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Comparative analyses of B cell populations in trout kidney and mouse bone marrow; establishing “B cell signatures”

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Abstract

This study aimed to identify the frequency and distribution of developing B cell populations in the kidney of the rainbow trout, using four molecular B cell markers that are highly conserved between species, including two transcription factors, Pax5 and EBF1, recombination activating gene RAG1, and the immunoglobulin heavy chain mu. Three distinct B cell stages were defined: early developing B cells (CLP, pro-B, and early pre-B cells), late developing B cell (late pre-B, immature B, and mature B cells), and IgM-secreting cells. Developmental stage-specific, combinatorial expression of Pax5, EBF1, RAG1 and immunoglobulin mu was determined in trout anterior kidney cells by flow cytometry. Trout staining patterns were compared to a well-defined primary immune tissue, mouse bone marrow, and using mouse surface markers B220 and CD43. A remarkable level of similarity was uncovered between the primary immune tissues of both species. Subsequent analysis of the entire trout kidney, divided into five contiguous segments K1-K5, revealed a complex pattern of early developing, late developing, and IgM-secreting B cells. Patterns in anterior kidney segment K1 were most similar to those of mouse bone marrow, while the most posterior part of the kidney, K5, had many IgM-secreting cells, but lacked early developing B cells. A potential second B lymphopoiesis site was uncovered in segment K4 of the kidney. The B cell patterns, or “B cell signatures” described here provide information on the relative abundance of distinct developing B cell populations in the trout kidney, and can be used in future studies on B cell development in other vertebrate species.

INTRODUCTION

The location of primary lymphoid tissues is quite varied among vertebrate species. In mammalian species, hematopoiesis initially takes place in the fetal liver and spleen, and after birth, moves to the bone marrow where it functions throughout life. Teleosts lack bone marrow and have alternative sites for lymphopoiesis. For example, rainbow trout use the anterior kidney as their main site for B lymphopoiesis (Irwin and Kaattari, 1986; Kaattari and Irwin, 1985; Murayama et al., 2006; Zapata and Cooper, 1990), while in zebrafish, the dorsal aorta is intimately associated with the kidney and together form “whole-kidney marrow” (Traver 2003).

Mammalian hematopoiesis has been studied extensively. The bone marrow environment is necessary and sufficient to generate all immune cells, including hematopoietic stem cells, lymphoid and myeloid progenitors, as well as their more differentiated immune cell

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descendants, with the exception of T cell development, which takes place in the thymus. Mature, surface-IgM expressing B cells are generated through the B lymphopoiesis pathway; earliest progenitors include the common lymphoid progenitor (CLP), followed by pro-B cell, pre-BI, large pre-BII, small pre-BII, and immature B cell stages (reviewed in Melchers and Kincade, 2004). Immature B cells leave the bone marrow for the spleen where they become fully mature B cells (Melchers and Kincade, 2004). Small populations of Ig-secreting cells, including plasmablasts (PB), pre-plasma cells (pre-PC), and plasma cells (PC), are also housed in the mammalian bone marrow (reviewed in Rajewski and Radbruch, 2004).

Very little is known about the presence and distribution of developing B cell populations in teleosts, as essential reagents for detection of developing B cell markers are not (yet) available. As an alternative approach, our lab uses highly conserved, B cell specific transcription factors as markers to explore B cell developmental and activation pathways in the rainbow trout.

A recent study from our lab used a combination of the B cell-specific transcription factor Paired box-5 (Pax5) and trout Ig heavy chain mu (HC mu)-specific antibodies to determine the frequency of mature B and IgM-secreting cells in two secondary immune sites of the rainbow trout: spleen and PBL (Zwollo et al., 2008). However, at that time it was not possible to dissect developing B cell populations in the trout's primary immune organ, the anterior kidney, as markers for the earliest stages of trout B cell development were not available.

