mesh (37 μm) filtered HKL tissue cell suspension in heparinised L-15 medium with bovine serum were purified by Percoll (34/51%) DCDGC at 3000 *g* for 30 min. Leucocytes were collected, washed with PBS (pH 7.6) and suspended in L-15 with 10% bovine serum.	Respiratory busrt Phagocytosis Immune gene expression	[146]
	PBL, SPL & HKL	PB diluted 1:3 in L-15 medium and cell strainer filtered (50–100 μm) homogenised tissue cell suspension were purified by Percoll (1.080 g mL^{-1}) DDGC for 40 min at 800 *g* and 4 °C. Leucocyte were collected, washed once in PBS for 10 min at 400 *g* and 4 °C. Cells were resuspended in L-15 medium, counted using a Kova slide chamber and adjusted to 10^7 mL^{-1}.	Antibody detection Flow cytometry characterisation	[147]
	PBL & HKL	PBL and HKL were isolated as described in Øverland, Pettersen, Rønneseth, and Wergeland [148]. Heparinised PB diluted in L-15 + medium and cell strainer (100 μm) filtered HK tissue cell suspension in heparinised L-15 + medium with gentamicin sulphate, L-glutamine, and HEPES were purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC for 35 min at 400 g and 4 °C.The cell pellet was resuspended in L-15 medium and counted by CASY Cell Counter.	Respiratory burst Microscopic characterisation	[149]
	PBL & HKL	Cell strainer (100 μm) filtered HK tissue cell suspension in heparinised L-15 medium with gentamicin sulphate, L-glutamine, and HEPES. Peripheral blood and HK leucocytes were isolated on Percoll (1·060/1·075 g mL^{-1}) DCDGC for 35 min at 400 g and 4 °C according to Pettersen et al. [142].	Phagocytosis Flow cytometry Immunofluorescence	[148]
	PBL & HKL	Heparinised PB diluted 1:4 in L-15 medium were purified by Histopaque 1077 CDGC at 400 g for 45 min. Leucocytes were collected using a Pasteur pipette. If erythrocyte contamination of PBL was considered to be excessive (> 2%), then the	Respiratory burst Total lipid analysis	[41]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		PBL fraction was centrifuged again on 4 mL of fresh Histopaque 1077. Cells were washed twice in 0.1% L-15 medium, at 600 g for 10 min and stored at −20 °C. Nylon mesh (100 μm) filtered HKL suspension in heparinised L-15 medium were isolated and cells suspended in bijoux kept on ice until used.		
	PBL	Heparinised PBL were purified by Percoll (1.060/1.075 g mL^{-1}) DCDGC for 35 min at 400 g according to Pettersen et al. [142]. Heparinised PB was diluted 1:4 in PBS, and leucocytes purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC at 400 g for 30 min, and leucocyte fraction collected from the interface were washed in PBS by centrifugation at 200*g* for 10 min. The cell pellet was resuspended in PBS-380 and kept on ice.	Nitrite oxide production Respiratory burst Phagocytosis Immune gene expression Bactericidal assay Flow cytometry Immunofluorescence Sudan Black B PBL myeloperoxidase	[150]
	PBL	PBL were isolated by Histopaque (1.077 g mL^{-1}) CDGC at unspecified settings and stored at −80 °C.	Same assays as for *O. mykiss* above	[95]
	HKL & PBL	Heparinised PB was diluted to 2 mL in PBS and cell strainer (100 μm) filtered HK tissue cell suspension in RPMI 1640 with L-glutamine and gentamicin. Leucocytes from PB and HK were purified by Percoll (1.060/1.075 g mL^{-1}) DCDGC for 35 min at 400 g according to Pettersen et al. [142].	MAbs labelling Flow cytometry characterisation	[151]
	HKL & PBL	Unfiltered HK tissue cell suspension in RPMI 1640 with gentamicin sulphate, L-glutamin and heparinised PB diluted 1:3 in PBS (pH 7.3) on ice were isolated by discontinuous Percoll (1.060/1.075 g mL^{-1}) centrifugation for 35 min at 400 g according to Pettersen et al. [142]. Leucocyte at the interface were collected and washed with PBS at 200 g for 10 min, and cells adjusted to 10^6 mL^{-1} in PBS with 1% BSA, 0.1% sodium azide and EDTA.	MAbs labelling Flow cytometry characterisation	[152]
	HK-L, PBL	Mesh filtered HK tissue in heparinised L-15 medium with 2% FCS and antibiotic solution on ice were purified by Percoll (51%) CDGC according to Secombes [83]; adjusted to 10^7 mL^{-1}. For PBL, PB pellet was resuspended 1:2 in PBS and centrifuged at 100 g for 10 min. The buffy coat was aspirated, diluted in PBS, and leucocytes purified by	Phagocytosis Respiratory burst PBL proliferation	[153]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		Histopaque (1.077/0.001 g mL^{-1}) DCDGC for 30 min at 400 g and 15 °C. Leucocytes were collected, washed twice in PBS for 10 min at 400 g and 4 °C and once in modified phenol red-free L-15 medium with antibiotic solution.		
	PBL & HKL	Cell strainer (100 μm) filtered HK tissue cell suspension in cold wash medium and PB diluted to 2 mL in PBS. HKL and PBL were isolated by Percoll (1.060/1.075 g mL^{-1}) DCDGC for 35 min at 400 g according to Pettersen et al. [142]. The leucocyte suspension was washed at 200 g for 10 min in PBS and resuspended in EDTA PBS with 1% BSA, 0.1% sodium azide at 10^6 mL^{-1}.	MAb assay Flow cytometry characterisation	[154]
	HKL	Sterile steel mesh (100 μm) filtered HK tissue cell suspension in RPMI 1640 were purified by Percoll (34/51%) DCDGC for 30 min at 400 g and 4 °C. Leucocytes were aspirated at the interface and washed twice for 5 min at 1200 g and 4 °C. The pellet was resuspended in Cortland saline at 1200 g for 5 min at 4 °C, and the cell pellets diluted at 10^7 mL^{-1}, with the help of a Thomas' haemocytometer.	Phagocytosis	[48]
	PBL & HKL	Heparinised PB was diluted 1:4 in PBS, were purified by Percoll (1.060/1.075 g mL^{-1}) DDGC at 400 *g* for 30 min. PBL were collected from the interface, washed with PBS at 200 *g* for 10 min, cells were resuspended in PBS with 1% BSA, sodium azide and Titriplex III and kept on ice until used. Unfiltered HKL suspension in RPMI 1640 with gentamycin, L-glutamine and 10% FBS were purified by Percoll CDGC for 30 min at 400 *g* and 4 °C. Cells were washed once in RPMI 1640, at 200 g for 10 min at 4 °C, resuspended in TCM with RPMI 1640, gentamicin, L-glutamine, mercaptoethanol and 10% FCS.	PBL MAb characterisation PBL flow cytometry characterisation HKL MAb characterisation	[155]
	HK-L-TO cells	Unfiltered HK tissue suspension in RPMI 1640 with gentamicin sulphate, L-glutamine, and 10% FCS were purified by Ficoll CDGC for 30 min at 1000 g and 4 °C. HKL were suspended in RPMI 1640, centrifuged for 10 min at 900 g and 4 °C. The collected cells were diluted in Eagle's MEM, containing Earle's BSS, gentamicin	Virus production	[156]

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Species	Cells	Technique	Intended assays	Reference
		sulphate, fungizone, L-glutamine, mercaptoethanol, 1% MEM Eagle non-essential amino acid and 10% FCS.		
	HKL	Cell strainer (100 μm) filtered HK tissue cell suspension in heparinised L-15 medium with antibiotic solution and 2% FCS were purified by Percoll (25/54%) DCDGC at 400 g for 40 min at 4 °C with minor modification to the protocol by Graham and Secombes [157]. Leucocytes at the interface were collected and washed twice in L-15 medium, and seeded at 10^6 mL^{-1} in L-15 medium with 5% FCS, and antibiotic solution.	IFN cytokine like activity	[140]
	HKL	Nylon mesh (70 μm) filtered HK tissue cell suspension in heparinised L-15 medium with antibiotic solution and 2% heat-inactivated FCS were purified by Percoll (37%/51%) DCDGC for 35 min at 400 *g* according to Braun-Nesje et al. [89]. Cells at the interface were collected and washed in heparinised L-15 medium with 0·1% FCS at 200 g, for 10 min, and finally resuspended in medium, at 10^6 μL^{-1}.	HKL lysozyme activity Northern blotting	[158]
	PBL	Heparinised PB was diluted 1:4 in PBS, and leucocytes purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC at 400 g for 30 min, according to Pettersen et al. [144]. The leucocyte fraction collected from the interface were washed in PBS by centrifugation at 200*g* for 10 min. The cell pellet was resuspended in PBS-380 and kept on ice.	MAb assay Immunofluorescence double staining Flow cytometry	[142]
	HKL (SHK-1) cell line	Unfiltered HK tissue suspension in HEPES-buffered saline with collagenase were purified by Percoll (25–42%, 42–50%, & 50–58%) DCDGC at 400 g for 60 min as previously described [159,160]. Leucocytes were washed by centrifugation and resuspended in L-15 medium with 5% FCS, gentamycin and mercaptoethanol until needed for assays.	Phagocytosis Bacterial killing Enzyme cytochemistry Immunohistochemistry	[161]
	HKL	Cell strainer (70 μm) filtered HK tissue suspension in L-15 medium with antibiotic solution and 2% FCS were washed twice in L-15 medium counted and resuspended to a concentration of 10^6 mL^{-1} in L-15 + ab and 5% FCS.	Leucocyte proliferation	[162]
	PBL	Heparinised PB was diluted 1:2 in L-15 medium with	Lymphocyte proliferation	[163]

