Aquaculture and Fisheries xxx (xxxx) xxx

Contents lists available at ScienceDirect

Aquaculture and Fisheries

journal homepage: www.keaipublishing.com/en/journals/aquaculture-and-fisheries

Original research article

Neutrophils in Atlantic salmon (*Salmo salar* L.) are MHC class II^{+} and secret IL-12p40 upon bacterial exposure

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ARTICLE INFO

Keywords:
Granulocytes
Antigen presenting cells
Major histocompatibility complex
T cell activation
B cells
CD83
CD80/86
MHC class II

ABSTRACT

Antigen-presentation via major histocompatibility complex (MHC) to T cells is the key event to initiate adaptive immune responses. In teleosts, as in mammals, the main types of professional antigen-presenting cells (APCs) are dendritic cells (DCs), monocytes/macrophages, and B cells. In the current study, flow cytometry, immunostaining and qPCR have been used to show that neutrophils in the teleost fish Atlantic salmon (*Salmo salar* L.) have antigen-presenting properties. The neutrophils were positive for MHC class II, CD83 and CD80/86, and upon *in vitro* bacterial exposure, gene expression analysis of purified neutrophils showed that IL-12p40, which is essential for proliferation of naïve T cells, was highly upregulated at both 6 and 24 h post bacterial exposure. Based on presence of MHC class II and upregulation of molecules involved in antigen presentation and T cell activation, we suggest that neutrophils in Atlantic salmon have potential to function as professional APCs. This work makes an important basis for further exploring the potential of using neutrophils to develop new, targeted immunoprophylactic measures.

1. Introduction

Neutrophils are phagocytic cells important in innate immunity where they play a primary role in resistance to extracellular pathogens, in acute inflammation and maintenance of homeostasis through clearance of damaged tissue (reviewed in (Mantovani, Cassatella, Costantini, & Jaillon, 2011)). They are professional phagocytes harbouring potent antimicrobial functions and killing abilities. Their primary function in the fight against microbes are phagocytosis, but in contrast to other professional phagocytic cells like macrophages, dendritic cells (DC) and B cells in fish (Banchereau & Steinman, 1998; Kordon, Karsi, & Pinchuk, 2018; Wu et al., 2020), they have not been fully recognized as antigen presenting cells (APCs). There is, however, some accumulating evidence that neutrophils also have a differentiated role in antigen presentation in humans and mice (Abi Abdallah, Egan, Butcher, & Denkers, 2011; Li et al., 2019; Meinderts et al., 2019; Vono et al., 2017). Neutrophils are the most abundant leukocyte cell type in human blood (70%), compared to lower levels in rodents (20%). The percentages of neutrophils in blood in teleost species varies from only 5% in the cichlid (*Cichlasoma dimerus*Henckel, 1840) (Rey Vazquez & Guerrero, 2007) and goldfish (*Carassius auratus* L.), to about 15–20% in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) (Hamdani et al., 1998; Pettersen, Bjorlow, Hagland, & Wergeland, 2005; Pettersen, Ulvenes, Melingen, & Wergeland, 2003) and as much as 80% in Atlantic cod (*Gadus morhua*, L.) (Rønneseth, Wergeland, & Pettersen, 2007). Among the head and trunk kidney leukocytes of several fish species, neutrophils are dominant.

An astonishing immunological diversification is shown among teleosts, e.g. some species lack MHC class II and CD4, the hallmark molecules for induction of adaptive immune responses. These molecules are missing in all members of the order Gadiformes investigated so far (Malmstrøm et al., 2016; Pilstrom, Warr, & Stromberg, 2005; Star et al., 2011), and in the phylogenetically distant species *Syngnathus typhle* belonging to the order Syngnathiformes (Haase et al., 2013) and some species of the order Lophiiformes (Dubin, Jorgensen, Moum, Johansen, & Jakt, 2019; Swann, Holland, Petersen, Pietsch, & Boehm, 2020). All other fish species studied to date have MHC class II and CD4. The lack of these key molecules is likely the reason why cod show low adaptive immune response upon antigen stimulation compared to e.g. salmon (Pilstrom et al., 2005). Neutrophils in fish are, as their mammalian homologues, the first cells migrating to the site of inflammation as a response to chemokines and other signalling molecules released from

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https://doi.org/10.1016/j.aaf.2022.07.002
Received 13 June 2022; Received in revised form 21 July 2022; Accepted 22 July 2022

macrophages and they are essential in the acute inflammatory response as killers of intruding microbes (Havixbeck, Rieger, Wong, Hodgkinson, & Barreda, 2016; Lin & Lore, 2017; Takashima & Yao, 2015). The roles of the neutrophils as phagocytic cells, their development, migration and innate functions like ingestion and degradation of microbes and their participation in the initiation and regulation of adaptive immunity are described in a recent review (Li et al., 2019).

In humans and mice, several studies suggest that neutrophils are also involved in initiation and regulation of adaptive immunity, including priming of Th cell differentiation through antigen presentation via MHC class II (Abi Abdallah et al., 2011; Culshaw, Millington, Brewer, & McInnes, 2008; Li et al., 2019; Lin & Lore, 2017; Takashima & Yao, 2015). The plasticity of mammalian neutrophils and their role as APC has been described in the review by Takashima and Yao (2015). In this review, it is reported that neutrophils from both human and mouse can acquire properties of dendritic cells (DCs) both in culture and at sites of inflammation. They also describe a neutrophil-DC hybrid cell type with surface markers and properties of both neutrophils and DCs. Despite detailed knowlegde of MHC class I and II molecules in teleosts (Edholm, Pasquier, Wiegertjes, & Pierre, 2021; Grimholt, 2016) and T cell activation upon bacterial and viral challenge in various species (reviewed in (Castro & Tafalla, 2015), in-depth knowlegde of APC subsets in fish is in its infancy. The possible role of fish neutrophils in antigen-presentation is largely unknown. The description by Cuesta, Angeles Esteban, and Meseguer (2006) of MHC class II expression by acidophilic granulocytes (eosinophils) is to our knowledge the only study in fish suggesting an antigen presenting role of a polymorphonuclear leukocyte. Since teleosts is the first evolutionary group with an adaptive immune system, it is interesting, both from an evolutionary and functional point of view, to investigate if neutrophils in teleost fish play a role in antigen presentation.

In the present study, we have performed immunostaining of leukocytes using an anti-salmon MHC class II antiserum in combination with a monoclonal antibody (mAb) against neutrophils and analyzed leukocytes by flow cytometry and fluorescent microscopy. The neutrophils were sorted, and gene expression of molecules involved in antigen-presentation, as well as responses after *in vitro* bacterial challenge, were measured by quantitative PCR (qPCR). This is, to our knowlegde, the first study showing that salmon neutrophils have potential acting as profession APCs, making it an important foundation for further functional analyses of neutrophils and other subsets of APCs in this species.

2. Materials and methods

2.1. Fish and rearing conditions

Non-vaccinated, healthy Atlantic salmon (body weight 53.8 ± 11.2 g and average length 17.4 ± 1.4 cm) were obtained from The Aquatic and Industrial Laboratory (ILAB) at the High Technology Centre in Bergen, Norway. The fish were kept under normal optimal rearing conditions at a temperature of 8 °C, salinity of 34‰ and 12:12 h light/dark. The water flow was 1200 L per hour which gave a minimum of 70% oxygen saturation in the outlet water. The fish were fed commercial dry feed obtained from Skretting, Norway.

