 doi:10.1111/jfd.12058

Short Communication

Differential occurrence of immune cells in the primary and secondary vascular systems in rainbow trout, *Oncorhynchus mykiss* (Walbaum)

K J Rasmussen[1], J F Steffensen[2] and K Buchmann[3]

1 Department for Cancer and Inflammation Research, University of Southern Denmark, Odense, Denmark
2 Faculty of Science, Marine Biological Laboratory, University of Copenhagen, Copenhagen, Denmark
3 Section of Biomedicine, Faculty of Health and Medical sciences, University of Copenhagen, Frederiksberg, Denmark

Keywords: fish, immunity, rainbow trout, vascular system.

The vascular system in fish is divided into two components, the blood circulation and a secondary circulatory system, which is a vessel system referred to by earlier authors as 'lymphatics'. Also, Wardle (1971) used the latter term for his description of the secondary system. However, true lymphatics are defined solely anatomically (and not functionally) as a system of vessels without direct vascular connection to the arterial part of the blood circulation. Based on newer studies pointing to deviations from this picture in fish, the term secondary vascular system (SVS) was proposed for these special vessels, and accordingly, the normal blood circulation system was termed the primary vascular system (PVS) (Vogel 1985a, b). The SVS was described in some detail in ray-finned fishes (Actinopterygii) by Vogel & Claeviez (1981), Vogel (1985a,b), Steffensen & Vogel (1986) and Steffensen & Lomholt (1992) confirming that this system described thus far lacked the characteristic anatomy of lymphatics normally seen in other vertebrates, especially mammals. The latter authors showed that the SVS was in communication with the systemic arteries via a large number of anastomoses of capillary dimensions. The SVS forms capillary beds situated in the outer surfaces of the fish, such as the skin, gills, mouth and pharynx (Vogel 1985a,b), but appears to be absent in regions of the mesenteric and renal tissues, where the lymphatic system is normally found in mammals. In addition, red blood cells may, under certain circumstances, be present in the SVS, which was also shown by Wardle (1971) when describing lymphatic-like vessel systems in the plaice. Although this author interpreted this as an artefact (due to puncturing of vessels or abnormal blood pressure), the latter study does comply with the notion of the existence of a communication between PVS and SVS. These clear deviations from the structure in higher vertebrates advocate for a reassessment of whether the SVS has functions related to lymphatic functions in higher vertebrates – including immune-related elements – or not (Vogel 1985a,b; Steffensen & Lomholt 1992; Skov & Bennett 2004). As a first elucidation of this question, we have investigated whether the composition of immunologically central elements (various leucocytes) in SVS of rainbow trout, *Oncorhynchus mykiss* (Walbaum), differs from that of PVS.

Rainbow trout (total body weight 300–400 g), were collected from the fish farm 'Hvilested Dambrug', Kolding, Denmark, and brought to the laboratory where they were kept in 1500-L tanks and provided with continuously aerated recirculated fresh water (10 °C). Fish were acclimatized to laboratory conditions for 3 weeks, and remained unfed for at least 1 week before sampling to minimize physiological changes induced by differences in feed uptake. A total of five fish

Correspondence *K Buchmann, Section of Biomedicine, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark (e-mail: kub@sund.ku.dk)*

Journal of Fish Diseases

were killed by a sharp blow to the head, and fluid in the secondary system was collected according to the method of Maule *et al.* (1996) with minor modifications. A scalpel blade was used to make a shallow incision, perpendicular to and across the anterior end of the lateral line. From here, the SVS fluid could be extracted by inserting a heparin-coated PE-10 tube into the lateral cutaneous vessel that is dorsal of (and runs parallel to) the lateral line. The PE-10 tube was further joined with a small piece of PE-50 tube connected to a blunt-end cannula and syringe. Fluid could then be collected by applying a finger pressure along the lateral line from tail to head, together with the vacuum from the syringe. Fluid in the SVS was generally clear, indicating a low number of erythrocytes. Prolonged sampling gave fluid with red colour, possibly due to contamination of the blood, but this part was excluded from the investigation. Blood from the PVS was collected from the caudal vein using a heparin-coated syringe.