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Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		glutamate and sodium bicarbonate. Medium osmolality was adjusted to 380 mOsm. The suspension leucocytes were purified by 54% Percoll CDGC at 400 *g* for 25 min. The leucocytes were washed with medium and adjusted to 10^6 mL^{-1}.	Leucocyte IgM detection	
	PBL	Heparinised PB diluted 1:1 in PBS (pH 7·2), were purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC at 400 g, for 40 min, according to Braun-Nesje et al. [89]. Cellular fractions were collected for assaying following suspension in PBS with 0.1% BSA.	MAb assay Antibody inhibition Immunofluorescence Western blotting Deglycosylation of Igm	[144]
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK tissue cell suspension in L-15 medium with FCS and antibiotic solution were purified by Percoll (37%/51%) DCDGC for 30 min at 400 g. Cells were adjusted to the right density in L-15 medium with 0.1% FCS, and antibiotic solution.	Respiratory burst Bactericidal assay Superoxide inhibitors	[164]
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK tissue cell suspension were purified by Percoll (37%/51%) DCDGC for 30 min at 400 g, according to Braun-Nesje et al. [89]. Cells at the interface were collected adjusted to 10^7 mL in L-15 medium with 0.1% FCS, antibiotic solution.	Respiratory burst Phagocytosis Bacterial killing Hydrogen peroxide production NADPH-oxidase inhibition	[165]
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK tissue cell suspension in L-15 medium with glucose, gentamycin and 2% FCS were purified by Percoll DCDGC for 20 min at 400 *g* and 4 °C, following the protocol by Braun-Nesje et al. [89]. Cells at the interface were collected, washed in pure medium, and adjusted to 10^6 mL^{-1}.	Phagocytosis Pinocytosis Superoxide anion production Cellular lysosomal enzyme activity Cellular acid phosphatase	[166]
	HKL	Metal nets pressed HKL in medium were purified by 54% Percoll CDGC at 400 g according to Reitan and Thuvander [125]. Cells at the interface were washed, counted and resuspended in RPMI 1640 with HEPES, glutamine, antibiotic solution, gentamicin and 5% FCS and used for later assays.	ELISA Leucocyte proliferation	[167]
	HK-L, SPL, TH-L, PBL	PB diluted 1:5 in RPMI 1640, and metal nets pressed HK, SP and TH tissue suspensions in medium were purified by 54% Percoll CDGC at 400 g as described by Thuvander et al. [122]. Cells at the interface were washed, counted and resuspended in	Same assays as for *O. mykiss* above	[125]

(*continued on next page*)

Table 2 (*continued*)

Species	Cells	Technique	Intended assays	Reference
		RPMI 1640 with HEPES, glutamine, antibiotic solution, gentamicin and 5% FCS and used for later assays.		
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK tissue suspension in heparinised L-15 medium with 10% FCS, glucose and antibiotic solution were sedimented to remove debris and cell viability was done via Trypan Blue exclusion (0.5%). Leucocytes were purified by Percoll (1.060/1.068 g mL^{-1}) DCDGC for 40 min at 400 g at 5 °C in the laboratory or on ice. Cell suspensions were washed in L-15 medium with FCS, counted and adjusted to the appropriate density.	Same assays as for *O. mykiss* above	[89]
S. fontinalis	HKL	Unfiltered HK tissue cell suspensions in DMEM with 10% FCS, glutamine and antibiotic solution were sedimented and the cellular supernatant was recovered, washed in PBS and leucocytes were purified by Ficoll-Histopaque CDGC at unspecified parameters. Collected cells were resuspended in DMEM with 10% heat-inactivated FCS at a density of 10^6 mL^{-1} for cultures.	Northern analysis Phagocytosis Flow cytometry characterisation	[168]
S. trutta	HKL	Unfiltered HK tissue cell suspensions were purified according to MacKenzie et al. [168] above.	Same assays as for *S. fontinalis* above	[168]
	HK-L, SPL	Metallic filtered (100 μm) issue cell suspension were purified by Ficoll-paque (Histopaque 1.077 g mL^{-1}) CDGC at 600 g for 30 min at 20 °C. Lymphocytes were collected at the RPMI/Ficoll interface, washed twice, and adjusted to 10^6 viable lymphocytes mL^{-1} in RPMI1640 with 2 mM L-glutamine and 10% FCS.	Phagocytosis Lymphocyte cytotoxicity	[169]

accross seven salmonid species, including Coho salmon (*Oncorhynchus kisutch*), *O. mykiss*, *O. tshawytscha*, sockeye salmon (*Oncorhynchus nerka*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), and *S. salar*. Most of the immunological studies were carried out using farmed *O. mykiss* and *S. salar* (Table 1). Although lymphoid tissues, including SP, HK, and the TH have been used to illustrate immunological functions of isolated leucocytes among different salmonids, the majority of immunological investigations involved isolating cells from the HK, PB and SP in order of popularity are listed in Table 1.

Indeed, teleost HK is the most important lymphoid tissue as it is comparable to adult mammalian bone marrow [37]. In fish, the HK is the primary haematopoietic tissue where all blood cells form during larval and adult stages; blood forms in the liver in the mammalian foetus, and the bone marrow in adults [68,69].

2.1. Techniques used to isolate salmonid leucocytes

The use of fish lymphoid and PBL requires isolation from a mass of red blood cells present in PB, the HK, TH and SP, through density gradient centrifugation and hypotonic lysis [38,39]. The present review found that most studies used discontinuous density gradient centrifugation (DDGC) (44%) and continuous density gradient centrifugation (CDGC) (41%) to isolate fish leucocytes form lymphoid tissues and PB (Fig. 2). Generally, DDGC involves layering density gradients of at least two different densities in a centrifugation vessel, usually a 15 mL centrifuge tube and topping up with buffer diluted PB or tissue single cell suspension to be separated into red blood cells and leucocytes. For instance, we found that Percoll density gradient was commonly layered in the range 1.060 g mL^{-1} over 1.075 g mL^{-1} and 34% over 51% for *S. salar* and *O. mykiss*, respectively.

The centrifugation speed, duration and temperature settings varied

Table 3
Commonly studied immunological tests in salmonid fish immunology. LM: Light microscopy, TEM: Transmission electron microscopy, SEM: Scanning electron microscopy. Data comes from 114 reviewed studies. Same assays reported by the same author in one paper on different species are reported once.

Immunological assays	Assay counts	% of assays
Flow cytometry characterisation	21	9.5
Microscopy (LM, TEM, SEM)	5	2.3
Leucocyte differential counts	1	0.5
Bacterial killing/Cytotoxicity	6	2.7
Phagocytosis, phagocytic index	41	18.6
Pinocytosis	3	1.4
Respiratory burst, superoxide anion and hydrogen peroxide production	29	13.2
Nitric or Nitrite oxide production	3	1.4
Lysozyme activity	4	1.8
Acid phosphatase	2	0.9
NADPH-Oxidase	1	0.5
Superoxide dismutase	1	0.5
Cell proliferation	20	9.1
Cell migration	1	0.5
Monoclonal antibodies	10	4.5
Antibody production or detection	6	2.7
Immunoglobulins	7	3.2
Plaque forming cells	8	3.6
Peroxidase and myeloperoxidase	3	1.4
Immunohistochemistry, immunofluorescence, epifluorescence, chemiluminescence	8	3.6
Enzyme cytochemistry	1	0.5
ELISA	1	0.5
Immune gene expression, cytokine activity	23	10.5
Northern or western blot	3	1.4
Sudan Black B	2	0.9
Mitogen stimulation	3	1.4
Virus production	1	0.5
Total lipid analysis	1	0.5
Cell viability, apoptosis	4	1.8
Binding capacity	1	0.5
Total assays reviewed	**220**	**100.0**

greatly among studies and species, although all protocols yielded fish leucocytes (Table 2). Overall, the density gradient centrifugation process mostly lasted between 15 and 40 min at 400 g, and at 4–15 °C (Table 2). CDGC involved layering buffer diluted blood or PB or tissue suspension over a pure layer of density gradient medium ($1.077\ g\ mL^{-1}$) or a dilute density gradient layer (e.g. 50–54%) followed by centrifugation as detailed below (Table 2).

The most commonly used density gradient medium was Percoll (GE Healthcare) by 63 of the studies, although other media such as Histopaque 1077 (Sigma-Aldrich Co. LLC) was used by 14 of the studies, Ficoll or Ficoll-Paque PLUS (GE Healthcare), and Lymphoprep (STEMCELL Technologies) were also applied (Table 2). All isolation gradient products have a standard density of $1.077\ g\ mL^{-1}$ and are designed to separate cells based on weight by centrifugation. Percoll density gradient is described by GE Healthcare as a low-viscosity, nontoxic medium suitable for density gradient centrifugation of cells, viruses and subcellular particles. Similarly, Histopaque 1077 is a sterile, endotoxin-tested solution of polysucrose and sodium diatrizoate. In addition, Ficoll-Paque PLUS is a sterile, ready-to-use aqueous medium composed of a mixture of Ficoll PM400 and sodium diatrizoate, for high yield isolation of lymphocytes from peripheral blood. However, Ficoll use in higher vertebrate leucocyte isolation alters cellular surface markers, functional responses and morphology [170]. Density gradient isolation of leucocytes also limits granulocyte recovery, with consequences for adaptive immune responses when compared to hypotonic lysis, and has been reported to increase respiratory burst in PBL [38,171].

Despite the dominance of density gradient centrifugation as a technique for leucocyte isolation, these commercial reagents are expensive. Development of modified micro-scale techniques that require a minimal density gradient medium and < 300 μL of PB is a cost-effective solution, avoids euthanisation, in some cases, and can be applied on small fish [30]. Similarly, the hypotonic lysis technique originally developed by Crippen et al. [37] has been recently improved by Hu et al. [38] to facilitate fast and affordable isolation of lymphoid tissue and PBL for fish immunological studies. The technique has recently gained use [38,75–78] and reported to give superior cellular composition, with limited erythrocyte contamination [38] allowing a holistic assessment of fish immune parameters at innate and adaptive levels in immune and vaccine studies [38,171].