2.2. Growth of bacteria

Vibrio anguillarum serotype O1 isolated from lumpfish was used for *in vitro* challenge of neutrophils. Prior to exposure, the bacteria were grown in tryptic soy broth (Bencton Dickinson) containing 2% NaCl (Sigma) until late log phase (OD_{600} = 1.0), washed twice in PBS and finally resuspended in PBS. The corresponding number of bacteria was calculated using an OD_{600}/CFU correlation.

2.3. Sampling and isolation of leukocytes

Leukocytes were isolated from healthy, non-vaccinated salmon. The fish were quickly netted and killed by a sharp blow to the head. Peripheral blood (up to 0.7 mL) was collected from *Vena caudalis* using a syringe and transferred to BD heparinized containers (Fisher scientific) and diluted to a total volume of 2 mL with Leibovitz L-15+ medium (L-15 (Cambrex) (adjusted to 370 mOsm by adding a solution consisting of 5% (v/v) 0.41 M NaCl, 0.33 M $NaHCO_3$ (Sigma) and 0.66% (w/v) D-glucose (Sigma)), supplemented with 100 μg/mL gentamicin sulphate (Cambrex), 2 mM L-glutamine (Cambrex) and 15 mM HEPES (Sigma)). The kidney was sectioned into four parts, termed HK, K1, K2, K3 (head kidney to posterior kidney, see Supplemental Fig. 1). The average weight of the tissue samples for the kidney was 63.2 ± 5.6 mg for head kidney fragment (HK), 76.3 ± 11.8 mg for first kidney fragment (K1), 111.6 ± 8.3 mg for second kidney fragment (K2) and 114.8 ± 16.0 mg for the most anterior kidney fragment (K3). The whole spleen, with an average weight of 37.0 ± 7.7 mg, was used for leukocyte isolation. Cell suspensions from the kidney fragments were obtained using GentleMACS (Milteny Biotec) as described previously (Rønneseth, Haugland, & Wergeland, 2013). Spleens were homogenised using program A in the GentleMACS. Peripheral blood leukocytes (PBLs), head kidney leukocytes (HKLs), kidney leukocytes (K1Ls, K2Ls and K3Ls) and spleen leukocytes (SLs) were isolated on discontinous percoll gradients as described by (Pettersen et al., 1995). Further, the isolated leukocytes were diluted to 500 μL in PBS + E (PBS supplemented with 1% (w/v) BSA (Sigma) and 25 mM EDTA (Sigma), pH 7.3) and cell numbers, aggregation factor and viability were analyzed in a CASY-TT cell counter (Innovatis). Cell suspensions were diluted in PBS + E to 5×10^6 or 1×10^6 cells mL^{-1} based on viable cell counts in the CASY-TT prior to flow analyses and cytospin preparations, respectively.

2.4. Antibodies

For flow cytometry, monoclonal antibodies (mAbs) reacting with IgM^+ B cells (C7G7, 2 μg mL^{-1}) and neutrophils (E3D9, 2 μg mL^{-1}) were used. The reactivity and specificity of the mAbs are described previously (Haugland, Pettersen, Sviland, Rønneseth, & Wergeland, 2010; Pettersen, Bjerknes, & Wergeland, 2000; Pettersen et al., 1995). A rabbit polyclonal serum against MHCII was produced by super speedy protocol with four immunizations (Eurogenitec, Belgium) using the synthetic peptide DGREVKSDVTSTEEL conjugated to keyhole limpet haemocyanin (KLH) via m-maleimidobenzoyl-*N*-hydroxysuccinimide ester. This peptide has previously been similarly used to generate MHCII antibodies to salmon MHCII (Iliev et al., 2010). Serum sampled from the rabbit prior to immunization is hereafter termed pre-serum. The reactivity of the MHCII antiserum was verified in ELISA using MHCII peptide and KLH as antigens (Fig. 1C). For flow cytometry analyses, the *anti*-MHCII antiserum was diluted 1:2500, while a 1:1000 dilution was used for immunostaining of cytospin preparations. Negative controls were leukocyte samples without primary antibodies and an isotype control using a non-salmon reactive mAb (1-D4) of same isotype as test mAbs, at a concentration of 1 μg mL^{-1} and pre-serum from rabbit. Secondary antibodies used in the flow cytometry analyses were R-phycoerythrin (RPE)-conjugated $F(ab')_2$-fragment of goat-anti rabbit IgG (Molecular probes, Invitrogen) and Alexa Fluor (AF) 647 $F(ab')_2$ fragment of goat anti-mouse IgG (H + L) (Molecular probes, Invitrogen), both diluted 1:400. For immunostaining, goat-anti rabbit Alexa Fluor 488 (Molecular probes, Invitrogen) and goat-anti mouse Alexa Fluor 555 (Molecular probes, Invitrogen) were used as conjugates.

2.5. Flow cytometry analyses

Isolated leukocytes, 1×10^6 cells per polystyrene tube in 200 μL PBS + E, were labelled with 250 μL primary antibody cocktail mix consisting of MHC class II antiserum together with C7G7 (reacting with B cells) or

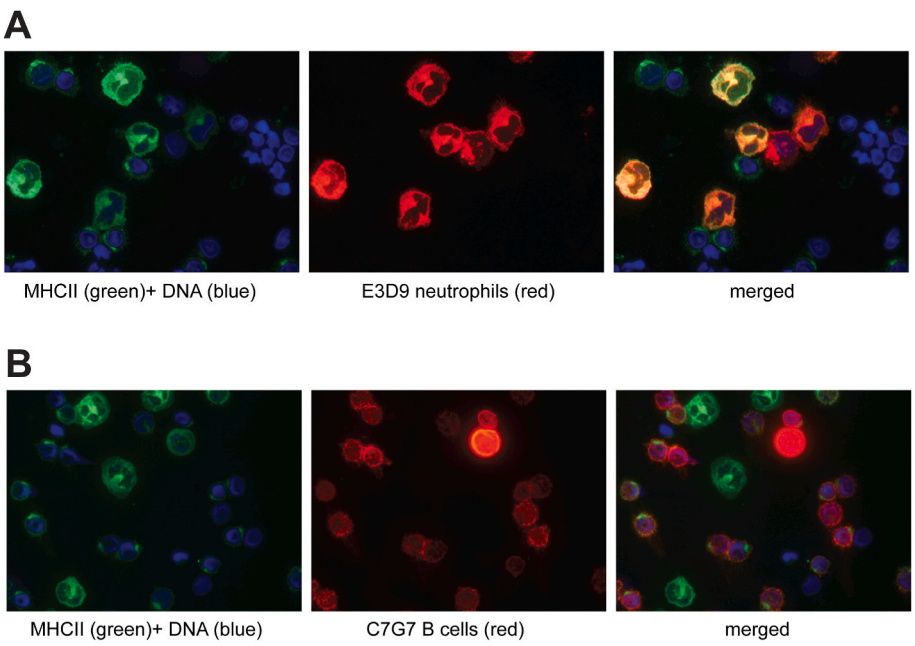

Fig. 1. Immunostaining of PBL cell reactivity of anti-salmon MHC class II antibody. **(A, B)** Left panel shows cells stained with *anti*-MHCII serum and antibodies conjugated with Alexa Fluor 488 ($MHCII^+$ cells appear as green). **(A)** In the middle panel, the leukocytes are stained with the mAb E3D9 reacting with neutrophils and antibodies conjugated with Alexa Fluor 555 (neutrophils appear as red). In the right panel, an overlay of the two pictures is shown. **(B)** In the middle panel, leukocytes are stained with the mAb C7G7 which react with IgM on B cells and Alexa Fluor 555 (B cells appear as red). The right panel is an overlay of the two aforementioned pictures. Cell nucleus DNA is stained blue by DAPI. (C) ELISA for testing reactivity of rabbit antiserum from the final bleed against the MHC class II peptide and the carrier protein. Serum collected from the rabbit (pre-serum) showed no reactivity against the peptide or the carrier in contrast to the immune serum.