The quantification and distribution of cells (percentage erythrocytes and leucocytes) in the primary and secondary circulatory systems were estimated using a counting chamber (haemocytometer) in which a sample was added consisting 10 μL of the cell containing fluid (from PVS or SVS) mixed with 10 μL 0.4% Trypan blue (Invitrogen). Cells were counted in four selected areas, each subdivided into 16 squares. A total count exceeding 100 cells in each area was achieved.

Fluid samples withdrawn from the PVS and the SVS were centrifuged at 300 ***g*** for 10 min. Cells from the SVS were isolated by aspiration of the plasma, and the buffy coat was isolated from the PVS. The cells were then fixed for 1 h by adding 1 mL of 4% neutral formaldehyde to the cells. Then, the cells were carefully centrifuged at 100 ***g*** for 10 min and redissolved in TBS to achieve 20 000 cells mL^{-1}. A drop of 100 μL was applied to a microscope slide and allowed to air-dry overnight.

Fixed cells from the SVS and PVS applied on microscope slides were transferred to TBS for 5 min. Antigen retrieval was performed as described for immunohistochemical procedures (Olsen *et al.* 2011). The endogenous peroxidase activity was blocked with a preparation of 0.3% H_2O_2 in TBS for 5 min. The slides were then washed twice in TBS. Afterwards, the slides were washed twice and placed in blocking buffer (2% BSA in TBS) for 15 min. Monoclonal antibodies (Table 1) diluted in 1% BSA in TBS were added to each slide to identify lymphocytes (B cells reactive for IgM or IgT and T cells reactive for CD8) and antigen-presenting cells (positive for MHC II). Control slides with 1% BSA in TBS without primary antibody were also prepared. Incubation was carried out overnight at 4 °C, after which the slides were washed and incubated for 1 h at room temperature with secondary antibody EnVision+ System-HRP, labelled Polymer anti-mouse (DAKO). Slides were incubated with a carbazole solution (Chettri *et al.* 2012) for 20 min, and development of red colour signified binding of primary antibody to epitopes on cells. The reaction was stopped by washing sections for 5 min in tap water. Staining of the cell nucleus was carried out by counterstaining with Mayer's haematoxylin for 5 min. Finally, slides were washed in tap water and mounted in Aquatex (Merck). Blind test counting was performed on approximately 500 cells from each sample. Statistical analysis was performed using the Wilcoxon pairwise comparison test applying a probability level of 0.05.

The mean density of cells (erythrocytes and leucocytes) in PVS was estimated to be 8.7×10^8 cells mL^{-1}; the mean density of leucocytes in the PVS was 3.8×10^7 cells mL^{-1} giving a relative density of leucocytes of 4.8% ± 2.6%. The mean density of cells (all cells) in the SVS was estimated to be 4.8×10^6 cells mL^{-1}, and the leucocyte count was 3.1×10^6 cells mL^{-1} giving a relative density of 66% ± 20% (Table 2).

A significant difference in cell composition was found with regard to putative T cells (CD8-positive cells) (Fig. 1) and IgT-positive cells, with

Table 1 Monoclonal antibodies used for immunocytochemical characterization of cells in primary vascular system and secondary vascular system of rainbow trout. Reactivity, target cells and reference for characterization of monoclonal antibody

Antibody reactivity	Reference for production and characterization of antibody	Target cell
Rainbow trout IgT	Olsen *et al.* (2011) and Chettri *et al.* (2012)	IgT-positive B-lymphocytes
Rainbow trout IgM	Jørgensen *et al.* (2011)	IgM+B-lymphocytes
Rainbow trout CD8	Hetland *et al.* (2010), Olsen *et al.* (2011) and Chettri *et al.* (2012)	CD8-positive T-lymphocytes
Rainbow trout MHCII	Hetland *et al.* (2010) and Olsen *et al.* (2011)	MHCII-positive antigen-presenting cells

Table 2 Densities of all cells and leucocytes (cells mL^{-1}, mean and SD) and relative occurrence of different leucocytes (percentage in relation to all leucocytes, mean and SD) in rainbow trout PVS and SVS