3. Popular immunological assays in salmonid leucocyte immunology

Within the 114 reviewed articles, over 200 immunological tests were conducted to study isolated lymphoid tissue and PBL in salmonids

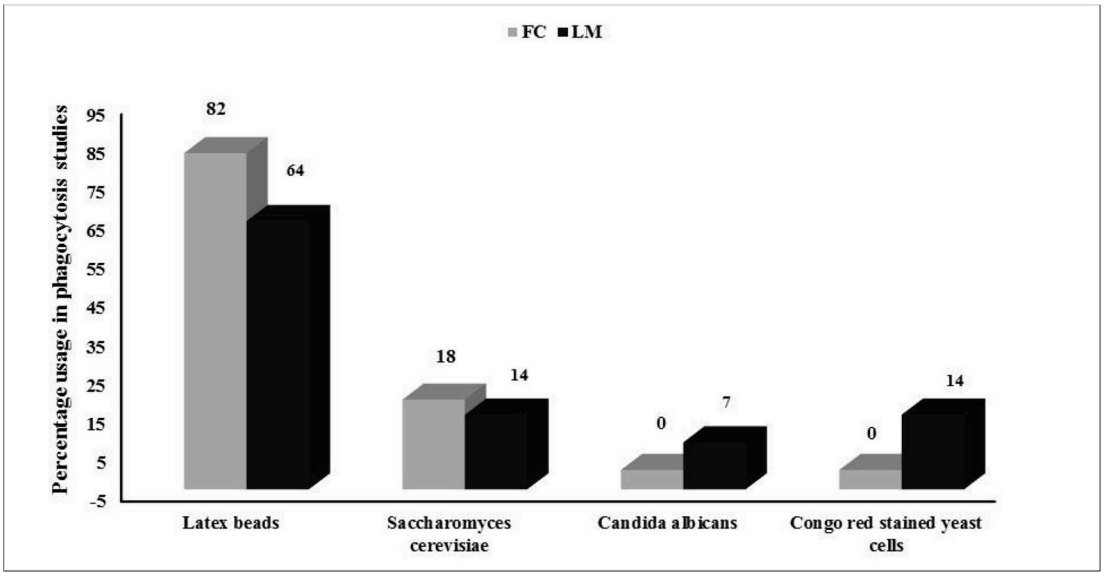

Fig. 3. Techniques commonly used to study phagocytosis involving isolated salmonid fish leucocytes. FC: Flow cytometry, LM: Light microscopy. Data comes from 114 reviewed studies. [1.5].

(Table 3). Findings showed that phagocytosis was the most applied assay to investigate cellular immunological functional capacity, with this technique being followed by respiratory burst activity (Table 3). We briefly discuss the most popular assays and give appropriate examples, where possible, below.

3.1. Phagocytosis assay

Phagocytosis is one of the most important fish immunological responses to pathogens, making its characterisation a good indicator of cellular defence capability. Previous studies show that phagocytosis is the most important innate immune defence mechanisms in poikilothermic fish, as it is the least affected parameter by temperature [reviewed in Ref. [5]]. The phagocytic process involves pathogen uptake via engulfment into phagosomes [148]. Phagocytes can take up harmful foreign particles as well as dead cells, macromolecules and cellular debris [5,172,173]. Among leucocytes, phagocytic cell-types include B lymphocytes, monocytes, macrophages, neutrophils, MC, DC and non-specific cytotoxic cells [5,174,175].

Macrophages and neutrophils are professional phagocytes [176,177], with a high phagocytic ability and capacity [177]. Professional phagocytes eliminate bacterial pathogens through reactive oxygen species (ROS) production during respiratory burst [5] and reactive nitrogen intermediates (RNI) via pattern recognition receptors (PRR) and cytokines [172]. Inside macrophages, lysosomes are well-equipped with bactericidal molecules, including lysozyme, nucleases, proteases, lipases, and hydrogen peroxide, among others [177]. Neutrophils contain granular myeloperoxidase which kills bacteria via halogenation of cell walls in the presence of hydrogen peroxide and halides, and lysozymes with hydrolytic enzymes [178].

The phagocytic process is initiated by receptor-mediated endocytosis or through non-specific hydrophobic interactions of the cell membrane with the target particle [177,179] through pathogen associated molecular patterns (PAMPs). The steps involved in neutrophil and macrophage destruction of pathogens sequentially involve: pathogen recognition via chemotaxis, pathogen adhesion, pathogen ingestion and formation of a phagosome, followed by a phagolysosome, digestion and exocytosis of residues [177].

In this paper, leucocyte phagocytosis was the most applied immunological assay to illustrate immunomodulation in farmed salmonids, reported in 40 studies. The most commonly used technique to study phagocytosis was flow cytometry, followed by light microscopy,

Table 4
Techniques used to study phagocytosis using isolated salmonid lymphoid tissue and PBL. CL: calorimetry, FC: flow cytometry, PCM: phase contrast microscopy, LM: light microscopy, EFM; epifluorescence microscopy, FM: fluorescence microscopy, SEM: scanning electron microscopy, SP: spectrophotometer, MMR: multimode microplate reader, LU: Luminometer, CC: colony count, MPF: Microplate fluorometer; ER: ELISA reader. For blood tissues, HK: head kidney, PB: peripheral blood, MK: Middle kidney, and SP: Spleen.

Species	Tissue	Targeted cells	Inducers	Technique	Reference
O. mykiss	PB	Leucocytes	Fluorescent latex beads 1.0 μm	FC	[38,75,76]
	HK,SP,MK	Leucocytes	Green fluorescent 1.0 μm latex beads	FC	[82]
	HK	Macrophages	Latex beads	LM	[85]
	HK	Macrophages	Latex beads	LM	[86]
	HK	Macrophages	Zymosan A	SP	[88]
	HK	Leucocytes	Pathogenic bacteria (*Streptococcus iniae, Lactococcus garvieae, A. salmonicida, Yersinia ruckeri, Vibrio anguillarum* and *Vibrio ordalii*)	CC	[91]
	HK	Leucocytes	Latex beads 0.85 μm	LM	[92]
	HK	Leucocytes	Latex beads 0.8 μm	LM	[98]
	HK	Leucocytes	*S. cerevisiae*	LM	[97]
	HK	Macrophages	Fluorescent latex beads 2.0 μm	LM	[99]
	HK	Macrophages	Fluorescein-labelled *Escherichia coli*	MPF	[100]
	PB	Macrophages	*S. cerevisiae*	SP	[105]
	HK, PB	Leucocytes Free erythrocytes	*C. albicans*	LM	[106]
	HK	Leucocytes	Latex beads 0.8 μm	LM	[107]
	HK	Leucocytes Macrophages	*S. cerevisiae*	LM	[109]
	HK	Leucocytes	Fluorescent latex beads 1.03 μm	FC	[112]
	PB, HK	Leucocytes	Fluoresceinated (FITC) latex beads	FC	[113]
	HK	Leucocytes	Latex particles (0.85 μm)	LM	[84]
	SP	Leucocytes	Fluoresceinated (FITC) latex beads, 1.72 μm	FCM,	[127]
	PB	Leucocytes	Fluoresceinated (FITC) latex beads, 1.72 μm	FC, FCM, SEM	[122]
	HK	Macrophages	SRBC, latex beads, heat-killed *Candida*, live yeast cells & *V. anguillarum*	PCM	[89]
O. *nerka*	HK	Leucocytes	FITC-labelled *Staphylococcus aureus*	FM	[132]
O.tshawytscha	PB	Leucocytes	Fluorescent latex beads 1.0–1.3 μm	LM	[133]
	HK	Macrophages	SRBC	SP	[43]
	HK	Macrophages	SRBC	SP	[53]
	HK	Leucocytes	FITC-labelled *S. aureus*	FM	[47]
S. salar	HK	Macrophages	Yeast cells	LM	[143]
	HK	Macrophages	Fluorescein-labelled *E. coli*	ER	[146]
	PB	Leucocytes	Fluorescent latex beads 1.00 μm	FC	[145]
	PB, HK	Leucocytes	Fluorescent latex beads 1.00 μm	FM, FC	[148]
	HK	Macrophages	Fluorescent latex beads 2 or 3 μm	LM, FC	[150]
	HK	Phagocytes	Yeast cells	LM	[153]
	HK	Macrophages	Opsonised zymosan	LU	[48]
	HK	Macrophages	*A. salmonicida*	EFM	[161]
	HK	Macrophages	Glutaraldehyde-fixed sheep red blood cells	CL	[165]
	HK	Macrophages	Fluorescein labelled Latex microbeads	PCM	[180]
	HK	Leucocytes and macrophages	Fixed sheep red blood cells, latex beads, heat-killed *Candida*, live yeast cells & *V. anguillarum*	PCM	[89]
S. fontinalis	HK	Macrophages	*S. cerevisiae* FITC	FC	[168]
S. trutta	HK	Macrophages	*S. cerevisiae* FITC	FC	[168]
	HK	Leucocyte	Opsonised zymosan	LU	[169]

using fluorescent latex beads (Fig. 3). In addition, studied leucocytes were mostly isolated from the HK and PB (Table 1); while the most studied species were *O. mykiss* and *S. salar* (Table 1). Recent examples that employed isolated leucocyte phagocytosis to investigate salmonid immunological responses included engulfment of fluorescence-emitting beads by *O. mykiss* PBL, following stimulation with interleukin-1β (IL-1β) and interleukin-6 (IL-6) [38]. In addition, sheep red blood cells (SRBC) were phagocytosed by *O. tshawytscha* HKL [43], and yeast cells by *S. salar* HK [143]. The complete list of particles employed in assessing isolated leucocyte phagocytosis among salmonids is included in Table 4.