E3D9 (reacting with neutrophils) diluted in PBS containing 0.5% BSA. Negative controls were leukocyte samples without primary antibodies and leukocytes samples with pre-serum and leukocyte samples with mAb 1-D4. The samples were incubated with primary antibodies for 45 min at 4 °C, washed twice with PBS + E, incubated with a cocktail mix consisting of goat-anti rabbit- R-phycoerythrin (RPE) (Molecular probes) and goat anti-mouse Alexa Fluor 647 (Molecular probes) conjugates, both diluted 1:400 in PBS + E, for 45 min at 4 °C and finally washed twice with PBS + E. The cells were resuspended in 500 µL PBS + E and analyzed in a BD FACSCalibur flow cytometer (BD biosciences) using Cell Quest version 3.1 software (Becton Dickinson). Ten thousand cells were recorded in each sample and the cells were analyzed for forward scatter (FSC) and sideward scatter (SSC) patterns, representing the size and granularity of the cells, respectively, and for yellow/orange fluorescence detecting $MHCII^+$ cells in fluorescence channel FL2 (detecting emitted light excited by argon laser, 488 nm) and red fluorescence detecting B cells and neutrophils in FL4 (detecting emitted light excited by red diode laser, 635 nm). Double positive cells were detected in both channels. Further data analyses were performed using FCS express 3 (De Novo Software). Dead cells and debris were excluded from the analyses by gating. By using the isotype and pre-serum controls, the limit between true positive and negative cells was set to 1% of the controls for both single and double positive cells.

2.6. Immunostaining

Immunostaining was performed on cytospun leukocytes isolated from peripheral blood and tissues. The cytospin slides were made using a Shandon Cytospin III cytocentrifuge and Colorrapid staining was performed as described previously (Haugland, Rønneseth, & Wergeland, 2014). The cells were fixed in acetone/methanol bath, washed once in PBS-bath and blocked with PBS containing 0.5% BSA to avoid background binding. The slides were further incubated with a mix of MHCII antiserum (1:1000) together with C7G7 (2 µg mL^{-1}) or E3D9 (2 µg mL^{-1}) in a humidity chamber for 1 h at room temperature. Subsequently, the slides were washed three times in PBS-baths and incubated with a mix of Alexa Fluor 488-goat anti-rabbit and Alexa Fluor 555-goat anti-mouse conjugates (Molecular probes). The slides were cover slipped with Prolong Gold Antifade Reagent with DAPI (Molecular probes). For negative controls, the primary antibodies were omitted, and a mix of rabbit pre-serum and 1D-4 was used as primary antibody.

2.7. Isolation of B cells and neutrophils using MACS LS column

B cells and neutrophils were isolated with MACS LS columns (Miltenyi Biotec) as described previously (Haugland, Jordal, & Wergeland, 2012) by use of the mAbs C7G7 and E3D9, respectively, and goat anti-mouse IgG Microbeads (Miltenyi Biotec). For isolation of B cells 4 µg mL^{-1} C7G7 per 6.4×10^6 PBLs, while 8 µg mL^{-1} E3D9 per 7.1×10^6

HKLs was used for isolation of neutrophils. Prior to elution of bound cells (B cells and neutrophils), the columns were washed three times with 3 mL MACS buffer (1 × PBS, 0.5% BSA and 2 mM EDTA, pH 7.3). Sorted cells were centrifuged, resuspended in lysis buffer, and stored at −80 °C until RNA isolation.

2.8. In vitro challenge of sorted neutrophils with bacteria

Sorted neutrophils from three fish were resuspended in L-15+ medium and added to 24 well plates (2.5×10^6 neutrophils per well) and challenged with *V.ang.* harvested in log phase (section 2.2.) at a multiplicity of infection (MOI) 1:10 neutrophils: bacteria in a total volume of 500 μL. Neutrophils without bacteria were negative controls. The plates were centrifuged for 1 min at 1280×*g* (Allegra X-15R centrifuge) and incubated at 15 °C. After 90 min, 1% penicillin -streptomycin (Sigma) was added to all wells and the plates were further incubated at 15 °C for (in total) 6 and 24 h. Following incubation, the plated were centrifuged for 10 min at 200×*g*, the supernatant were carefully removed and 350 μL lysis buffer was added to each well and the samples were stored at −80 °C until RNA isolation.

2.9. Isolation of RNA and cDNA synthesis

Total RNA was isolated from B cells and neutrophils using GeneElute Mammalian Total RNA miniprep kit (Sigma) as described previously (Haugland et al., 2012) and the concentration was determined by NanoDrop ND-1000 UV–Vis Spectrophotometer (Nanodrop Technologies). The total RNA was treated with DNase I (Sigma) to remove traces of genomic DNA, quantified in the Nanodrop and transcribed into cDNA using qScript cDNA synthesis kit (Quanta BioScience) according to the manufacturer's instructions using a maximum of 1000 ng per reaction.

2.10. RT-PCR

RT-PCR was performed to investigate the phenotype of B cells isolated with C7G7. The genes included in the analyses was membrane bound IgM (mIgM), soluble IgM (sIgM), IgD, IgT, CD3, TcRα, FcεRγ and MCSF. The HPLC purified oligonucleotides used as primers are listed in Table 1. The PCR mix (25 μL) contained 2.5 μL 10 × Buffer for DyNAzyme™ DNA polymerase, 0.5 μL dNTP mix (10 mM), 1 μL forward primer (10 μM), 1 μL revers primer (10 μM), template (20 ng in 2.5 μL), 0.5 μL DyNAzyme II (Finnzymes) and 17 μL nuclease-free water (Sigma). The RT-PCR was performed in an Applied Biosystem 2720 Thermal cycler. The numbers of PCR cycles are given in Fig. 2B. The PCR products were analyzed on a 1% agarose gel.

2.11. Quantitative PCR (qPCR)

qPCR was performed in a CFX96™ Real-Time system and C1000 Thermal cycler (BioRad) using SYBR Green Jumpstart Taq ready mix kit as described previously (Rønneseth et al., 2013) and HPLC purified primers (Sigma-Genosys). The primers are listed in Table 1. The PCR reactions (25 μL) contained 5 μL (4 ng/μL), 12.5 μL 2 × SYBR green jumpstart Taq Ready Mix, 1 μL (0.4 mM) forward primer, 1 μL (0.4 mM) reverse primer and 5.5 μL nuclease-free water. Three parallels were performed for all genes. After the run, melting curve analyses were performed of each amplicon to ensure the specificity of the primers and the qPCR products were visualized on a 2% agarose gel for size determination. Negative controls: PCR-reaction without template (non-template control, NTC) and cDNA reaction without reverse transcriptase as template (-RT control) were included for each master mix. Ct-values for the target genes were normalized against RPS20 by using Q-Gene.