	Cell density (all cells) Cells mL^{-1} Mean (SD)	Leucocyte density Cells mL^{-1} Mean (SD)	MHCII cells Relative occurrence % Mean (SD)	IgM cells Relative occurrence % Mean (SD)	IgT cells Relative occurrence % Mean (SD)	CD8 cells Relative occurrence % Mean (SD)
PVS	$8.7 \times 10^{8*}$ (4.2×10^{8})	$3.8 \times 10^{7*}$ (1.6×10^{7})	38% (21.1)	41.1% (18.7)	7.6% (2.3)*	3.7% (1.5)*
SVS	4.8×10^{6} (2.1×10^{6})	3.1×10^{6} (8.8×10^{5})	26.4% (17.0)	39.4% (10.8)	14% (1.0)	35.4% (5.1)

PVS, primary vascular system; SVS, secondary vascular system.
*PVS is significantly different compared to SVS, $P < 0.05$ (Wilcoxon test for pairwise comparison).

a higher relative occurrence in SVS. No difference with regard to MHC II and IgM-reactive cells (Fig. 2) was found although the occurrence of MHC II positive cells was slightly increased in PVS (Table 2).

Although the rainbow trout SVS does not represent a true lymphatic system, as defined by a clear anatomical separation from the arterial blood circulation (Vogel 1985a,b; Steffensen & Lomholt 1992), this work indicates that SVS has a number of characteristics that separate it functionally from the PVS, and it cannot be excluded that this gives SVS a special role during host immune responses. Thus, the relative composition of leucocytes including B and T lymphocytes differed markedly between these two vascular components in rainbow trout. Leucocytes in the PVS accounted for only 4.8% of all cells, but 66% in the SVS, reflecting that red blood cells are less prevalent in the SVS.

The frequency of PVS leucocytes reported here is consistent with other comparable studies ($4–8 \times 10^{7}$ cells mL^{-1}) performed on trout (Lowe-Jinde & Niimi 1986; Tillmann & Biron 2000). Due to previous lack of cellular markers for identification of the different cell types in fish, most of the differential counts performed by other fish physiologists have been based on cell morphology. Further, cell evaluation was mainly performed on cells derived from blood circulation, whereas only a few previous studies reported cell counts from the lymphatic ducts (SVS) (Wardle 1971; Lowe-Jinde & Niimi 1986; Tillmann & Biron 2000). These authors suggested that lymphatics in fish contain leucocytes in the same proportions as in serum, although in much smaller number. This contrasts with the data presented in this work and may be explained by the improved resolution and identification using monoclonal antibodies for immunocytochemistry as presented here. Accordingly, when we analysed the relative occurrence of specific cell types in relation to all leucocytes in

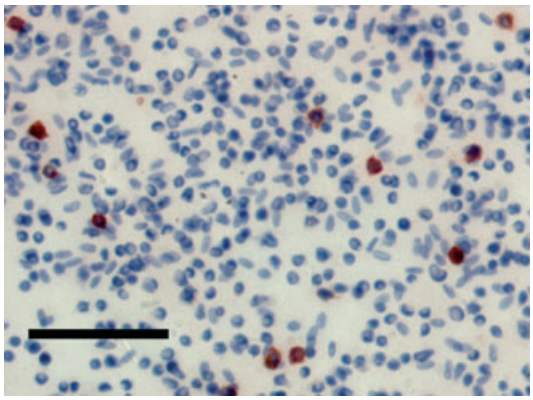

Figure 1 Micrograph showing cells from the primary vascular system of rainbow trout. Immunocytochemically stained cells are CD8-positive lymphocytes among numerous unstained erythrocytes. Scale bar: 100 μm.

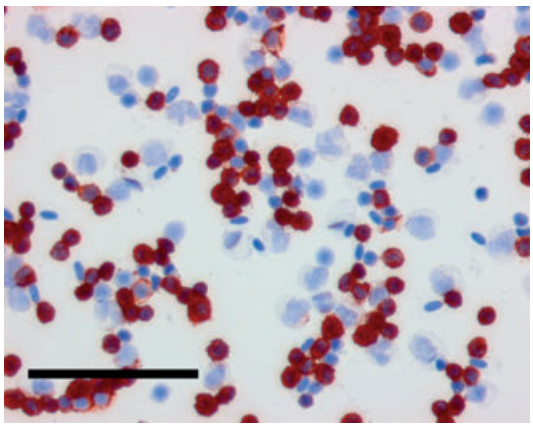

Figure 2 Micrograph showing cells from the secondary vascular system of rainbow trout. Stained cells are IgM-positive lymphocytes among unstained leucocytes and a few erythrocytes. Scale bar: 50 μm.