This study aimed to identify the distribution of developing B cell populations in the kidney of the rainbow trout using other molecular markers that are highly conserved between species. A combination of 4 molecular markers was employed, in addition to Pax5 we employed Early B cell Factor (EBF1), the Recombination-Activating Gene (RAG1), and HC mu (Adams et al., 1992; Hagman et al., 1993; Riblet, 2004; Schatz et al., 1989). The B cell-specific transcription factor Pax5, which has been studied extensively in several species including mouse and trout, provides a reliable vertebrate B cell marker (Adams et al., 1992; Urbanek et al., 1994; Zwollo et al., 2005, 2008). Pax5 is expressed from pro-B through plasmablast stages, but is absent in plasma cells and non-B cells (Adams et al., 1992). The transcription factor EBF1 is highly expressed during CLP and pro-B cell stages but expression is reduced in pre-B and mature B cells and absent in the terminal differentiation stages (Northrup and Allman, 2008; Zandi et al., 2008). EBF1 has been identified in a number of species including mice, chicken, and clearnose skate (Anderson et al., 2004; Garcia-Dominguez et al., 2004; Northrup and Allman, 2008; Strausberg et al., 2003; Zandi et al., 2008), and encodes a highly conserved protein (Liberg, 2002). The lymphoid-specific recombinase RAG1 is highly expressed in mammalian CLP and pro-B/T cells, and is re-expressed briefly during the pre-BII stage (Igarashi et al., 2002; Kranhel and Schlissel, 2004; Liberg et al., 2002). The RAG1 gene has also been isolated from rainbow trout, and is expressed in surface-IgM negative thymic cells and anterior kidney, supportive of a role during early lymphopoiesis (Hansen and Kaattari, 1996). Murine and human cytoplasmic HC mu protein is first expressed during the large pre-BII cell stage (Hoffman et al., 2002), and expression is greatly increased during the B-terminal differentiation stages (plasmablast, pre-plasma cell, and plasma cell stages; Tarte et al., 2003). Several studies in rainbow trout using a trout-specific anti-HC mu antibody have already shown that surface IgM+ B cells and IgM-secreting cells are present in the anterior kidney (Bromage et al., 2004; Zwollo et al., 2005, 2008), but no data are available as yet on the frequency of B cell subsets in this tissue.

The combinatorial expression of Pax5, EBF1, RAG1 and HC mu in mouse BM cells and trout anterior kidney cells was determined using flow cytometry. Those patterns were then validated in mouse BM, using mouse surface markers B220 and CD43. The approach was then applied to the entire length of the trout kidney, and revealed complex, site-specific patterns of both developing and Ig-secreting B cells.

EXPERIMENTAL PROCEDURES

Animals and facilities

Outbred rainbow trout (*Onchorhynchus mykiss*) (8–10 inch, or 11–15 inch), were purchased from Casta Line Trout Farms (Goshen, VA), or were a gift from Dr. Steve Kaattari (The College of William and Mary). Fish were maintained in a 100-gallon tank with a recirculating system employing biologically filtered well water cooled to 12 C. Fish were fed dry, floating pellets (Aqua Max Grower 600). C57BL/6J mice were between 3–5 months of age, and were obtained from the Jackson Laboratories.

Tissue collection

Trout kidney cells were collected from kidney as described previously (Zwollo et al., 2005); the kidney was divided into five sections of 7 vertebrate lengths each, K1–K5, with K1 being the most anterior section, and K5 the most posterior (Zwollo et al., 2005). Hence, K1 is equivalent to head kidney or anterior kidney, but with a highly defined border. Mouse bone marrow cell suspensions were isolated from C57BL/6 mice by flushing the femurs and tibias with HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM Na₂HP0₄.2H₂O, and 20 mM Hepes at pH 7.05). Trout kidney tissues were collected in 5 mls sterile HBSS. Single cell suspensions for each tissue were obtained by repeated uptake and release through a 10 ml syringe followed by forcing cells through a 40 nm nylon cell strainer (Falcon; BD Biosciences). Cells were pelleted and resuspended in cold HBSS. Trout kidney cells were layered onto Histopaque 1077 cushions (Sigma Aldrich), and spun at 400 g at 4C for 30 min to remove erythrocytes. Next, trout or mouse cells were washed in 50 mls of HBSS, spun at 250 g for 10 min, and pellets resuspended in PBS (1.9 mM NaH₂P0₄.H₂O, 8.1 mM Na₂HP0₄.7H₂O, 137 mM NaCl, and 2.6 mM KCl, pH 7.4) containing 0.02% sodium azide.

Fixation and permeabilization of cells for flow cytometry

Fixing and permeabilization of cells has been described previously (Tarte et al., 2003). Briefly, cell suspensions were washed in PBS plus 0.02% sodium azide, and cells were fixed in 1% paraformaldehyde (10% stock, EM-grade; Electron Microscopy Sciences) in PBS on ice for 15 minutes, and then permeabilized in ice-cold 80% methanol and stored for at least 16 hours at –20C before use.