3.2. Respiratory burst

Respiratory burst or oxidative burst involves rapid production of ROS (superoxide molecules and hydrogen peroxide) by different leucocytes during innate immune responses to pathogens. The technique is one of the most important salmonid immunological assays, frequently assessed together with phagocytosis. This technique may require stimulation with PAMPs and macrophage-activating factors (MAF), such as phorbol myristate acetate (PMA) or concanavalin A (con A) either in synergy or singly. In this review, respiratory burst was the second most studied cellular innate immunological assay employed in innate immune responses of salmonids (Table 3). During fish immunological responses to pathogens, respiratory burst plays an important biochemical role that facilitates successful phagocytosis and eventual pathogen destruction. The technique is a monopoly of phagocytic neutrophils, monocytes and macrophages, and aims at ROS production, catalysed by NADPH oxidase complex that lines the inner phagocyte cell membranes and cytoplasm following stimulation [181]. However, as monocytes differentiate into macrophages, they lose capacity to produce ROS [182].

The process of respiratory burst has been reviewed thoroughly by Grayfer et al. [172] and the process for ROS production was illustrated earlier in a review in teleosts by Neumann et al. [177]. Briefly, the reaction starts with formation of superoxide anion (O_2^-), which eventually gets catalysed into hydroxyl radical (OH.), hydrogen peroxide (H_2H_2), hypochlorus acid (OCl^-) and peroxynitrite ($ONOO^-$) [reviewed in Ref. [172]]. In salmonids, genes for the enzyme NADPH oxidase complex have been linked with ROS production in *O. mykiss*, *S. salar*; and were cloned and sequenced in *O. mykiss* [reviewed in Ref. [172]]. In addition Grayfer et al. [172], provided numerous evidences of the induction of phagocyte ROS production by cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) and IL-1β in *O. mykiss*.

Consequently, leucocyte respiratory burst has been widely used in several *in vitro* and *in vivo* experiments to illustrate immunomodulatory effects induced by dietary manipulations in *O. mykiss* [49,85], *S. salar* [46]; immunostimulants in *O. mykiss* [98]; *O. tshawytscha* [133] and in *S. salar* [143]. Respiratory burst has also been used to assess: immunomodulatory effects induced by chemical and physical stressors in *O. tshawytscha* [43], in *O. mykiss* [40,55]; vaccines efficacy in *O. mykiss* [115,119]; and effects of pathogens in *O. mykiss* [45] and *S. salar* [48].

3.3. Flow cytometry assays

Flow cytometry is a popular laser-based technology that allows multiple characterisation of cells in biofluids and has been used in fish to rapidly, accurately, and consistently analyse fish leucocyte subpopulations and cellular immune functions [113]. For example, flow cytometry has been used to characterise fish leucocytes in *O. mykiss* [37,78,81,129,142,183,184], *O. tshawytscha* [30], *S. salar* [141,154,155,168]. Flow cytometry has also been used to assess cellular functional characteristics [113] of respiratory burst [149], antibody production [44,136,147,150,152,154], and phagocytosis [122,145,148] among others. Leucocyte flow cytometry has also been used to assess salmonid fish immune response to pathogens [45,113,151]; and chemical stressors [134,135]. Recently, a portable flow cytometer, the Muse® Cell Analyser, has been used in aquaculture as an accurate and fast quantitative assessment tool of single cells compared to traditional manual methods [30].

3.4. Immune gene and cytokine expression

Detection of immune genes is yet another important technique used to assess immunomodulation in aquaculture, via leucocyte cytokine transcript expression. Zhang and An [185] described cytokines as proteins released by cells and have a specific effect on the interactions and communications between cells. Depending on the cells that produce them, cytokines are called lymphokine, monokine, or chemokine if they have chemotactic capacity and interleukin (IL) if produced by a specific leucocyte type and act on another leucocyte type. Stimulated macrophages produce pro-inflammatory cytokines, which accelerate inflammatory reactions during pathological or stressful conditions. Pro-inflammatory cytokines of IL-1β, IL-6, and TNF-α, are involved in pathological pain; while anti-inflammatory cytokines such as IL-1 receptor antagonist, IL- 4, IL-10, IL-11, and IL-13 are produced to control actions of the pro-inflammatory cytokines [185].

Fish have major pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, downstream effectors IL-8 and IL-12; and the classical anti-inflammatory IL-10 and transforming growth factor (TGF-β1), but lack most of the mammalian TNF-β and IL-1 members, such as IL-1F1, IL-1F3 and IL-1F5-11 [reviewed in Ref. [64]]. In fish, IL-1β stimulates T-lymphocytes [reviewed in Ref. [6]], while IL-1 receptors have been suggested to regulate IL-1β in stress responses, as they have been consistently regulated in *O. mykiss* and *S. salar* HK and SP following LPS and TNF-α stimulation [186,187]. Recently, PBL were used to demonstrate IL-2 modulated gene expression and cytokine induced cellular proliferation [75,76]. Thus, gene expression has been employed in salmonids to characterise *S. salar* TO cell line, small mononuclear cells for dendritic like properties [145,150], *O. mykiss* HK and PB leucocytes for immune response [38,80] and viral pathogen transcript detection [45,52]. Immune gene expression has also been used in salmonids to assess immunomodulatory effects induced by steroid hormones [50,88,94,102,146], LPS and β-glucan administration [46,94,103], and temperature perturbations [95,96] in farmed salmonids. However, the potential effects of nucleic acids and lipoproteins as crude LPS contaminants may be co-inducers of pro-inflammatory activity [188].

3.5. Cell proliferation assay

The cell proliferation assay is a measure of an increase in cell population, considered an indicator of cell health, and is commonly used to monitor the response of cells following immunostimulation [189]. In salmonids, isolated leucocyte proliferation is one of the important immunological assays widely applied. The technique was recently used to characterise *O. mykiss* PBL following isolation by hypotonic lysis and density gradient centrifugation [38]. Frequently, leucocyte proliferation was used to assess effects induced by steroid hormones [74,102,163], pathogens [113,117,119,153], mitogens and or pathogens [125,129], vaccines [114,118,162,167], vitamin C [121], hydrocarbon creosote [112], sewage effluents [100], and long term UVB irradiation [40] in *O. mykiss* and *S. salar*.

3.6. Antibody production assay

Antibodies (Ab) or immunoglobulins (Ig) are surface cellular markers that provide a suitable surface for attachment of specific pathogens to B-lymphocytes. They form part of the adaptive immune system that is very pathogen specific, thanks to their high affinity [reviewed in Ref. [190]]. Antibodies help to facilitate a proper response to pathogens [191] in association with the complement system. In teleosts, the

complement system is composed of different serum proteins that play a vital role in fish defence and is divided into three different pathways. These include the classical complement activity, which is triggered by antibody binding to cell surfaces [192]. The alternative pathway is directly activated by foreign pathogens [193], and the lectin pathway is activated by binding of a protein complex made of mannose or mannan binding lectin in bacterial cells [194]. In fish, the alternative complement pathway is the most important innate immune defence mechanism against pathogens [193], through pathogen killing via opsonisation and phagocyte activation, usually through Gram-negative bacterial cell wall LPS [195]. In their review, Uribe et al. [5] noted that the complement proteins enhanced salmonid antibody neutralisation of encapsulated viruses; while LPS present in Gram-negative pathogenic bacteria stimulated complement proteins to facilitate phagocytosis.

A number of studies characterised salmonid isolated leucocytes for the presence of Ig [71,81,82,136,142,144,147]. Isolated leucocyte Ig or Ab production was used to study immunomodulatory effects induced by recombinant interleukin-4/13A in *O. mykiss* HKL cultures [44] and rearing temperature [152]. Other leucocyte Ig or Ab production studies looked at immune effects of seawater and freshwater shifts on MAb + and IgM + cells in PB and HK leucocytes in *S. salar* [154], photoperiod and immune response in *S. salar* PB and HK leucocytes [155]. Isolated leucocyte MAbs production were also used to detect infectious pancreatic necrosis virus (IPNV) in vaccinated *S. salar* HK and PB leucocytes [45,151]. Other studies used leucocyte Ig and MAbs production to assess immunomodulatory effects of bovine lactoferrin [97], vaccines [118], mitogens and pathogens [125], hydrocarbon creosote [112], polychlorinated biphenol [123], tributyltin [134], and hormones [163].

4. Leucocyte application to assess effects of dietary manipulations

Intensive aquaculture produces fish under high density, and may subject animals to a multitude of stressors and conditions that support pathogenic infections. Good nutrition, and welfare must be ensured to support growth and enhance disease resistance [196]. Good nutrition prior to disease outbreaks increases fish resistance and reduces mortality [197]. Thus, immune system enhancement is a central aspect in aquaculture operations. In this regard, several *in vivo* and *in vitro* dietary manipulations using for instance probiotics, immunostimulants, including but not limited to nucleotides, β-glucans, LPS and polysaccharides have been commonly applied in aquaculture to enhance fish innate immunity and prevent infection [reviewed in Refs. [5,6]].

4.1. Illustrating immune benefits of probiotics

Probiotics are live microbial adjuvants which confer beneficial effects to the host when administered in sufficient quantities [198]. In the last three decades, several studies have used isolated leucocytes functional properties to assess effectiveness of singular and synergistic probiotics administration against pathogens. At a cellular immune functional level, singular oral administration of Kocuria SM1 in *O. mykiss* resulted in effective defence against *Vibriosis* via enhanced respiratory burst and HKL bacterial killing activities [49]. Offered synergistically, probiotics *Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33, significantly enhanced HK leucocyte phagocytosis and respiratory burst, respectively, against *A. salmonicida* and *Y. ruckeri* [92]. In addition, enhanced HKL phagocytic and respiratory burst activities, resistance against *L. garvieae* and *S. iniae* were reported following probiotics *Aeromonas sobria* GC2 administration in *O. mykiss* [98]. Also, oral administration of probiotics JB-1 and GC2, equated to *Bacillus* sp. and *A. sobria*, respectively, stimulated HKL phagocytosis and respiratory burst activities against infectious *A. salmonicida, L. garvieae, S. iniae, V. anguillarum, V. ordalii* and *Y. ruckeri* in *O. mykiss* [91]. Besides, a potential probiotic *Lactobacillus rhamnosus* (JCM 1136) induced increased HKL phagocytosis in *O. mykiss* [99].