2.12. Statistics

For statistical analyses two-tailed unpaired *t*-test was performed. Results were considered significant at $p \leq 0.05$. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

3. Results

3.1. Neutrophils and B cells are MHC class II $^{+}$ cells

A polyclonal anti-salmon MHC class II serum was used to characterize different subtypes of APC in Atlantic salmon. The antiserum was used for immunostaining of fixed cells on cytospin slides and non-fixated cells for flow cytometry analyses. Immunostaining of cytospun leukocytes showed that the polymorphonucleated cells stained positive, indicating that neutrophilic cells in Atlantic salmon are MHCII^{+} cells (Fig. 1). This was verified by double immunostaining with the mAb E3D9, specific for neutrophils (Fig. 1A). B cells, known to be MHCII^{+} in both fish and mammals, were included as a positive control (Fig. 1B). Strong reactivity against both the carrier and the peptide was measured by ELISA (Fig. 1C).

3.2. MACS sorted B cells from peripheral blood are IgM $^{+}$ IgD $^{+}$ IgT^{-}

C7G7 is a monoclonal antibody (mAb) that react with salmon IgM

Table 1
Oligonucleotides used in RT-PCR and qPCR.

Gene	Acc.No	Forward primer (5′-3′)	Revers primer (5′-3′)	Size	Application	Ref
EF1αO	NM_001123629.1	GGACGTTTACAAAATCGGTGGTAT	GTCTCCAGACTTGAGGAATTTGG	460	RT-PCR	This study
IgT	GQ907003.1	GTCATCAACACTAACTGGAACAACAA	ACACTGGACTTTGTTCACATTGGT	365	RT-PCR	This study
IgD	AF_141607	TCTATAAAGATAGTGAGACCATCAGTTTCTG	TGTAGGTGGTGCTAGTGTTGTTGTTc	483	RT-PCR	This study
mIgM	Y12391.2	CTTATGAACAAAAAAGCTGAGCTTGT	GACAGTCAGTCAACACGAGACACT	289	RT-PCR	This study
sIgM	Y12391.2	TTATGAACAAAAAAGCTGAGCTTGT	ATCAATAAGCCAAGCCACTAAAACTT	400	RT-PCR	This study
FcεRIγ	BT049732.1	CTCTTTAACCTTTCATTTTGTGCCT	TTACCACCACTCACACTACTGTCTTC	380	RT-PCR	This study
MCSF	NM_001171807.1	GACCATGTCTGATACAGGAAACTTC	CCGCTCGAGTCATCAGTGGTGGTGGTG	427	RT-PCR	This study
TCRα	AY552002.1	AGAGAAACATGAGCCATCCTACTACA	TCCAGACAATCAAGAGATTCCAAT	463	RT-PCR	This study
CD3γσ	EF421418.1	TGATTGGAGTAGCTGTCTACAGTATCG	AACTATTATTCTGTGGCGTGGTAAAAG	455	RT-PCR	This study
RPS20	BT060032	ATCACCACCAGAAAGACACCCT	GAGGTGATCTGCTTGACAATCTCA	122	qPCR	Rønneseth et al. (2013)
IL12p40c	BT049114	ATGGTTGATAAGGGGGACAGTTT	CCTTGGACCTGAGTTTTGTCTAAAT	110	qPCR	This study
IL12p40b2	BT049762.1/ BT049253.1	CATACCATCACCCAACAGAACG	GCCAAACACCTTATGTACTCATCTTC	81	qPCR	This study
IL12p40b1	HG917957	TTGTCCCGCATCCATCTCACTGTAT	ATCTTGTCTGGCTTCACTATTTCTTG	104	qPCR	This study
CD80/86	DW580717.1	TAGACCACACACAGGGAACAATG	ATTGAGATGTATGTTCTTGTCGTCG	116	qPCR	Haugland et al. (2012)
CD83	BT047309	GCACCTGTAGGAGAGCAGAACC	TCCCTTTCTTCTGATTGGTCTGT	89	qPCR	Haugland et al. (2012)
MHCII	X70165	GTGGAGCACATCAGCCTCACT	GACGCACCGATGGCTATCTTA	92	qPCR	Pettersen, Ingerslev, Stavang, Egenberg, and Wergeland (2008)
MHCI	AF504019	CCTGAGGAGTGGAAGAACAACAA	CATTCCAGTTGGTCTTGATCTCAG	109	qPCR	This study