Antibodies

The polyclonal rabbit anti-mouse antibody Pax5 (ED-1) has been described previously (Zwollo et al., 1998). The polyclonal rat anti-mouse-CD43-PE IgG, rat anti-mouse CD11b-Alexa647 IgG, rabbit-anti-human RAG1 IgG (H300; recognizing aa 744-1043 of the human RAG protein), and rabbit-anti-human EBF IgG (H300; detecting aa 1-300 of EBF1, and reactive with mouse/human EBF2, 3, and 4) were purchased from Santa Cruz Biotech. Rat-anti-mouse IgM-APC was purchased from BD Biosciences, the rat-anti-mouse/human CD45R/B220-PE monoclonal (cl. RA3-6B2) antibody was from eBiosciences. The mouse-anti-trout HC mu (I-14) monoclonal was a gift from Dr. Greg Warr (DeLuca et al., 1983). Isotype control antibodies included rabbit IgG, goat IgG (Molecular probes), rat IgG, or mouse IgG (eBiosciences) conjugated to Alexa 555 or Alexa 647. For flow cytometric analyses, unlabeled antibodies (including Pax5, RAG1, EBF, and 1–14 antibodies) were conjugated (Alexa Fluor 555 and/or Alexa Fluor 647) using protein labeling kits according to manufacturer's instructions (Molecular Probes). Antibody aliquots were stored in 1% BSA at –20C.

Western blot analysis

For RAG1 analysis, cells were collected in aliquots of 1•10⁶ cells and whole-cell protein lysates were prepared by resuspending cells in 40µl of a sample buffer containing 5% 2-ME (Zwollo

et al., 2005). 10^6 cells were loaded per well. For EBF analysis, tissues were collected in RNeasy lysis buffer, homogenized in buffer RLT with 1% BME according to instructions (RNeasy, Qiagen), and 100 μ g of homogenized protein sample loaded per well. Proteins were separated by size using denaturing 12% SDS-PAGE gels, as described previously (Zwollo et al., 2005) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). Membranes were incubated in blocking solution of 5% dry milk in PBS for 1 h, followed by a 1 h incubation in blocking solution in the presence of primary antibody: RAG-1 (1.3 μ g/ml) or EBF (1 μ g/ml). Four 5 min washes in PBS were then followed by a 1 h incubation with a goat anti-rabbit IgG-HRP conjugate (0.1 μ g/ml; Zymed Laboratories) in blocking solution, and membranes were washed four more times in PBS and developed using a chemiluminescence kit (ECL-Plus; GE Healthcare Life Sciences).

Flow cytometric analysis

Fixed and permeabilized cells were removed from -20°C and washed in 1 ml of permeabilizing solution (BD perm wash in PBS, BD-Biosciences) containing 2% FBS and then resuspended in perm wash + 5% FBS at a concentration of 10^7 cells/ml followed by a 15 min incubation at 4°C with gentle shaking. Fluorescent Abs were added to the cell suspensions to a final antibody concentration of 0.5–2 μ g/ $0.5 \times (10)^6$ cells/50 μ l final volume, and cells incubated at 4°C for 90 min in the dark with gentle shaking. Next, cells were washed in 1 ml of perm wash + 2% FBS followed by a 10 min incubation in the dark, shaking, at 4°C . The wash and incubation was repeated once. Cells were spun and pellets resuspended in 200 μ l perm wash containing 2% FBS, and transferred to a 96-well polystyrene round bottom plate (Fisher) for immediate analysis. 50,000 events were acquired per sample using a BD FACSArray (BD Biosciences). Duplicate samples were analyzed for each experiment. Experiments were repeated a minimum of four times. Contour graphs were generated using WinMDI 2–8 (J.Trotter 1993–1998) software, and are shown as log algorithms with intervals of 50%. Means and standard errors were calculated for each experiment.

RESULTS

The anterior kidney (referred to as K1 herein) of the rainbow trout is its main site for B lymphopoiesis, and is believed to be the functional equivalent of mouse BM. The approach used here focused on four differentially expressed B cell markers that are highly conserved among vertebrate species: immunoglobulin HC μ , Pax5, RAG1, and EBF (Adams et al., 1992; Hagman et al., 1993; Riblet, 2004; Schatz et al., 1989). Expression patterns for the four markers are well defined in mammalian species, including the mouse. As shown in Table I, their combinatorial expression is unique for each of the following six developmental and activation stages: CLP, pro-B cell, pre-BI/large pre-BII, small pre-BII/immature B/mature B, PB/pre-PC, and PC.