the different compartments, it was found that putative T cells positive for CD8 were more than nine times as prevalent in the SVS compared with the PVS. In addition, IgT-positive lymphocytes were almost twice as prevalent in the SVS. No major differences were found with regard to IgM-positive cells and the MHC II carrying cells were merely slightly more prevalent in the PVS. Previous MHC class II studies in teleost fish mainly focused on the DNA and RNA level (Randellia, Buonocore & Scapigliati 2008), and studies using phenotyping with MHC class II antibodies are few (Hetland *et al.* 2010; Olsen *et al.* 2011). The estimation of the relative density of IgM^+ B cells in the PVS and the SVS was 39–41%, corresponding to other studies made with mAbs against IgM, which stated that around 45% of the blood leucocytes in trout are IgM^+ B cells (Thuvander, Fossum & Lorenzen 1990). IgT^+ B cells in this study were estimated to be 8% in PVS and 14% in the SVS, which corresponds to the calculated 11% from previous work (Zhang *et al.* 2010). It has been estimated that the relative T-cell content in fish blood is 3% (Randellia *et al.* 2008), and our evaluation of $CD8^+$ T cells in the PVS indicated that 4% of the leucocytes were CD8 positive, as opposed to the SVS, where the prevalence was found to be 35% for T cells. The difference in the cell distribution for CD8-positive cells suggests that some selection of cells may occur before entering the SVS from the PVS.

The relatively high abundance of putative T cells and IgT-positive lymphocytes in the SVS, which is known to drain surface tissues of trout, suggests that the SVS could have a specific role to play in mucosal immunity. The immunoglobulin-type IgT has recently been designated as a functional equivalent to IgA found in mammals and birds. It occurs as a monomer in blood plasma, whereas it is present as a polymer in the gut mucus (Zhang *et al.* 2010). In rainbow trout, IgT-positive cells are prevalent in gill epithelia, and they have been suggested to play a protective role against the parasitic epithelium-invading ciliate *Ichthyophthirius multifiliis* (Olsen *et al.* 2011) and the bacterium *Yersinia ruckeri* (Chettri *et al.* 2012). Using different routes of immunization and challenge with these two pathogens, it has been indicated that trout has a communication between the systemic and mucosal protective systems (Jørgensen *et al.* 2008; 2011; Deshmukh *et al.* 2012). The higher abundance of IgT-positive cells and T lymphocytes in SVS (preferentially draining skin and gill epithelia) may therefore be one of the immunological elements of rainbow trout linking mucosal immunity to systemic immunity.

The differences between the PVS and SVS may be associated with and regulated by the special anatomical structure of the teleost circulatory system. The SVS originates from tiny irregularly curved vessels with a diameter of about 10–14 μm, branching from the primary arteries (Vogel 1985a,b). The small vessels immediately merge to form secondary arteries and are hence termed arterio-arterial anastomoses, as thoroughly described in long-finned eel, *Anguilla reinhardtii*, with 3–7-μm microvilli encircling the mouth of the anastomoses (Skov & Bennett 2004). It is assumed that the microvilli have a filtrating function, given that they have the ability to exclude erythrocytes. This will result in a positive selection for white blood cells, and this cell communication between PVS and SVS can be controlled by hypoxia, exercise, stress and catecholamines. Therefore, this study also suggests that environmental factors, which are known to affect the fish immune system (Bowden 2008), can at least partly influence host responses through influencing concentration and distribution of immune cells in SVS.

Acknowledgement

This work was in part supported by The Danish Council for Strategic Research through a grant to the Danish Fish Immunology Research Centre and Network (www.dafinet.dk).

References

Bowden T.J. (2008) Modulation of the immune system of fish by their environment. *Fish & Shellfish Immunology* **25**, 373–383.

Chettri J.K.C., Raida M.K.R., Kania P.W. & Buchmann K. (2012) Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*. *Developmental and Comparative Immunology* **36**, 463–474.