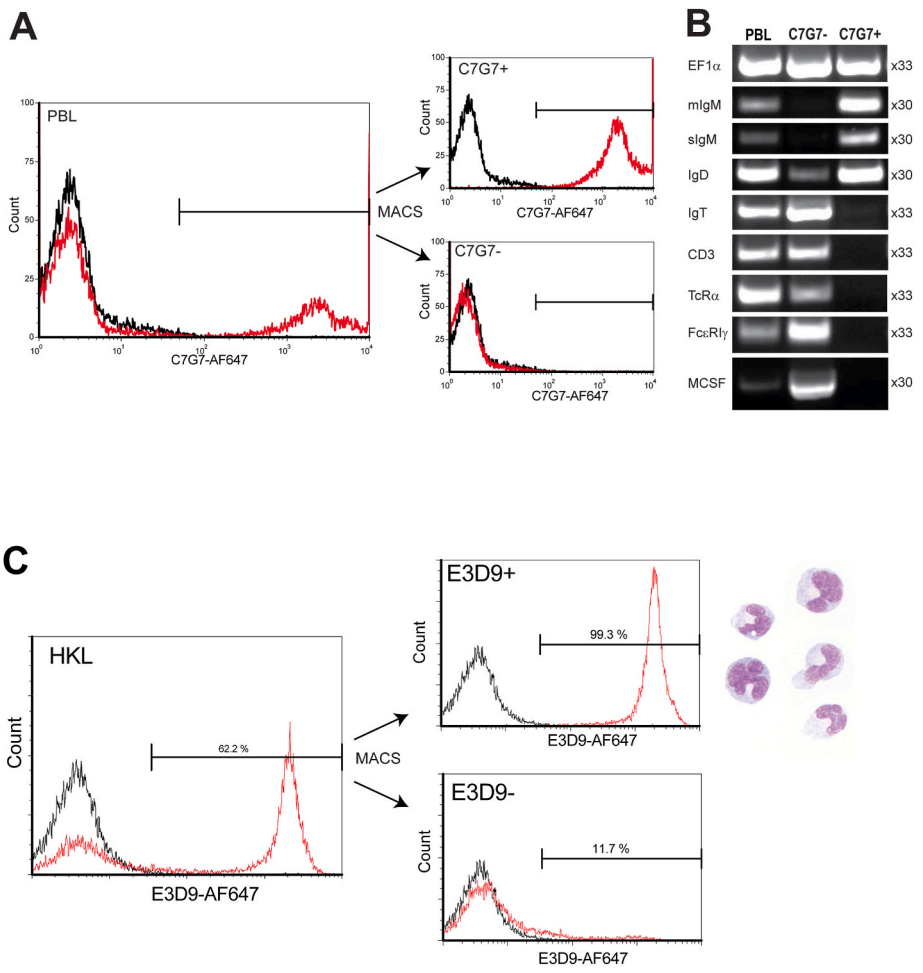

Fig. 2. Magnetic activated cell sorting (MACS) of B cells. **(A)** Representative histogram showing labelling of B cells with mAb C7G7 among isolated PBL. Black line shows all cells which is also not stained by the isotype control, red line show C7G7 labelled cells. Markers define C7G7 positive cells. The panels to the right show cell fractions after magnetic sorting. Upper, right panel shows cell fraction bound to C7G7 (C7G7$^+$). Lower, right panel show all cells in unbound fraction (C7G7$^-$). **(B)** Agarose gel of reverse transcriptase PCR products of PBL prior to MACS, unbound (C7G7$^-$) and bound (C7G7$^+$) fraction after sorting. The analyzed genes are listed on the left side and numbers of PCR cycles are listed on the right side of the agarose gel. **(C)** MACS of neutrophils from HKL using the mAb E3D9 and the response after *in vitro* bacterial challenge measured with RT-qPCR. **(A)** Representative histogram showing labelling of neutrophils with the mAb E3D9 among isolated HKL. Black line shows non-labelled cells and tested by isotype control, red line is mAb E3D9 labelled cells. Markers define E3D9$^+$ cells. The panels to the right show labelling of cells with mAbE3D9 after magnetic sorting. Upper, right panel shows the bound fraction (E3D9$^+$). Lower, right panel shows the unbound fraction (E3D9$^-$). Representative single cells in the bound fraction (neutrophils) stained with Colorrapid are shown. EF1α = elongation factor 1α, mIgM = membrane bound IgM, sIgM = soluble IgM, IgD = immunoglobulin D, IgT = immunoglobulin T, CD3 = cluster of differentiation 3, TcR α = T cell receptor α subunit, FcεRIγ = Fc epsilon receptor gamma subunit, MCSF = macrophage colony stimulating factor.

and IgM$^+$ B cells (Pettersen et al., 1995). To further determine the phenotype of these B cells, MACS was performed using C7G7. After two passages through the column, the purity of the isolated B cells was 98.3 ± 0.7% (Fig. 2A) and the isolated cells had a viability of 96.9 ± 1.4% (n = 3). In the unbound fraction only trace amounts of C7G7$^+$ B cells were present (0.2 ± 0.1%, n = 3) showing that the separation was successful. RT-PCR was performed to verify presence or absence of different markers on the bound fraction (C7G7$^+$). Unsorted PBLs and unbound fraction (C7G7$^-$) were included in the analysis for comparison. As shown in Fig. 2B, the isolated B cells (C7G7$^+$ cells) were positive for both membrane-bound IgM (mIgM), secreted IgM (sIgM) and IgD. IgT$^+$ B cells did not bind to C7G7 and were present in the unbound fraction. Also, a weak band for IgD was observed in the unbound fraction. The genes encoding CD3 and TCRα specific for T cells, FcεRIγ and MCSF in cells from monocyte/macrophage linage were also detected in the unbound fraction, but not in the bound fraction containing purified C7G7$^+$ B cells. These results show that the MACS protocol described yielded a pure B cell fraction with high viability. Neutrophils were isolated from HKLs using MACS (Fig. 2C). The bound fraction (E3D9$^+$) containing the neutrophils were purified to a level of 99%. The cell viability of the bound fraction was 95.8 ± 2%. Representative neutrophils with polymorphonuclear nuclei are shown.

3.3. *Proportion of neutrophils, B cells and total fraction of MHC class II$^+$ cells among HKLs, K1Ls, K2Ls, K3Ls, SLs and PBLs*

To investigate the proportions of different cell subsets among leukocytes isolated from kidney (HKLs, K1Ls, K2Ls, K3Ls), SLs and PBLs in healthy salmon, isolated leukocytes were analyzed for presence of neutrophils (E3D9$^+$), IgM$^+$ B cells (C7G7$^+$) and MHC class II using flow cytometry (Fig. 3). The results were in accordance with the scatter plot distribution of cells (Supplemental Fig. 1) as neutrophils were numerous in all parts of the kidney, but not in spleen and peripheral blood. Among HKLs, 44% were neutrophils (Fig. 3A and B), while in the different parts of the body kidney (K1, K2 and K3) the neutrophils constituted 62–68% of the isolated leukocytes. Among SLs and PBLs, B cells were more numerous than neutrophils (Fig. 3C and D). Further, the fractions of total MHCII$^+$ cells among isolated leukocytes were determined (Fig. 3E and F), as well as the fractions of MHCII$^+$ cells with low and high fluorescent intensity, termed MHCII^{low} and MHCII^{high}, respectively, which reflect the amount of MHC class II molecules at the cell surface. Among the MHCII$^+$ leukocytes isolated from all organs and peripheral blood, most of the MHCII $^+$ cells were MHCII^{low} (Fig. 3E and F).

3.4. *Most of the neutrophils are MHC class II$^+$ and they represent the main MHC class II $^+$ cell type in head kidney and body kidney, but not in spleen and peripheral blood*

To determine the fraction of MHCII$^+$ neutrophils and MHCII$^+$ B cells of total neutrophils and B cells, respectively, isolated leukocytes were double stained with *anti*-MHCII serum and mAbs specific for neutrophils (Fig. 4A and B) and B cells (Fig. 4C and D). A representative sample (HKL) is shown, but the same approach was used for K1Ls, K2Ls, K3Ls, SLs and PBLs. The negative control, an isotype control (1-D4) versus preserum is shown in the panels to the left (Fig. 4A, C). In Fig. 4A, MHCII$^+$ neutrophils are defined by the upper right square in the quadrant plot in the center panel, while in Fig. 4C, the upper right square represents MHCII$^+$ B cells. MHCII$^+$ neutrophils and MHCII $^+$ B cells were further