Antibodies for detection of Pax5 and HC μ had already been shown to be effective in detecting trout Pax5 and trout HC μ protein in trout immune tissues, using both western blot (Zwollo et al., 2005) and flow cytometric analyses (Zwollo et al., 2008). Specificity of two additional antibodies, anti-EBF and anti-RAG1, was tested by western blot analysis. The anti-RAG1 antibody was directed against amino-acids 744–1043 of human RAG1, and has a 90% homology to trout RAG1 protein (Hansen and Kaattari, 1996; Schatz et al., 1989). The EBF antibody was generated against amino-acids 1–300 of the human EBF1 protein. This region includes both the highly conserved DNA binding domain and the highly conserved dimerization domain of EBF1. The human EBF1 aa sequence is almost identical in this 300 aa region with both chicken and skate EBF1: it has only 5 amino acid differences with skate (NCBI# AAL86576.1) and 7 amino acid differences with chicken EBF1 (NCBI# NP990083). Hence, the anti-human EBF1 antibody should recognize trout EBF1 with high specificity.

As shown in Figure 1, a 120–130 kD protein was detected in trout PBL, spleen, and K1 cell lysates using the RAG1 antibody, which is the expected size published for murine and trout RAG1 protein (Hansen and Kaattari, 1996; Schatz et al., 1989). Using the EBF antibody, an approximately 80 kD protein was readily detectable in K1 and PBL, in agreement with its reported size in mice, skate, and chicken (Anderson et al., 2004; Garcia-Dominguez et al., 2003; Northrup and Allman, 2008; Strausberg et al., 2003; Zandi et al., 2008). EBF levels were high in K1, as expected based on high expression in early lymphoid progenitors (Table I, Northrup and Allman, 2008; Zandi et al., 2008). Together, the western blot data show that the selected EBF and RAG1 antibodies detect their respective target proteins in trout immune tissues.

Next, trout K1 and mouse BM cells were fixed, permeabilized, and analyzed by flow cytometry. Comparison of cellular characteristics are shown in Figure 2A. As is clear, trout K1 and mouse BM showed a remarkable similarity in size (forward scatter; FSC) and complexity (side scatter; SSC) distribution of their lymphoid populations. The lymphoid populations were gated for all subsequent experiments (Fig. 2A). Both trout K1 and mouse BM samples revealed two major (low SSC) cell populations: small cells (low FSC), and large cells (high FSC), as shown in contour graphs in Figures 2B and 2C (left panels).

Next, expression patterns for Pax5, HCmu, EBF1, and RAG1 were compared between mouse BM and trout K1. The four antibodies were conjugated directly to fluorochrome Alexa555 or Alexa647. The same Pax5, RAG1 and EBF antibodies were used for both species, while for HCmu, species-specific trout or mouse antibodies were used. Comparisons between trout K1 and mouse BM initially focused on the relationship between cell size (FSC) and one marker. Using the anti-HCmu antibodies, it was found that trout K1 contained an average of 10.8% HCmu+/low FSC cells (Figure 2B). The means and SE of all samples are summarized in Table II. Mouse BM contained a remarkably similar frequency of HCmu+/low FSC cells (Figure 2C). Based on the expected expression patterns as shown in Table I, small, mu+ cells represent the sum of all small pre-BII and (im)mature B cells in each primary immune tissue. A second, minor population of larger size (high FSC), HCmu+ cells was detected in both trout K1 and mouse BM (averaging 2.5% and 4.2% respectively), which likely represents early pre-B cells which already express the HC mu chain (Hardy et al., 1991; Hoffman et al., 2002).