4.2. Studying immune benefits of immunostimulant and MAFs effects

Evolutionally important, PAMPs are conserved signal molecules found in bacteria and viruses [199]. They include viral double stranded ribonucleic acid (dsRNA) and bacterial deoxyribonucleic acid (DNA), fungal β1,3-glucans (β-glucan), bacterial cell wall peptidoglycans, polysaccharides and Gram-negative bacterial endotoxin or lipopolysaccharides (LPS), not expressed in multicellular life forms [200]. PAMPs are recognised by the vertebrate immune system through the pattern recognition receptors (PRRs) on cell surfaces, following breach of physical barriers. The binding of the host cellular PRRs and the PAMPs initiates cellular responses specifically designed to kill and eliminate the microbial pathogen [201]. MAFs are receptor based signals that initiates pathogen clearance [202]. Indeed, salmonid isolated leucocytes have been used to assess immunostimulatory effects of PAMPs, such as viral double stranded (dsRNA), LPS, β-glucan, cytokines, MAFs such as PMA, con A and phytohaemagglutinin (PHA) following singular or synergistic administration *in vivo* and *in vitro*.

Functionally, singular administration of LPS *in vivo* enhanced oxidative burst, phagocytosis, and bactericidal activities against pathogenic *Aeromonas hydrophila* in *O. mykiss* [85]. However, conflicting results were obtained as singular LPS did not alter phagocytic capacity in *O. mykiss* HK and PB macrophages *in vitro* [203]. Furthermore, *in vitro* exposure to different bacterial LPS products enhanced respiratory burst activity, phagocytic activity and ability to kill an avirulent A-layer lacking strain of *A. salmonicida* in *S. salar* HKL macrophages and enhanced pathogen resistance [165]. Using β-glucan *in vitro*, singular administration induced higher ROS production compared to singular LPS stimulation in *S. salar* HKL macrophages [46], and *in vitro*, administration of β-glucan enhanced respiratory burst in *S. salar* HK macrophages, but did not induce increased bactericidal activity against avirulent and virulent strains of *A. salmonicida* [164]. Offered synergistically, LPS and β-glucan enhanced phagocytosis, pinocytosis and superoxide anion production in *S. salar* HKL macrophages [166]. Similarly, combined yeast β-glucans and bacterial LPS enhanced respiratory burst activity in *S. salar* HKL macrophages [46,158].

While applying MAFs, *O. mykiss* HKL macrophages were stimulated into respiratory burst with PMA to produce oxygen and hydrogen peroxide, which was catalysed by exogenous superoxide dismutase (SOD) and its inhibitor (diethyldithiocarbamate; DDC) reduced hydrogen peroxide production [79]. Similarly, *O. mykiss* HKL macrophages, stimulation with PMA induced higher respiratory burst activity than stimulation with LPS, TNF-α or β-glucans, while a synergistic response was even higher [116]. Likewise, MAF con A induced stronger *O. mykiss* PBL proliferation than did LPS, PHA or pokeweed mitogen (PKWM) *in vitro* [129] and earlier work had revealed that *O. mykiss* leucocytes isolated from HK, TH, PB and SP, responded very well to LPS and con A *in vitro* [131].

Also, in *O. kisutch*, IgM production was induced following PHA treatment, which suggested that this PAMP may not be limited to T cell activation in salmonids, but may alternatively induce the production of lymphokines capable of polyclonal activation of B cells, while fetal calf serum (FCS) induced production of large amounts of IgM without antigenic stimulation [71]. In addition, *O. mykiss* SPLs were stimulated *in vitro* for antibody production following antigen B-lymphocyte stimulation with Trinitrophenylated (TNP) forms of *E. coli* LPS and keyhole limpet haemocyanin (KLH) [130].

At the gene level, isolated leucocytes have been used to demonstrate that synergistic β-glucan and bacterial LPS administration enhanced accumulation of lysozyme gene transcripts in *S. salar* HK macrophages [158]. In addition, *S. salar in vitro* combined stimulation with bacterial LPS and β-glucan highly induced gene expression for arginase-1 and pro-inflammatory IL-1β in the stimulated cells [46]. In established *S. salar* TO cell line, LPS induced significant upregulation of cluster of differentiation 83 (CD83)gene transcript, indicative of DC origin, while expression of TCR-α or the macrophage marker M-CSFR were not

detected *in vitro* [150]. Using bacterial LPS *O. mykiss* macrophages stimulated *in vitro* by *E. coli* LPS resulted into upregulation of genes for vascular cell adhesion molecule, the CCAAT/enhancer binding protein β, nuclear factor kappa B (NF-kB α) inhibitor, DCs cell restricted marker (CD209e), major histocompatibility complex class II (MHC II), cyclin L1, acute phase serum amyloid A, and prostaglandin endoperoxide synthase 2 [103]. Finally, plasmid DNA and synthetic oligodeoxynucleotides (ODNs) with unmethylated CpG induced antiviral IFN cytokine activity in *S. salar* HKL; particularly among adherent macrophages than suspension leucocytes [140].

Fish leucocytes have been used to study and classify different cytokines in *O. mykiss* HKL incubated with LPS and Poly I:C or the pro-inflammatory cytokine IFN-γ, which induced class 2 cytokine receptors named R1 type receptors (IL-10R1/CRFB7, IL-20R1a/CRFB8a and IL-20R1b/CRFB8b) and one R2 type receptor (IL-10R2/CRFB4), as antiviral molecules against VHSV [80]. In addition, isolated leucocytes were applied to assess directional immune response as *O.mykiss*, RTS 11 cells, recombinant IL-1β and IFN-γ reportedly induced upregulation of genes involved in inflammation and major histocompatibility complex class I (MHC I) antigen presentation pathway respectively [204].

4.3. Illustration of immunomodulatory benefits of plant and animal extracts

During the 1990s and early 2000s, salmonid isolated leucocytes were used to demonstrate immune effects of plant- and animal-based extracts at cellular immune functional levels. For example, PBL showed that dietary plant extracts from mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*) in *O. mykiss* significantly enhanced phagocytosis and extracellular respiratory burst in fish fed a diet containing 1% powdered ginger root extracts [105]. In addition, enhanced HKL phagocytosis, respiratory burst and bactericidal activities demonstrated enhanced immunity following oral administration of Garlic (*Allium sativum*) in *O. mykiss* [86]. Enhanced HKL phagocytes chemiluminescence and phagocytosis in *O. mykiss*, administrated *in vivo* and *in vitro* with bovine lactoferrin was demonstrated [84], including increased HKL endocytosis in *O. mykiss* administered with bovine lactoferrin [97]. Conversely, the administration of bovine lactoferrin did not affect HKL antibody production against human-γ-globulins (HGG) *in vivo* in *O. mykiss* [97].

In vitamin C deficient *O. mykiss*, isolated HKL showed that *in vitro* supplementation enhanced leucocyte proliferation and response to PAMPs, while intraperitoneal (i.p.) administration enhanced the same parameters and *in vitro* supplementation enhanced leucocyte proliferation in fish fed a commercial diet [121]. In addition, *in vivo* supplementation or *in vitro* HKL incubated with or without pantothenic acid (vitamin B_5) induced strong cell mediated migration against infection with *Cryptobia salmositic* in *O. mykiss* [126].

Similarly, in *O. tshawytscha* fed with experimental feeds with either fish meal as a control, fish meal plus cooked fish by-products, or fish meal plus hydrolyzed fish protein alone, or with hydrolyzed fish protein and processed fish bones, there was enhanced phagocytosis, respiratory burst and myeloperoxidase activity in all fed groups [205]. Moreover, feeding level affected *O. tshawytscha* cellular function, as phagocytic activity appeared to be inversely proportional to feeding level [47].

4.4. Understanding the effects caused by hormonal supplements

Hormonal supplements are applied in animal production to enhance growth [206] and enhance immune function [143]. Indeed, cultured and isolated salmonid leucocytes have been used to demonstrate immune enhancement both *in vitro* and *in vivo*. For example, *S. salar* SHK-1 cell line and HKL exhibited increased ROS production and phagocytosis following synthetic prolactin (PRL)-releasing peptide (PrRP) administration [146]. Administered *in vitro* and *in vivo* HK macrophages treated with PRL resulted into enhanced ROS production and cellular phagocytosis [143]. In addition, *in vitro* administration of insulin-like growth factor-I (IGF-I) stimulated increased superoxide production in zymosan-stimulated *O. mykiss* HKL [88]. Synergistic *in vitro* PRL and growth hormone (GH) administration did not affect cellular proliferation [102]; while *in vivo* singular i.p. administered of GH induced production of superoxide anions in *O. mykiss* PBL in both freshwater and seawater [108].