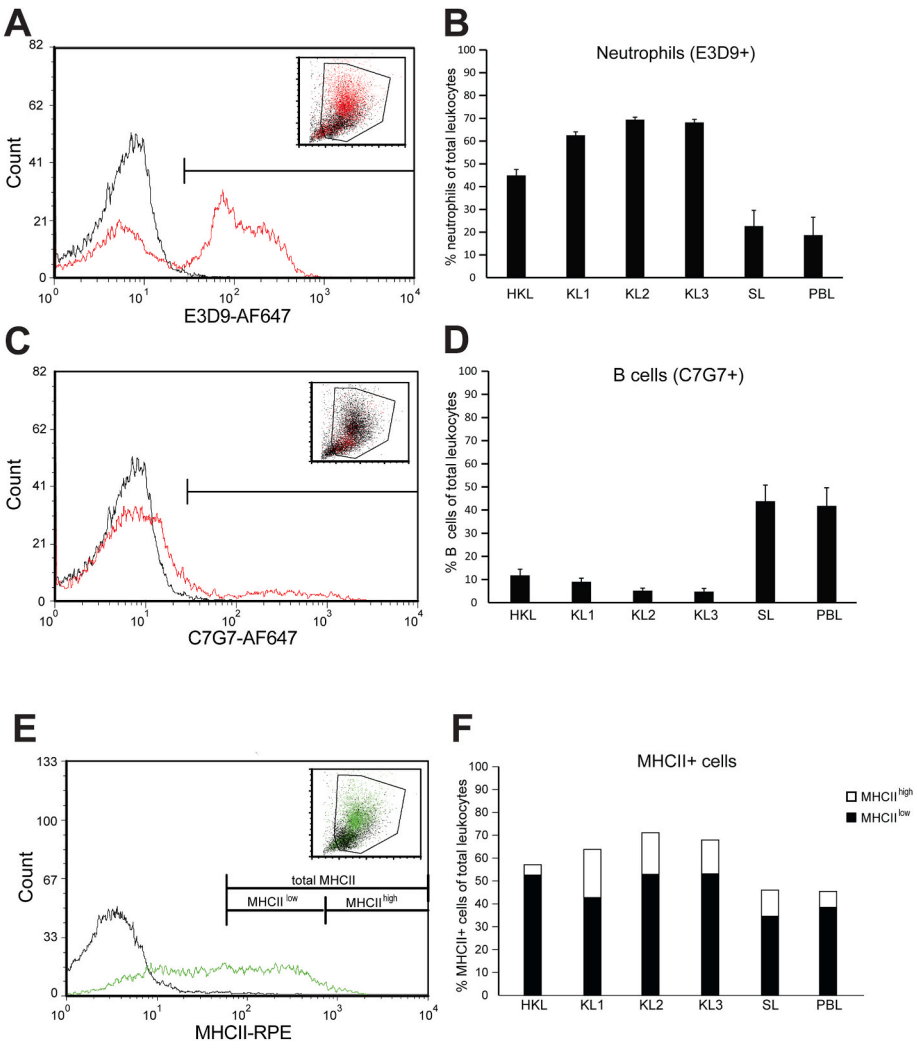

Fig. 3. Quantification of neutrophils (E3D9$^+$), B cells (C7G7$^+$) and MHCII$^+$ cells among isolated head kidney leukocytes (HKL), kidney part 1 leukocytes (K1L), kidney part 2 leukocytes (K2L), kidney part 3 leukocytes (K3L), spleen leukocytes (SL) and peripheral blood leukocytes (PBL). **(A)** Representative histogram of HKL labelled with mAb E3D9. Distribution of the positive cells is shown as red dots in the dot plot. The marker defines positive cells (neutrophils). **(B)** Diagram showing the percentage of neutrophils among leukocytes isolated from the different organs. The bars show average (n = 8) ± S.D. **(C)** Representative histogram of HKL labelled with mAb C7G7. Distribution of the positive cells is shown as red dots in the dot plot. The marker defines positive cells (B cells). **(D)** Diagram showing the percentage of B cells among leukocytes isolated from the different organs. The bars show average (n = 8) ± S.D. **(E)** Representative histogram of HKL labelled with MHCII antiserum. Distribution of the positive cells is shown as red dots in the dot plot. The markers represent positive cells (total MHCII), and MHCII^{low} and MHCII^{high}. **(F)** Diagram showing the percentage of MHCII$^+$, MHCII^{low} (white bar) and MHCII^{high} (black bar) among leukocytes isolated from the different organs. The bars show average values (n = 8).

divided into MHCII^{low} and MHCII^{high} based on fluorescent intensity. The flow analyses showed that the majority (80–90%) of the neutrophils were MHCII$^+$, also among SLs, although the percentage of MHCII$^+$ neutrophils were lower (63%) compared to HKLs, K1Ls, K2Ls, K3Ls and PBLs. Except for K1L, the major fractions of MHCII $^+$ neutrophils were MHCII^{low} (Fig. 4B). Around 60% of the B cells were MHCII$^+$ and of these, 3–18% was MHCII^{high} (Fig. 4D). Further, the percentages of neutrophils and B cells of total MHCII$^+$ leukocytes were calculated. As shown in Fig. 4E, the neutrophils constituted the major cell type among MHCII$^+$ HKL and body kidney leukocytes (K1Ls, K2Ls, K3Ls), while among the MHCII$^+$ leukocytes in SLs and PBLs, B cells were the most numerous cell type.

3.5. Gene expression analyses for sorted neutrophils show high level of MHCII, CD80/86 and CD83, and upregulation of IL-12p40 upon bacterial exposure

Basal expression levels of markers for professional APC, revealed that the neutrophils (sorted by MACS) had high level of MHC class II and CD83, as well as CD80/86 (Fig. 5A). Atlantic salmon has three homologues of IL-12p40 (b1, b2 and c) (Wang & Husain, 2014). These were all detected, but at low levels as expected for proinflammatory cytokines in healthy individuals (Fig. 5A). To investigate the immune response of neutrophils upon bacteria exposure, sorted neutrophils were exposed for bacteria for 6 and 24 h. The transcript levels of CD80/86 and CD83 were significantly upregulated at 6 h post challenge (hpc), 2.55 and 4.68-fold respectively, compared with the control. The transcriptional levels of the IL-12p40-b1 and IL-12p40-b2, but not IL-12p40-c, were highly upregulated at both 6 h and 24 h post challenge (hpc) (Fig. 5B).

4. Discussion

The most prominent feature of professional antigen presenting cells (APCs) is the constituent expression of MHC class II molecules. APCs take up soluble antigens that are further processed and presented on MHC class II molecules to T cells. However, to activate naïve T cells, more signals delivered from the APC are required, including co-stimulatory molecules and cytokines like CD80, CD86 and IL-12 (Banchereau & Steinman, 1998; Kambayashi & Laufer, 2014; Moini, Badolato, & Ahangari, 2020).

The data presented here show that salmon neutrophils express MHCII as shown both at the protein level, by immunostaining and by gene expression analyses. Neutrophils were easily observed among isolated leukocytes due to the characteristic polymorphic nucleus and they were identified and isolated by use of a neutrophil specific mAb. When combining the mAb with MHCII antiserum, it was shown that neutrophils were highly positive for MHCII. Similarly, B cells, identified with a mAb to salmon IgM, were positive for MHCII as expected as these are known to be APCs.

Purification of IgM$^+$ B cells and neutrophils by MACS yielded highly purified cell subtypes based on both flow cytometry analyses and gene expression profiles. The isolated B cells were both IgM$^+$ and IgD$^+$ which