The majority of Pax5+ cells were small (low FSC) cells in both mouse and trout, and must represent small pre-BII or (im)mature B cells. We will refer to this cell population as “late developing B cells” (see Tables I and II). Although expression of Pax5 was similar between trout K1 and mouse BM, trout had more Pax5+ cells (mean values of 18.2% low FSC/Pax5+ cells for trout, compared to 13.4% in mouse BM, Figure 2 and Table II). A minor population of high FSC, Pax5+ cells was also present in both species (Figure 2), and likely represents pro-B, pre-BI, or large pre-BII cells

In mice, the transcription factor EBF1 is highly expressed in a number of early B cell progenitors, but expression is reduced in the later stages of development (Hardy et al., 1991; Kondo et al., 1997). As expected, EBF did not stain with small (low FSC), late developing B cells (Figure 2B, C), but stained a population of high FSC cells in both species, which should include CLPs, pro-B cells, and early pre-B cells. Mouse BM had significantly more FSC high/EBF+ cells as compared to trout K1 (Figure 2). Based on the high frequency of EBF+ cells, especially in mouse BM, cannot be ruled out that the EBF antibody also stained with certain non-immune proteins that are quite abundant in the bone marrow, including adipocytes, osteoblasts, chondrocytes, myocytes, stromal cells, and olfactory epithelium cells (reviewed in Liberg et al., 2002), and similar cell types may be present in the trout. In the absence of an EBF1-specific antibody, this could not be determined.

Mouse RAG1 is expressed in CLPs, pro-B, pre-BII cells and early developing T cells (Igarashi et al., 2002; Kranhel and Schlissel, 2002; Liberg et al., 2002). Data in Figure 2 show that trout K1 possessed a significantly lower frequency of FSC high, RAG1+ cells than mouse, with means and averages shown in Table I.

As negative controls, cells from trout K1 and mouse BM were incubated with isotype control antibodies (see methods), as shown in the right panels (C555, Figure 2, or C647, not shown), and as expected, only low levels of non-specific staining were detected. In conclusion, there appears to be a remarkable conservation of both early developing and late developing B cell populations, when comparing the primary immune tissues of mouse and trout. However, trout K1 appears to possess more *late* developing B cells, while mouse BM possesses more *early* developing B cells.

Next, to confirm that Pax5, EBF1, and RAG1 markers detect the actual B cell populations as predicted in Table I, important control experiments were performed in mouse BM. Two-color flow cytometry was performed using two well-defined cell surface markers for mouse: CD45R/B220, and CD43 (Coffman, 1982; Hardy et al., 1991). These markers are not available for trout. Figure 3A shows two-color staining patterns using the CD45R/B220 marker. B220 is expressed on all mouse B cells, including pro-B, pre-B, mature B, and activated B cells, but not plasma cells (PC) or non-B cells (Coffman, 1982; Hardy et al., 1991). A number of B220-positive populations were detected in mouse BM, with low FSC cells being most abundant (Figure 3A). The majority of EBF+ cells lacked B220, and such cells must include CLPs and other cell types that express EBF proteins. A small percentage of cells co-stained for B220 and EBF, and are most likely pro-B or early pre-B cells, as such cells are B220+. Staining of BM cells with both B220 and RAG1 showed similar results, with the majority of RAG1+ cells being B220-, and only few cells expressing both RAG1 and B220 (Figure 3A). In contrast, the majority of B220+ cells co-stained with Pax5, as expected, because both markers are expressed in late developing B cells but not plasma cells (Figure 3A; ref Coffman, 1982; Hardy et al., 1991).

CD43 is an early B cell marker in mice, and is expressed in large number of cells, including granulocytes, T cells, early developing B cells, (including CLPs, pro-B cells, and early pre-B cells) and PCs (Dragone et al., 1995; Hardy et al., 1991). Late developing B cells with low FSC do not express CD43 (Hardy et al., 1991). An average of 9.5% of the cells were found to be CD43-/HCmu+, confirming that HCmu-expressing cells have differentiated past the CD43+ early developing B cell stages (Figure 3B, middle panel). A similar pattern of mutually exclusive staining was seen when CD43 was used in combination with Pax5 (Figure 3B, bottom panel), with Pax5+ cells typically lacking CD43. The monocyte/macrophage marker CD11b detects early developing B cells in mouse BM (CLP and pro-B; ref. Bin-Wakkach et al., 2006; Kansas et al., 1990). As expected, the majority of CD43+ cells co-stained with the CD11b marker (Figure 3B, third panel). Together, this set of data confirmed that the 4 markers HCmu, Pax5, RAG1, and EBF1, had the expected cell staining patterns in mouse BM, when used in combination with mouse B cell surface markers B220 and CD43.