At the gene expression level, isolated salmonid leucocytes have been used to validate immunostimulatory and immunosuppression effects of hormones through assessment of immune related cytokine expression. For instance, PRL supplementation immediately upregulated expression of toll-like receptors (TLRs), myeloid differentiation factor 88 (MyD88) gene transcripts and prolonged the expression of IL-1β to regulate long term immune response in *P. salmonis* infected *O.mykiss* [50]. Similarly, PRL upregulated the expression of genes for IL-1β, inhibitor of kappa B (IκBα), TLR1, and TLR5M (membrane-bound form) in *S. salar* SHK-1 cells infected with *P. salmonis* [51], and supplementary PRL-releasing peptide (PrRP) induced expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-12 and PRL in SHK-1 cells and *S. salar* HKL [146]. Conversely, cortisol administration significantly inhibited the LPS-dependent upregulation for expression of TNF-α2, a pro-inflammatory cytokine in *O. mykiss* macrophages [94]. Furthermore, salmon insulin like growth factor-1 (sIGF-I), induced expression of insulin like growth receptor (IGFR) type Iβ in *O. mykiss* HKL *in vitro* but did not enhanced expression of another IGFR type Iα [88]. Administered synergistically, PRL and GH did not affect PBL lysozyme gene transcript in *O. mykiss* [102].

4.5. Exemplary case studies

To demonstrate the application of fish leucocytes in illustrating immunomodulatory effects of dietary manipulation, an *in vitro* study by Ulvestad et al. [46] and an *in vivo* one by Nya and Austin [85] are used. The *in vitro* study investigated the effects of singular and synergistic *in vitro* LPS and β-glucan administration on *S. salar* HK macrophages respiratory burst and immune gene expression. HK were aseptically obtained from healthy and unvaccinated *S. salar* and leucocytes were isolated by discontinuous Percoll (GE Healthcare, Sweden) according to the protocol by Braun-Nesje et al. [89]. Isolated cells were adjusted to 10^5 mL^{-1} using a NucleoCounter® NC-200™ (Chemometec, Denmark). Cells were plated and kept in medium for 24-h, to separate adherent from suspension cells. Isolated by washing, adherent cells were cultivated in cell medium supplemented with antibiotic solution and 2% FBS. 24-hours post isolation, HK macrophages were stimulated with singular LPS/β-glucan or synergistically for 24-h (single stimulation) or 48-h (multiple stimulation), and control cells received only the cell medium. Functionally, cellular HK macrophages treated *in vitro* by LPS and β-glucan exhibited enhanced ROS production compared to the control, while stimulation and costimulation by β-glucan prompted higher ROS production than with LPS [46]. At the gene level, the arginase-1 and IL-1β gene transcripts were highly expressed in treated *S. salar* HK macrophages than in the control fish leucocytes [46].

Secondly, the *in vivo* study applied isolated leucocytes to demonstrate immunomodulatory effects of immunostimulant LPS supplementation [85]. The authors investigated the efficacy of supplementary LPS against infectious *A. hydrophila* in O. *mykiss* fingerlings. Fish fingerlings were fed a commercial diet supplemented with zero (control), 1·875, 3·75, 7·5 and 15.0 mg LPS 100 g^{-1} of feed. Five groups of 20 fish each were fed twice daily to satiety for two weeks. 24-hours post the dietary feeding schedule, fish were challenged by i.p. injection with 0.1 mL suspensions of *A. hydrophila.* To assess the efficacy of LPS as an immunostimulant, HKL were isolated by discontinuous Percoll gradient (Sigma-Aldrich) [84] to assess innate cellular immune functions of phagocytosis, respiratory burst, lysozyme activity and bactericidal activity from groups of 10 fish. Isolated HKL demonstrated that LPS administration *in vivo*, effectively enhanced the fish innate immune capacity against *A. hydrophila* via enhanced phagocytosis and phagocytic index in supplemented *O.* mykiss [85].

5. Leucocytes used to assess effects of physical and chemical stressors

Farmed fish and shellfish may face a multitude of intrinsic and extrinsic stressors that influence the innate immune capacity [reviewed in Ref. [5]]. Major extrinsic factors that modulate fish innate immune capacity include changes in temperature [207]; variation in oxygen levels, suspended solids, pH, and salinity [208], photoperiod [209], handling stress and stocking density [210]. However, temperature is even more important due to fish's poikilothermic lifestyle [211]. Relatedly, ultraviolet radiations have also been reported to modulate salmonid immunity [40,93,212,213]. Furthermore, immune effects of environmental contaminants have been reviewed by Rehberger, Werner, Hitzfeld, Segner, and Baumann [214] and include pesticides, organic compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs), polybrominated biphenyl ethers (PBDEs), heavy metals, pharmaceutical compounds and natural hormones [50].

5.1. Studying the effects induced by physical stressors

Among salmonids, isolated leucocytes have been applied to illustrate immunomodulatory effects of physical stressors, including but not limited to temperature, osmotic shock, ultraviolent (UV) irradiation at cellular functional and gene levels. For example, isolated HK macrophages showed that cold stress in *O. nerka* reared at 8 °C resulted into increased HK macrophage phagocytosis than in fish at kept at 12 °C [132] and fish reared at 6 °C exhibited higher percentages of Ig + cells in PBL compared to fish reared at 18 °C in *S. salar* post-smolts [152]. At the molecular level, cold stress at 2 °C downregulated the expression of genes for MHC-II β and α genes in *O. mykiss* [96], confinement in shallow water downregulated *O. mykiss* PBL corticosteroid receptors [74], while fish maintenance at 2 °C did not affect expression of beta-2 microglobulin (β_2m) in *S. salar* and *O. mykiss* [95].

In addition, osmotic stress during transfer from freshwater to seawater significantly enhanced HK macrophage respiratory burst activity in non-smolting *O. mykiss* [55] and in *S. trutta* [169]. Also, immunomodulatory effects of ultraviolet B (UVB) irradiation perturbed immune functions in salmonids and increased fish susceptibility to pathogens [40,93]. Conversely, long term UVB exposure did not affect PHA induced lymphocyte proliferation in *O. mykis* [40], high ultraviolet B (UVB) irradiation (1000 mJ cm^{-2}) exposure supressed respiratory burst following exposure in *O. mykiss* HKL [87].

5.2. Studying the effects caused by chemical stressors

Other than physical stressors, isolated salmonid leucocytes are also employed in revealing immunosuppressive effects of environmental contaminants, such as heavy metals, sewage effluents, polyaromatic hydrocarbons (PAHs). For example, isolated leucocytes showed that heavy metals cadmium, mercury and zinc altered cellular phagocytosis, respiratory burst, and lymphocyte differentiation [reviewed in Ref. [214]]. In *O. mykiss* exposed to lower cadmium (2 ppb), leucocytes exhibited altered macrophage phagocytosis and ROS production, in a time-dependent manner [215]; while *O. mykiss* PBL characterised as phagocytic by flow cytometry and electron microscopy, showed enhanced phagocytosis in a cellular function validation test with an arsenic [122]. In *O. mykiss* exposed to treated sewage effluents, increased *in vitro* lymphocyte proliferation was noted; while HKL oxidative burst, phagocytosis were unaffected [100].

Regarding immunological effects of PAHs, different concentrations of dichlorodiphenyltrichloroethane (DDT) metabolite 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p,p′*-DDE) significantly reduced cell proliferation in *O. tshawytscha* [135] and tributyltin induced apoptosis in SP and HK leucocytes [134]. For PBDEs, dietary manipulations containing binary combinations of the contaminant congers BDE-47 and BDE-99 induced increased HK macrophage phagocytosis, superoxide anions and ROS production in *O. tshawytscha* fed the binary diet compared to the control diet, while susceptibility to *V. anguillarum* infection was higher in the test group [43]. Similarly, oral exposure of *O. tshawytscha* to PBDE congeners (BDE-47 and BDE-99) resulted in reduced HK macrophages phagocytosis in fish exposed to BDE-99, while both congers induced increased production of superoxide anion, and salmon exposed to the pollutant exhibited increased susceptibility to *Listonella anguillarum* [53]. In *O. mykiss*, i.p. administration with polychlorinated biphenol (PCB) mixture Clophen A50, SP leucocyte response to both T and B cells to LPS and PHA increased significantly in the PCB exposed fish after immunisation with keyhole limpet haemocyanin (KLH) [120].

In *O. mykiss* exposed to PCB mixture Clophen A50, antibody levels to *V. anguillarum* were significantly lower in the groups that had been fed the Clophen A50-containing diet, while IgM levels remained unaffected [123]. In addition SP and HK leucocytes isolated from *O. tshawytscha* exposed to 59 ppm p,p′-DDE *in vitro* showed significantly lower percentage of Ig + blasting cells than controls, although the response was biphasic [135] and comparable findings were noted following tributyltin (TBT) exposure *in vitro* [134]. Similarly, creosote PAH exposure in *O. mykiss*, resulted into PBL dose dependent reduction in the number of sIg + cells following treatment with different mixtures [112]; and i.p. administration of stress hormone, cortisol downregulated the number of Ig + leucocytes in *S. salar* [163].

5.3. Exemplary case studies

To exemplify the application of salmonid isolated leucocytes in studying immunomodulatory effects of environmental stressors, two studies by Arkoosh et al. [53]; Arkoosh et al. [43] are used. In the first study, authors investigated the independent effects of two of the most predominant PBDE congeners, BDE-47 and BDE-99, on the health and survival of juvenile *O. tshawytscha*. Health fish were held in 300 L tanks with flow-through seawater and fed daily rations of BDE-47 or BDE-99 at 2% body weight for 40 days. Experimental diets contained environmentally acceptable levels at low (32), medium (100), and high (280) ng BDE-47 g^{-1} of food; and low (40), medium (120), and high (300) ng BDE-99 g^{-1} of food. The control group received food with 0.4 ng BDE-47 g^{-1} food and 0.1 ng of BDE-99 g^{-1} food as well as 0.1 ng of BDE-49 g^{-1} and 0.1 ng of BDE-100 g^{-1} food. 24-hours post feeding, subsets of treated fish were sampled, HKs aseptically removed, and HKL isolated by Histopaque 1077 CDGC according to Solem et al. [165]. Isolated HK macrophages were separated from suspension cells by plastic adherence according to Secombes [83]; incubated for 18-h prior to assay use. Using HK macrophages, immunosuppression was demonstrated in fish exposed to BDE-99, but not BDE-47 via reduced phagocytic capacity against SRBCs, although both congers enhanced HKL macrophage *in vitro* superoxide anion production, survival in fish was compromised following challenge with pathogenic *V. anguillarum* [53].