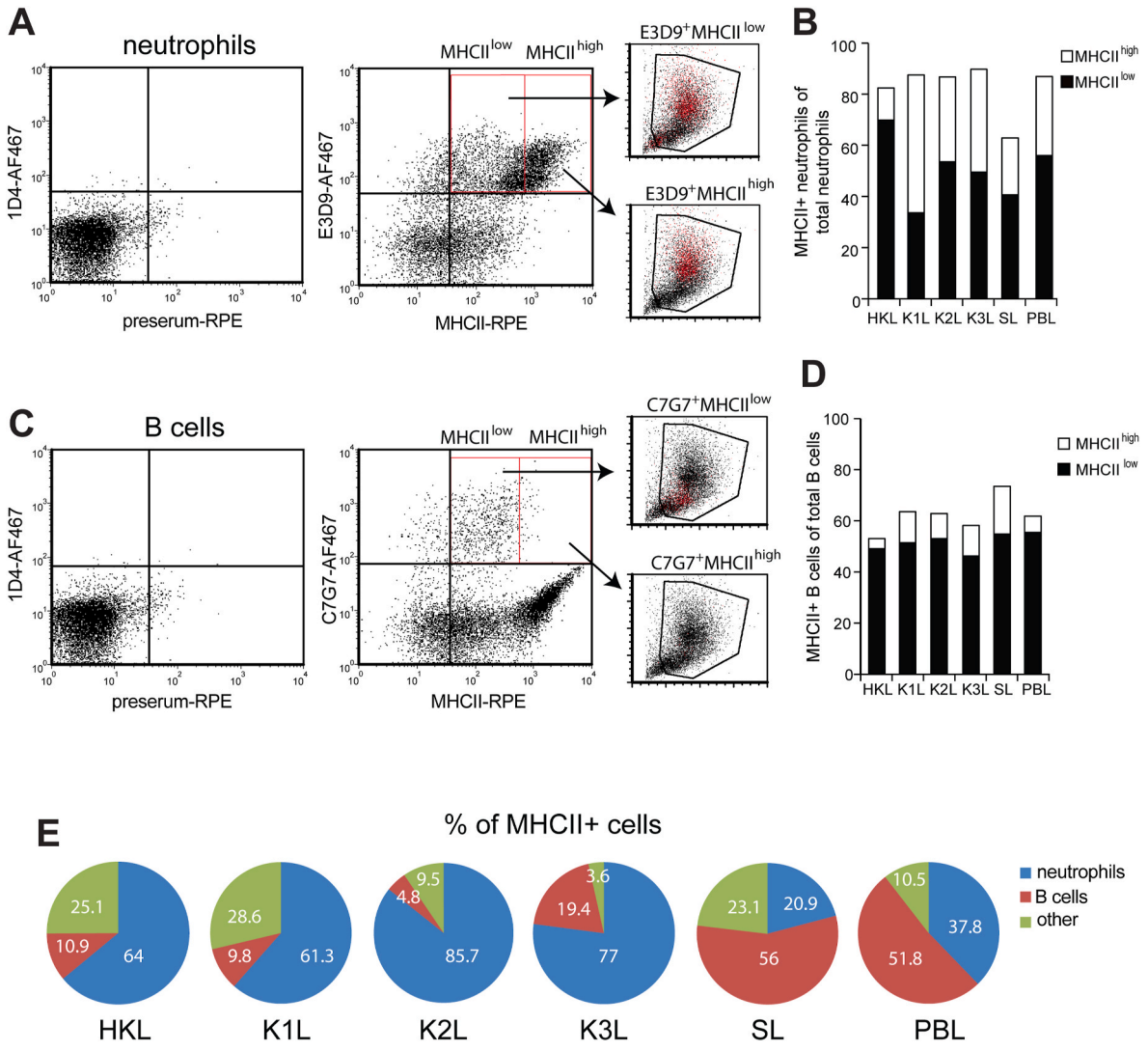

Fig. 4. Flow cytometry analyses of double stained leukocytes with cell-specific mAbs and *anti*-MHCII antiserum. Staining of HKL is shown as an example, but K1L, K2L, K3L, SL and PBL were analyzed in the same way. **(A)** Left panel, histogram shows the negative control (mAb 1-D4 versus pre-serum), central panel shows labelling with mAb E3D9 (neutrophils) versus MHC II. MHCII $^{+}$ neutrophils were further divided into MHCII^{low} (E3D9^{+}MHCII^{low}) and MHCII^{high} (E3D9^{+}MHCII^{high}) (red dots in the scatter plots). **(B)** The diagram shows the percentage of neutrophils that are MHCII^{+}, and the fraction of the MCHII $^{+}$ that are MHCII^{low} (black bar) and MHCII^{high} (white bar). **(C)** Left panel, histogram shows the negative control (mAb 1-D4 versus preserum), central panel shows labelling with mAb C7G7 (B cells) versus MHCII antiserum. MHCII $^{+}$ B cells were further divided into MHCII^{low} (C7G7^{+}MHCII^{low}) and MHCII^{high} (C7G7^{+}MHCII^{high}) (red dots in the scatter plots). **(D)** The diagram shows the percentage of B-cells that are MHCII^{+}, and the fraction of the MCHII $^{+}$ that are MHCII^{low} (black bar) and MHCII^{high} (white bar). **(E)** The proportion of the MHCII^{+} cells among HKL, K1L, K2L, K3L, SL and PBL that are neutrophils (blue region), B cells (red region) and other cells (green region).

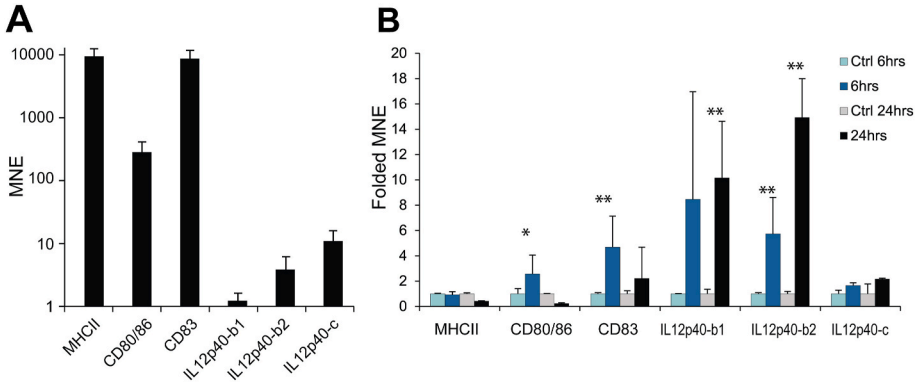

Fig. 5. **(A)** Basal expression of markers characteristic for antigen presenting cells and antigen presentation in isolated neutrophils. MNE = mean normalized expression using RPS20 as a reference gene. **(B)** Gene expression of isolated neutrophils 6 h (dark blue bars) and 24 h (black bars) after bacterial challenge. Light blue bars show non-challenged controls (Ctrl) 6 h post challenge, grey bars show non-challenged controls (Ctlr) 24 h post challenge. The results are shown as folded mean normalized expression (MNE) values using RPS20 as the reference gene and folded against the non-treated cells at each time points. The MNE-values of these controls are set to 1. Average ± S.D. is shown. Stars show significant change compared with the control (*$p < 0.05$, **$p < 0.01$).

is in accordance with previous analyses of B cells from other fish species (reviewed in (Sunyer, 2013)). IgM^+/IgD^+ B cells are the most common peripheral B cell subtype in mammals and in systemic lymphoid organs in fish (Sunyer, 2012). In the fraction of cells not bound by the B cell specific antibody, some cells were positive for IgD, but negative for both membrane bound and secretory IgM. Since IgM/IgD and IgT are expressed on two mutually exclusive B cell linages, this indicates that a small population of cells in PBL in Atlantic salmon express IgD solely. $IgM^-/IgD^+/IgT^-$ cells have been described in channel catfish (Edholm et al., 2010). In the unbound fraction depleted of the IgM^+/IgD^+ B cells, gene expression analyses showed the presence of IgT^+ B cells, T cells by TcRα and CD3 and myeloid cells by presence of FcεRIγ and MCSF. The presence of $IgT^+/IgM^-/IgD^-$ cells in PBL supports the findings of a B cell linage expressing only IgT also in Atlantic salmon. IgT in Atlantic salmon has been characterized by Tadiso, Lie, and Hordvik (2011), but functional analyses of IgT^+ B cells has not yet been described in Atlantic salmon. It is known from other fish species that IgT^+ B cells is the major B cell type in mucosal immunity (Abos et al., 2013; Parra, Reyes-Lopez, & Tort, 2015; Tacchi et al., 2014; Tongsri et al., 2020; Xu et al., 2013, 2016; Zhang et al., 2010; Zwollo, Cole, Bromage, & Kaattari, 2005).