One goal of this study was to identify developing B cell populations in the rainbow trout K1 by using two-color combinations of the molecular markers HCmu, Pax5, EBF1 and RAG1. To this end, flow cytometric analysis was performed in trout K1 and patterns compared to those of the well-defined mouse BM. Figure 4A shows results from mouse BM cells. EBF and Pax5 staining yielded mostly single-stained cells (either EBF+/Pax5- or EBF-/Pax5+), with very few EBF+/Pax5+ cells. In contrast, HCmu and Pax5 antibodies mostly co-stained (Figure 4A), while a minor population of BM cells was also detected that stained with Pax5 but not HCmu. Lastly, as expected there was strong RAG1 and EBF co-staining; such cells must represent CLPs, proB, or early pre-B cells (Figure 4A). We will refer to this population of RAG1+/EBF

+ /high FSC cells, which comprise CLPs, pro-B cells and early pre-B cells, as “early developing B cells”.

Figure 4B shows the patterns for trout K1, using the same combinations of markers. As observed in mouse BM, a lack of co-staining was observed using EBF and Pax5 antibodies, while strong co-staining was seen using Pax5 and HCmu antibodies; the latter population must represent pre-B or (im)mature B cells. As was observed in the mouse BM, an additional population of Pax5+/HCmu– cells was detectable using Pax5 and HCmu, although it was more prominent in the trout (8.1% in trout K1, and 1.7% in mouse BM). Furthermore, the combination of RAG1 and EBF antibodies provided a strong marker for early B cell development, with strong co-staining in both species. Frequencies for the relevant staining patterns for both mouse and trout are summarized in Table II. In summary, the data show that the markers HCmu, Pax5, RAG1 and EBF1 can be used to obtain specific patterns for both early developing B cells and late developing B cell populations in the rainbow trout, and that the pattern for each set of markers is remarkably similar to that of mouse BM.

Next we compared the frequency of IgM-secreting plasmablast/pre-plasma cells (Pax5+/HCmu++) and plasma cells (Pax5–/HCmu++) cells in both species. IgM-secreting cells can readily be identified by flow cytometry using permeabilized cells, because of the high concentrations of cytoplasmic antibody (“HCmu++”) in such cells (compared to mature B cells). It has been established previously that, (presumably long-lived) Ig-secreting cells are stored in primary immune tissues of mouse (Rajewski and Radbruch, 2004) and trout (Bromage et al., 2004; Zwollo et al., 2005, 2008). As shown in Table II, both mouse and trout store PCs (Pax5–) and non-PC (Pax5+) cells in their primary immune organ, with trout K1 having more IgM+ plasma cells than mouse BM.

Once the distribution of developing B cell populations was established in trout K1, we wished to test our novel approach by addressing the following question: what is the distribution of (developing) B cell populations throughout the entire trout kidney? Previously, our group had hypothesized that the trout kidney has a “B cell maturation gradient”, in which K1 is the only site for B lymphopoiesis, while mature/activated B cell populations are found towards the posterior end of the kidney, with the posterior kidney (K5) containing many activated and/or Ig-secreting cells (Zwollo et al., 2005). This model could now be tested.

Trout kidney was divided into five sections (K1-K5) of 7 vertebrae lengths each (starting on the posterior side), as reported by us previously (Zwollo et al., 2005). The total number of lymphoid cells was highest for sections K1 and K4 ($4.3 \times (10)^7 \pm 1$, and $4.3 \times (10)^7 \pm 1.5$ respectively), with much lower cell numbers in K2 ($3.6 \times (10)^6 \pm 0.78$), K3 ($11.3 \times (10)^6 \pm 1.5$), and K5 ($7 \times (10)^6 \pm 2.8$). The cells were fixed and permeabilized as before, and two-color flow cytometry performed.

The presence of late developing B cell populations (pre-B, (im)mature B) and IgM+ plasma cells was determined using a combination of HC mu and Pax5 antibodies. The frequency of HCmu+/Pax5+ cells was highest in K1, decreased from K1 (K1) through K4, with K4 having the lowest frequency (3.4%), and an increase again in K5 (Figure 5). Values for K2 showed high variation between fish, and this was investigated further. Interestingly, the frequency of mu+/Pax5+ cells, but only in K2, was significantly higher when larger fish were used. To investigate this further, three large fish (14–20 inch; typically older fish, from different parents) were analyzed independently and showed an average frequency of 22.4% (± 2.3) HCmu+/Pax5+ cells, as compared to 10.1% (± 1.1) in all other fish (<10 inches; different parents). The frequencies of HCmu+/Pax5+ cells in kidney sections K1, K3, K4 or K5 were independent of the size of the fish. Using the same two markers, the frequency of IgM+ plasma cells (HCmu++/Pax5–) was determined for each section. K1 and K5 had the highest frequency of plasma

cells, and K3 the lowest (Figure 5). Together, the data show that both K1 and K5 possessed the highest frequency of *late* developing B cells as well as PCs.