In the second study, investigators studied the effects of a synergistic formulation of the most predominant PBDEs congers (BDE-47/99 mixture) on the innate immune system and disease susceptibility of juvenile *O. tshawytscha*. Five BDE-47/99 mixture diets and a control were produced and labelled as control zero, and one, two, three, four to five for the treatments. Fish were fed daily on the BDE-47/99 mixture diets, thrice a day for 39 days in triplicates of 285 animals. At the end of the experiment, fish HK were sampled aseptically and HKL and adherent macrophages isolated according to Arkoosh et al. [53] and assayed for phagocytosis and respiratory burst. Isolated HK macrophages demonstrated enhanced cellular functional properties of phagocytosis, respiratory burst in fish that received the BDE-47/99 mixture diets than control diet fed fish. However, the mechanism for this enhanced innate immune function in HK macrophages following PBDE exposure remains a mystery although it has been suggested to result from the ability of PBDEs to act as an endocrine receptor agonist and/or antagonist. Conversely, the risk of mortality in BDE-47/99 mixture diets fed fish

was higher than in the control fish following challenged with pathogenic *V. anguillarum* [43].

6. Study of pathogen and parasite effects, vaccine design, and assessment of efficacy and vaccination strategies

From a pathological and immunostimulation perspective, isolated leucocytes have also been used to assess vaccine efficacy, immune response to parasites, bacterial and viral pathogens, design vaccines and vaccination strategies in farmed salmonids as illustrated in the subsequent sections.

6.1. Studying the effects of parasites and bacterial pathogens

Among salmonids, the cellular functional immunosuppression has been reported in fish following exposure to parasites. For instance, parasitic Myxosporean (PKX), the causative agent for proliferative kidney disease (PKD) led to impaired phagocytosis but not respiratory burst in *O. mykiss*, HK and PB leucocytes [113], while *Neoparamoeba* sp reinfection in *S. salar* previously infected with gill disease, depressed phagocytic activity of HK and PB leucocytes [153]. Other than parasitic infestations, isolated leucocytes have also been used to study immunological effects induced by bacterial challenges and infections. For instance, *V. anguillarum*, *in vitro* challenge in *O. kisutch*, *O. mykiss* and *O. tshawytscha*, induced mitogens and polyclonal activities in SP and HK lymphocytes [72], *A. salmonicida* or *Y. ruckeri* induced IL-22 production in PBL [78]; while unchallenged *O. mykiss,* HK and PB leucocytes *in vitro* resulted into noticeable antibacterial activity against Gram-positive bacteria [111].

6.2. Aiding vaccine design, assess efficacy and vaccination strategies

Isolated leucocytes have also aided design of bacterial vaccines, as *O. mykiss* injected with formalin-killed *V. anguillarum*, HK and PB leucocyte proliferated following challenge with formalin-killed bacteria outer membrane proteins (OMP) preparation [114]. Using *A. salmonicida* strain (MT004) preparations, the vaccine prepared in PBS (MT004/PBS) exhibited higher antigen specific proliferation and MAF production than A10H adjuvant (MT004/A10H) preparation in *O. mykiss* PBL following i.p. injection [115]. Recently, Attaya et al. [77] used *O. mykiss* PBL to screen for *A. salmonicida* based vaccines.

Vaccine efficacy has also been assessed using isolated leucocytes as an earlier study with *A. salmonicida* vaccine induced continued production of MAF in *O. mykiss* HKL post-immunisation, and the trend correlated very well with lymphocyte proliferation [119]. Relatedly, *O. mykiss* challenged with *A. salmonicida* antigens *in vitro*, all HKL primed demonstrated enhanced responses to *A. salmonicida* antigens, indicative of both T and B memory cell formation in vaccinated individuals. Furthermore, T and B cells from primed fish were able to respond to an A-layer positive strain (MT423) of the antigen, hence a shared common immunogenic T and B cell epitopes by both antigens [117]. In addition, vaccination with a mutant *A. salmonicida* vaccine against furunculosis in *O. mykiss*, live and not heat killed vaccine, significantly enhanced T-cell proliferation compared to B-cells [118]. In addition, vaccination against furunculosis in *S. salar* and *O. mykiss* PB, SP and HK leucocytes enhanced antibody production following stimulation with *E. coli* LPS and PHA [125], and a test for a mutant *A. salmonicida* vaccine against furunculosis in *O. mykiss*, showed that live bacteria significantly primed lymphocyte antibody production [118].

Regarding vaccination strategy choices, *S. salar* vaccinated by single and repeated administration by i.p. injection, immersion or oral route, and revaccination by combinations of these methods, against furunculosis: the i.p. injection strategy resulted into higher *in vitro* proliferation of HKL [162]. In addition, i.p. administration of adjuvant vaccine, preferably in a polyvalent formulation, was optimal to stimulate immunity and thus recommended for immunoprophylaxis against furunculosis [162]. Similarly, *S. salar* i.p. administered with an adjuvant furunculosis vaccine, HKL from vaccinated fish showed significantly stronger response to whole *A. salmonicida* than did unvaccinated controls, with minimal differences to response among the different mitogens [167].

6.3. Studying the effects of viral pathogens

Isolated leucocyte experimental studies involving viral pathogens have demonstrated impaired and enhanced cellular functional immune parameters. Viral haemorrhagic septicaemia virus (VHSV) impaired phagocytosis, but not respiratory burst in *O. mykiss*, HK and PB leucocytes [113], while i.p. injection of *S. salar* parr with salmon pancreas disease virus (SPDV) (P42p isolate of SPDV) invoked stimulation of innate immunity via increased HKL phagocytosis [48]. In addition, *in vitro S. salar* TO cells exhibited capacity to provide a high yield of infectious salmon anaemia virus (ISAV) and showed cytopathic effects (CPE) within 9 days; *S. salar* injected with diluted virus supernatant exhibited mortalities, hematocrit values and clinical signs in accordance with ISAV infection [156]. Besides, leucocytes flow cytometry analysis aided detection of IPNV in HK and PB leucocytes from IPNV vaccinated, non-vaccinated and i.p. infected *S. salar* (virus shedders) and carrier fish [45]. Furthermore, both flow cytometry and quantitative real time polymerase chain reaction (qRT-PCR) indicated that i.p. infected fish were carriers as B-cells and neutrophils and other leucocytes harboured IPVN following cohabitation challenge, hence enhanced protection against future infection [45].

Isolated salmonid leucocytes have also been employed to assess gene expression following viral pathogen challenges. To illustrate, *S. salar*, challenged with a high virulence (HV) and low virulence (LV) IPNV, exhibited higher levels of viral transcripts in HK of fish infected with LV IPNV compared to HV during the freshwater phase [52]. *In vitro*, *S. salar* HKL derieved cell lines, SHK-1 and TO, challenged with Salmon AlphaVirus (SAV) an aetiological agent of SPD, SAV nsP1 gene transcripts for strain P42P increased rapidly in TO cells with subsequent development of CPE, but not in SHK-1 cells [216]. However, SAV P42P strongly upregulated expression of type I interferon (IFN) and IFN-induced antiviral Mx protein transcripts in SHK-1 cells. However, higher IFN expression appeared in TO cells than in SHK-1 cells, and lower Mx gene transcripts in TO cells [216].

6.4. Exemplary case study

To demonstrate the application of fish isolated leucocytes in studying immunological effects of pathogens, a study by Desvignes et al. [48] is adapted. The study investigated the host immunological response following experimental infection with SPDV cultured in *O. tshawytscha* embryo (CHSE-214) cell line. 75 healthy *S. salar* yearling parr, were i.p. injected with 100 μL of an infectious dose of 10^5 tissue culture infectious dose 50% ($TCID_{50}$). The control group consisted of 75 healthy fish i.p. injected with 100 μL of virus free CHSE-214 cell culture supernatant. Treated and control fish were separated into triplicate groups, kept in covered 50 L tanks, at a density of 25 fish, supplied with oxygenated filtered water at 14 °C. Fish were sampled at 2, 4, 9, 16 and 30 days post infection (dpi) using five fish per tank.

HK tissues were sampled and leucocytes isolated by Percoll (Sigma, U.S.A.) DCDGC, washed twice, and viable cells resuspended in Cortland saline at $10^7\ mL^{-1}$ with the help of a Thomas' haemocytometer until needed for immunological assay, while blood plasma was used for humoral assessments. Overall, *in vivo* infection of *S. salar* with cultured SPDV induced an immune response at specific and non-specific levels. Isolated HKL exhibited higher phagocytosis in infected fish compared to control fish, while plasma lysozyme and complement increased in the treatment group. In addition, neutralising antibodies against SPDV appeared two weeks post infection and increased with time in content and prevalence among treated fish, although SPDV infection did not induce IFN activity in treated fish [48].

7. Salmonid fish immunological cell development

As earlier illustrated, fish immune cells can be isolated from

Table 5
Vertebrate fish lymphoid tissue and peripheral blood developed immunological cell lines: ATK: anterior part of trunk kidney, SP: Spleen, TH: thymus, HK: Head kidney, PB: peripheral blood.