To provide more information about subtypes of MHC class II^+ leukocytes in immune organs in Atlantic salmon under normal conditions and during bacterial challenge, isolated leukocytes were double strained with polyclonal rabbit antisera against salmon MHC class II beta chain and mAb E3D9 (specific for neutrophils) and MHC class II antiserum and mAb C7G7 (specific for B cells) and analyzed by flow cytometry and fluorescent microscopy. Immunostained cell preparations clearly showed the reactivity of the MHC class II antibody to leucocytes where MHCII positive B cells and neutrophils were shown by double staining.

In non-treated healthy salmon, the head kidney contained the lowest percentage neutrophils of total leukocytes (44%) compared to the other parts of the kidney. Among leukocytes isolated from the two most posterior parts, 60–68% were neutrophils, while about 20–25% among SL and PBL. This is in accordance with previous studies of neutrophils in salmon (Overland, Pettersen, Rønneseth, & Wergeland, 2010; Pettersen et al., 2003). In accordance with previous studies in salmonid fish (Supplemental Fig. in (Rønneseth, Ghebretnsae, Wergeland, & Haugland, 2015)), trout (Abos et al., 2013) and lumpfish (Rønneseth et al., 2015), B cells were most frequent among SL and PBL. All kidney sections contained B cells, from approximately 10% among HKL to 5% in leukocytes from the most posterior part (KL3). The anterior head kidney has been shown to be the site of B cell proliferation (Huttenhuis et al., 2005; Kibenge, Godoy, Fast, Workenhe, & Kibenge, 2012; Zwollo et al., 2005) and this can explain why B cells were most numerous in this part.

The frequencies of APCs, identified by specific antibody labelling of MHC class II^+ leukocytes among the total leukocytes in all analyzed tissues and peripheral blood in healthy (non-treated) fish, were similar to that of neutrophils (Fig. 4B and F). The positive cells grouped into $MHCII^{low}$ and $MHCII^{high}$ based on fluorescence intensity provided by cell surface binding of MHCII specific antibodies. The $MHCII^{high}$ proportion was present in SL and PBL and all kidney sections, up to 20% in K1, K2, K3 and lowest (5%) in HK, possibly reflecting that head kidney is a primary lymphoid organ in fish with high levels of immature cells.

When cell specific mAbs were combined with MHCII antiserum it clearly showed that a large proportion of neutrophils grouped into $MHCII^{high}$ among HKL, KLs, SL and PBL. In healthy fish, in the kidney sample termed KL1, $MHCII^{high}$ neutrophils constituted about 60% while lowest in HKL (about 10%). This might indicate that KL1 is a possible site for antigen presentation and for cells migrating to the more anterior HKL. For B cells the $MHCII^{high}$ fraction was highest in spleen and present in all leukocyte preparations of B cells analyzed, but lowest in HKL, where B cell development take place (Fillatreau et al., 2013). In fish the spleen is considered a secondary lymphoid organ where antigen presentation and activation of B cells takes place (reviwed in Castro et al., 2015; Kibenge et al., 2012) and the detection of high $MHCII^+$ B cells in spleen is therefore as expected.

Further, salmon neutrophils expressed markers characteristic for professional antigen presenting cells like MHCII, CD80/86, CD83 and expressed IL-12p40. Atlantic salmon has three different IL-12p40 termed p40a, p40b and p40c (Wang & Husain, 2014). It is now known that human and mouse neutrophils express MHCII and the costimulatory molecules CD80 and CD86 (Abi Abdallah et al., 2011; Culshaw et al., 2008). Neutrophils in humans also express the dendritic cell marker CD83 (Iking-Konert et al., 2002; Takashima & Yao, 2015) and T cell stimulatory behaviour (reviewed in (Oehler et al., 1998)). In the current study, we show that in salmon neutrophils stimulated *in vitro* with bacteria, these components were highly upregulated, at a transcriptional level, compared with non-stimulated cells. Although is seems to be generally accepted that neutrophils may have a role in adaptive immunity in mammals, the underlying mechanisms for antigen-presentation and T cell activation is largely unknown, as well as the regulatory effects of neutrophils on T and B cells (Li et al., 2019). Is it neither known if neutrophils in mammals have intrinsic ability for antigen presentation or if/how their antigen-presenting function is induced. One suggestion is that granulocytes acquire antigen-presenting cell feature via bystander activation from neighbouring profession APCs stimulating T cells (Lin et al., 2017).

It is well known that IL-12 is naturally produced by antigen presenting cells like DCs and macrophages and play an important role in activation of T cells. The high upregulation of the proinflammatory IL-12p40-b1 and -b2 by salmon neutrophils may indicate that they may have the ability to stimulate differentiation of naïve T cells into Th1 cells. Activation and proliferation of T cells is central for defining true APC. Without this compelling evidence for fish neutrophils yet, one could claim they are atypical APC as described by Kambayashi and Laufer (2014). However, the results obtained in this study of presence of hallmark molecules for APCs (MHC class II, CD80/86, CD83) combined with upregulation of IL-12p40, strongly support that salmon neutrophils can be considered as true APC. If fish neutrophils can be assumed to have dual properties of neutrophils and DC as described for murine neutrophils (Oehler et al., 1998; Takashima & Yao, 2015) and by production of IL-12 they might be important for protection during bacterial infections as well as stimulation of cellular immunity to virus infections. As Cuesta and co-authors (2006) also stated for granulocytes, more evidence should be provided about antigen presentation by neutrophils. The presented results on MHC class II^+ neutrophils in Atlantic salmon support that neutrophils might emerge from having a status of short-lived cells in innate immunity to key cells bridging innate and adaptive immunity.

5. Conclusion

In the current study we have shown that neutrophils in Atlantic salmon are MHC class II^+ cells secreting IL12p40 upon bacterial exposure. Our study provides novel insight into the repertoire of APCs in Atlantic salmon and make a solid basis for further elucidation of the neutrophils role in the immune system and the underlying mechanisms for antigen processing, antigen presentation and T cell activation. Such knowledge can also give insight into neutrophil functions in mammals and evolution of adaptive immunity in vertebrates. Furthermore, future functional studies of neutrophils in Atlantic salmon will unravel their potential as vaccine targets to shape tailored immune responses like dendritic cells in mammals.

Author contributions

GH, AR and HW planned the experiment, GH, AR, LG, HL and KN performed the experiment and the analyses. GH and HW wrote the initial draft of the manuscript. All co-authors contributed to proofreading and editing the manuscript.

Funding

Own funding

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Aknowlegdement

We want to thank Paul Løvik for technical help with the ELISA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aaf.2022.07.002.

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