Deshmukh S., Raida M.K., Dalsgaard I., Chettri J.K., Kania P.W. & Buchmann K. (2012) Comparative protection of two different commerciasl vaccines against *Yersinia ruckeri* serotype O1 and biotype 2 in rainbow trout (*Oncorhynchus mykiss*). *Veterinary Immunology and Immunopathology* **145**, 379–385.

Hetland D.L., Jørgensen S.M., Skjødt K., Dale O.B., Falk K., Xu C. & Koppang E. (2010) *In situ* localization of major histocompatibility complex class I and class II and CD8 positive cells in infectious salmon anaemia virus (ISAV)-infected Atlantic salmon. *Fish & Shellfish Immunology* **28**, 30–39.

Jørgensen L.v.G., Nemli E., Heinecke R.D., Raida M.K. & Buchmann K. (2008) Immune-relevant genes expressed in rainbow trout following immunisation with live vaccine against *Ichthyophthirius multifiliis*. *Diseases of Aquatic Organisms* **80**, 189–197.

Jørgensen L.v.G., Heinecke R.D., Skjødt K., Rasmussen K.J. & Buchmann K. (2011) Experimental evidence for direct *in situ* binding of IgM and IgT to early trophonts of *Ichthyophthirius multifiliis* (Fouquet) in the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **34**, 749–755.

Lowe-Jinde L. & Niimi A.J. (1986) Hematological characteristics of rainbow trout,*Salmo gairdneri* (Richardson), in response to cadmium exposure. *Bulletin of Environmental Contamination and Toxicology* **37**, 375–381.

Maule A.G., Schrock R., Slater C., Fitzpatrick M.S. & Schreck C.B. (1996) Immune and endocrine responses of adult chinook salmon during freshwater immigration and sexual maturation. *Fish & Shellfish Immunology* **6**, 221–233.

Olsen M.M., Heinecke R.D., Skjdt K., Rasmussen K.J., Kania P. & Buchmann K. (2011) Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: molecular and immunohistochemical studies. *Fish & Shellfish Immunology* **30**, 859–869.

Randellia E., Buonocore F. & Scapigliati G. (2008) Cell markers and determinants in fish immunology. *Fish & Shellfish Immunology* **25**, 326–340.

Skov P.V. & Bennett M.B. (2004) Structural basis for control of secondary vessels in the long-finned eel *Anguilla reinhardtii*. *Journal of Experimental Biology* **207**, 3339–3348.

Steffensen J.F. & Lomholt J.P. (1992) The secondary vascular system. In: *The Cardiovascular System*, vol. XIIA (ed. by W.S. Hoar, D.J. Randall & A.P. Farrell), pp. 185–217. Academic Press, Inc. San Diego, CA, USA.

Steffensen J.F. & Vogel W.O.P. (1986) *In vivo* observations on a specialized microvasculature, the primary and secondary vessels in fishes. *Acta Zoologica* **67**, 193–200.

Thuvander A., Fossum C. & Lorenzen N. (1990) Monoclonal antibodies to salmonid immunoglobulin: characterization and applicability in immunoassays. *Developmental & Comparative Immunology* **14**, 415–423.

Tillmann J.B. & Biron M. (2000) Acute stress response in triploid rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). *Aquaculture* **184**, 167–176.

Vogel W.O.P. (1985a). Systemic vascular anastomoses, primary and secondary vessels in fish, and the phylogeny of lymphatics. In: *Cardiovascular Shunts* (ed. by K. Johansen & W.W. Burggren). Alfred Benzon Symposium 21, pp. 143–159. Munksgaard, Copenhagen, Denmark.

Vogel W.O.P. (1985b) The caudal heart of fish: not a lymph heart. *Acta Anatomica* **121**, 41–45.

Vogel W.O.P. & Claeviez M. (1981) Vascular specialization in fish, but no evidence for lymphatics. *Zeitschrift fur Naturforschung* **36**, 490–492.

Wardle C.S. (1971) New observations on the lymph system of the plaice *Pleuronectes platessa* and other teleosts. *Journal of the Marine Biological Association of the UK* **51**, 977–990.

Zhang Y.-A., Salinas I., Li J., Parra D., Bjork S., Xu Z., LaPatra S.E., Bartholomew J. & Sunyer J.O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology* **11**, 827–835.

Received: 25 September 2012
Revision received: 18 October 2012
Accepted: 19 October 2012