Fish species	Origin	Cell line and immunological characterisation	Reference
1. Salmonid immunological cell lines (n = 10)			
O. mykiss	SP, HK, ATK	Short term *O. mykiss* trout DC cells: exhibited irregular membrane processes and expressed surface MHCII, had tree-like morphology, expressed DC markers, phagocytosed small particles, were activated by TLR-ligands, and migrated *in vivo*.	[82]
	HK	Long-term T-PKM cells: were positive for ROI and RNI negative with PMA, Con A and LPS.	[110]
	SP	Long-term *O. mykiss* cell line (RTS11): Majority of cells were small, non-adherent and a few larger, granular cells termed macrophages-like cells. Larger cells were phagocytic, and engulfed DiI-acetylated low-density lipoprotein and acridine orange and stain for non-specific esterase. Supernatants exhibited lysozyme activity. Small cells were induced to proliferate by RTS11 supernatant and LPS.	[217,218]
S. salar	HK	Long-term Salmonid cell line (TO): Cells showed quick adherence to the plastic surfaces, very phagocytic and bactericidal activity; no respiratory burst, and NO production; reacted positive to a leucocyte specific MAb but did not bind to neutrophil specific MAb or stain for myeloperoxidase. Cells also expressed CD83 gene but not the T-cell receptor alpha (TCR-α) or the macrophage marker (M-CSFR).	[150]
	PB	Short-term small, mononuclear blood cells from *S. salar*: highly expressed CD83 gene transcript and MHC-II; highly phagocytic, positive for acid phosphatase; lacked respiratory burst and myeloperoxidase activity; and have a high morphological plasticity.	[145]
	HK	Long-term Salmonid cell line (TO): Cells were cultured and passed more than 150 times and exhibited no changes in morphology, growth or virus production	[156]
	HK	Long-term Salmon HK, SHK-1 cell line: cells proliferated; negative for alkaline phosphatase; positive for acid phosphatase, non-specific esterase, and $Mg2^{+}$ dependent adenosine triphosphatase and 5′ nucleotidase. Cells reacted with MAb directed against *S.salar* PBL, and did not phagocytose *A. salmonicida*.	[161]
O. mykiss, S. salar	HK	Long-term HK macrophages: Cells were macrophage like and engulfed a range of particles	[89]
S. fontinalis, *S. trutta*	HK	Short-term primary trout monocytes and *in vitro* differentiated macrophages: showed phenotypic changes, strong phagocytosis of Zymosan; and LPS induced trout TNF-α (tTNF-α) mRNA expression.	[168]
2. Non-Salmonid immunological cell lines (n = 13)			
G. morhua	HK	Short-term HK macrophages: were highly phagocytic; acid phosphatase and non-specific esterase positive; d alkaline phosphatase and peroxidase negative; and enhanced respiratory burst by LPS.	[219]
Goldfish *(Carassius auratus)*	HK	Long-term *in vitro*-derived goldfish kidney macrophages (IVDKM): were positive for ROI and RNI with PMA, Con A and LPS.	[110]
C. auratus	HK	Long-term HK macrophages: stained positive for non-specific esterase; phagocytic on SRBC, amastigotes and promastigotes of Leishmania major; PMA & or LPS stimulated ROI & RNI.	[220]
	HK	Short-term HK macrophages: produced NO when stimulated by PMA, Con A or LPS, and co-stimulation with both factors induced synergistic NO production.	[221]
Catla catla	TH	Long-term Catla thymus macrophage (CTM) cell line: phagocytic on yeast cells and fluorescent latex beads; PMA & or LPS stimulated ROI & RNI; positive for alpha-naphthyl acetate esterase enzyme; and supernatant showed lysozyme activity.	[222]
Common carp (*Cyprinus carpio*) and Ginbuna (*Carassius auratus langsdorfii*)	TH	Long-term Carp thymus (KoT), and ginbuna thymus (GTS6 and GTS9): Cells proliferated and RT-PCR detected marker genes for myeloid/erythroid progenitors (*gata1*), haematopoietic stem cells (*gata2*), neutrophils (*mpx/mpo*), B-cells (*IgH*) and T-cells (*lck*, *TCRβ* and *gata3*) in primary cells, majority of which were lost after the third passage, except T-cell markers.	[223]
C. carpio	PB	Long-term Carp leucocyte cell line (CLC): reacted with MAbs against carp HK macrophages; binding to an antibody against complement component C3; had acid-phosphatase positive granule; phagocytic on SRBC; and LPS, PMA & co-culture with carp PBL enhanced respiratory burst; and secreted IL-1.	[224]
Rohu (*Labeo rohita*)	TH	Long-term *L. rohita* thymic macrophages (LRTM): tested positive for alpha naphthyl esterase acetate positive; exhibited nonspecific esterase and surface expression of Fc receptors for IgG; MHC-I, MHC-II antigens; engulfed yeast cells and latex beads; PMA and LPS induced ROI & RNI production;	[225]
L. rohita	HK	HK macrophages (HKM): Showed strong plastic adherence; phagocytosed yeast cells; LPS enhanced ROI & RNI, and lysozyme activity.	[226]
C. carpio	PB	Long-term Carp Leucocyte Culture (CLC): tested positive for peroxidase & periodic Acid Schiff (PAS); phagocytosed iron particles; cell growth enhanced with TNF-α, IL-2 and FBS enhanced multiplication and INF-γ depressed growth. Meanwhile, cell proliferation was highly enhanced by LPS than Con A; while PHA and SAC did not stimulate cells.	[227]
Channel catfish (*Chanos chanos*)	PB	Long-term monocyte like cell line from channel catfish: resembles mammalian monocytes or macrophages; stained positively for nonspecific esterase and peroxidase; phagocytosed latex beads; LPS induced IL-1 production; and showed effective antigen-presentation to autologous PBL for antigen specific *in vitro* proliferative and antibody responses.	[228]
Half smooth tongue sole (*Cynoglossus semilaevis*)	PB	Short-term monocyte-derived macrophages: Showed proliferative characteristics; highly enhanced respiratory burst against PMA, and *V. anguillarum* tests; highly phagocytic against latex beads and yeast cells; reacted positive to esterase activity; and enhanced expression of M-CSFR, MHC-II, IL-6, IL-10, TNF and arginase genes.	[229]
Nile tilapia (*Oreochromis niloticus*)	HK	Long-term Tilapia HK (THK): THK proliferated and THK cells ingested latex bead; HK cells exhibited monocytic leucocyte markers transcripts for CD33, CD53, CD82, MCSFR, and CD205; THK cells exhibited transcripts of haematopoietic stem cells (*gata2*) (GATA2), GATA4, and GATA6 but not myeloid/erythroid progenitors (GATA1).	[230]

lymphoid organs [36], by density gradient centrifugation and hypotonic lysis [37–39]. Indeed, several investigators have successfully isolated, cultured, maintained and characterised leucocytes from PB, HK, SP and TH tissues (Table 5), to aid farmed fish immunology understanding. Thus far, a number of cell lines have previously been developed from salmonid HKL, resulting into DC like TO cells in *S. salar* [156], trout primary kidney monocyte-like cultures (T-PKM) in *O. mykiss* [110] and *S. salar* SHK-1 [161]. Other HKL cell lines were isolated from *S. salar* and *O. mykiss* HK macrophages [89]; DC cells from *O. mykiss* [82], and HK macrophages in *S. fontinalis* and *S. trutta* [168].

In addition, salmonid leucocyte cell lines originating from the SP were developed for *O. mykiss* cell line (RTS11) in *O. mykiss* [217,218]; *O. Mykiss* PB, kidney and SP for DC cell lines [82], and small mononuclear cells with DC like characteristics from *S. salar* PBL [145]. Some investigators established long-term cell lines, and others short-term immune cell lines to enable fish immunological understanding using adherent and suspension cultures. These cell lines were characterised for cellular morphology, and functional properties of phagocytosis, respiratory burst, ROI and RNI production, immune enzyme activity, antibody production and immune gene expression (Table 5).

8. Future research and recommendations

In this review, we illustrated the techniques used to isolate salmonid immune leucocytes and their use to demonstrate immunomodulation following dietary manipulations, exposure to environmental stressors, effects of pathogens and parasites, vaccine design, efficacy and administration assessment. We also present findings on development of fish immune cell lines and their potential uses in aquaculture immunology. We mined 114 papers, among which, the most common technique used to isolate fish leucocytes was DDGC with Percoll density gradient.

Leucocytes were mostly isolated from fish HK and PB tissues, with *O. mykiss* and *S. salar* as the most studied species. Phagocytosis, followed by respiratory burst, were the most popular immunological assays employed in salmonid immunology. Regarding dietary manipulations, leucocyte commonly demonstrated enhanced cellular functions and immune gene expression following fish administration with probiotics, PAMPs, plant and animal extracts, and hormonal supplements. In addition, leucocytes demonstrated immunosuppressive effects following fish exposure to physical and chemical stressors, while parasites, viral and bacterial pathogens depressed fish immunity and upregulated pro-inflammatory cytokine transcripts. Overall, we found 23 fish lymphoid tissue developed and characterised cell lines, of which 10 originate from *S. salar* and *O. mykiss* HKL.

We observed in this review that most developed cell lines involved sacrificing fish. Also, large numbers of fish are sacrificed during vaccine trials, which can be avoided by using nonlethal PBL isolation techniques such as hypotonic lysis and micro-volume blood by density centrifugation. Hypotonic lysis gives a granulocyte enriched cell suspension compared to density gradient centrifugation, due to cell size fractionation. Thus, granulocyte quality and quantity in gradient isolated cells may affect assessment of adaptive immune parameters. We suggest that these observations be checked at species level for each method, as isolated leucocytes may be affected by fish gender, age, physiological state and isolation steps, among others. However, we suggest that techniques for leucocyte isolation need to move towards more affordable, time saving, nonlethal and ethically flexible methods such as hypotonic lysis in tandem with micro blood volumes use in the face of expensive gradient medium reagents. Finally, as this review retrieved no study of metabolomics using isolated leucocytes, we suggest that this approach be integrated with traditional techniques, and the newer novel Muse® Cell Analyser assays to open up new research avenues in fish physiology and immunology *in vitro*.

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