Next, the frequency of *early* developing B cell cells in each section was determined based on the frequency of EBF+/RAG+ cells. As expected, K1 had the highest frequency of such early developing B cells (an average of 8.8%), while low frequencies of such cells were present in K2, K3, and K5 (Figure 6A). Unexpectedly, K4 possessed a significant number of early developing B (EBF+/RAG+) cells, with 4.8% of cells carrying this phenotype (Figure 6A). To further assess whether EBF+/RAG+ cells in K4 represent early developing (μ^-) B cells, the experiment was repeated using HCmu, focusing on the RAG1+/HCmu – population. A similar pattern was seen as was observed using EBF and RAG1, namely two areas rich in early developing B cells, K1 and K4, as shown in Figure 6B. Figure 7 illustrates a model of the distribution of the three main B cell populations in the rainbow trout kidney.

DISCUSSION

The kidney of the rainbow trout is a complex and poorly defined immune organ. Several lines of evidence have supported an essential role for the anterior kidney in hematopoiesis, including expression of RAG1, TdT, and Ikaros (Hansen, 1997; Hansen and Kaattari, 1996; Hansen et al., 1997). Here, we used a combination of 4 molecular markers to explore the frequency of early and late developing B cell populations in the trout kidney. To establish developmental parameters, anterior kidney cell populations were extensively compared with cell populations from mouse BM. Flow cytometric analysis revealed that anterior kidney and its functional equivalent, the mouse bone marrow, were quite similar in terms of their B cell populations. Both shared large, *early* developing B cells (CLPs, pro-B cells, early pre-B) and small, *late* developing B cells (late pre-B cells and (im)mature B cells). A third population of B cells was also shared by mouse BM and trout K1, the IgM-secreting cells (plasmablast, pre-plasma cells, and plasma cells).

The frequency of the three populations was fairly similar between the two species, hence is likely to be conserved among vertebrate species. For example, the frequency of late developing (μ^+) B cells (HCmu+/Pax5+/EBF–/RAG1–/low FSC) was between 10–12% for both species. The frequency for this cell population is in agreement with reported frequencies of IgM+ cells in other primary immune organs; mouse BM has an average of 10% (Coffman, 1982; Hardy et al., 1991) small FSC, μ^+ cells, while rainbow trout K1 has a total of 12.4% μ^+ cells (which includes both small and large cells (DeLuca et al., 1983), and similarly, Japanese flounder K1 has 10.3% μ^+ cells (Tokuda et al., 2000).

Trout K1 had higher frequencies of low FSC Pax5+ B cells when compared to mouse BM. In fact, about one third of the Pax5+ cells in K1 (approximately 8%) lacked IgM (Table II). Of interest is that a similar Pax5+/IgM– population was noted by our group, in an earlier study on trout spleen and PBL (Zwollo et al., 2008). In that study, IgM–/Pax5+ cells also comprised approximately one third of the total Pax5+ cells in PBL and spleen tissue (28% and 32% respectively), with the frequency dropping slowly upon LPS activation. It was proposed that this population either represented IgT/IgD expressing B cells, or developing B cells (Zwollo et al., 2008). From the study here, we can certainly rule out the possibility that such cells are *early* developing B cells, as they lack expression of EBF and RAG1, and are small (low FSC), not larger size (high FSC), cells, hence they fall in the “*late* developing B cell” phenotype. In addition to late pre-BII and immature B cells, this phenotype also includes mature B, B1 cells, or memory-like B cells. In the absence of additional markers, μ^- /Pax5+/low FSC cells remain elusive.

Our data suggest that trout stores a significant number of PCs in both K1 and K5 (an average of 1.3% of lymphoid cells for each site). In contrast, only 0.16% of the mouse BM lymphoid cells carried the $\mu^{++}/\text{Pax5-PC}$ phenotype. Reportedly, approximately 1% of mouse BM are PCs (Haajjman et al., 1977), but the majority of these (long-lived) Ig secretors are of the IgG class rather than IgM (Slifka et al., 1998), which explains the difference in IgM+ PC abundance between the species. Trout lack the IgG class, and IgM-secreting cells make up the majority of Ig-secreting cells in the trout (Irwin and Kaattari, 1986; Kaattari and Irwin, 1985; Zapata and Cooper, 1990; Zwollo et al., 2005, 2008). Hence it is quite striking that trout K1 contains a similar frequency of PCs as mouse BM, and confirms the important role for both the K1 and K5 segments of the trout kidney in (long-lived) humoral immune protection, as suggested previously (Bromage et al., 2004; Kaattari et al., 2005; Zwollo et al., 2005, 2008).

Very little is known about the presence of B cell populations outside of the K1, including the mid and posterior areas of the kidney. When five contiguous kidney sections, K1–K5, were compared in this study, K1 clearly had the highest frequency of all three B cell populations measured: early developing B cells, late developing B cells, and (together with K5), IgM-secreting plasma cells. Hence, of the five segments tested, K1 is functionally most similar to the mouse bone marrow, as it contains similar frequencies of developing B cell populations and also stores Ig-secreting cells.

Kidney section K2 had a similar profile as K1 but had fewer immune cells. However, K2 was an interesting area of the trout kidney because of an apparent age/size-dependent increase of late developing B cells. Based on the preliminary data reported here, we propose that when rainbow trout mature or age, the number of late developing B cells, but not early progenitors, increases in K2. We speculate that such cells may include (im)mature B cells, or possibly memory-like B cells (Akroosh and Kaattari, 1991) that are stored in this area.

Like K1, K5 had high frequencies of both late developing B and IgM+ plasma cells, with one important difference, namely the absence of *early* developing B cells. These observations agree with an earlier hypothesis we proposed that posterior kidney functions as a secondary immune organ, where B cells become activated and differentiate into Ig-secreting cells (Zwollo et al., 2005). From the studies here, we can conclude that K5 lacks early developing B cells such as CLPs and pro-B cells, hence does not support B lymphopoiesis. In the absence of B lymphopoiesis, the “late developing B cell” population detected in K5 could either be late pre-BII cells, or (activated) mature B or memory-like B cells, which also carry the $\text{Hcmu}^{+}/\text{Pax5}^{+}$ phenotype. However, no markers are currently available to distinguish between such cell types.

Lastly, kidney segment K4 revealed an interesting and unexpected pattern: the site contained a relatively high level of early B progenitor cells; at the same time, late developing (μ^{+}) B cells were rare in K4. We speculate that K4 plays an important role in generating early lymphoid progenitors, which then migrate to K5 to complete their maturation and become activated. This is supported by an earlier study (Zwollo et al., 2005) which showed that both K1 and K4 had the highest rates of cell proliferation in the trout kidney; primary immune organs typically contain a large number of proliferating progenitor cells (Milano et al., 1997). If K4 truly contains a second site for B lymphopoiesis, the question remains why the kidney would need two distinct sites? In this regard, it is noteworthy that the posterior area of the kidney, including K4, lacks corticosteroid-secreting steroidogenic cells while such cells are abundant in K1 (Milano et al., 1997). The intimate intra-adrenal interactions between hematopoietic cells and interrenal tissues presumably have direct effects on K1 immune cells (Milano et al., 1997). In humans and mice, increased corticosteroid levels have been linked to depletion of early (including CLPs and pro-B cells), but not late developing B cells ((im)mature B cells) through induction of apoptosis (Igarashi et al., 2005). Based on these observations, we hypothesize that K4 provides an environment that is more resistant to cortisol-induced inhibition of B

lymphopoiesis compared to K1. Future immunohistochemical studies are needed to verify the function of K4 in B lymphopoiesis in greater depth.

In summary, we have developed a system to determine the frequency of three distinct B cell populations: early developing B cells, late developing B cells, and IgM-secreting cells, in the trout kidney. Based on our data, future analyses on trout kidney would benefit from focusing on the whole kidney, instead of only K1, as many important immune processes may take place outside the K1 region.

Further, we propose the term “B cell signatures” to refer to the specific combinatorial patterns of expression of selected B cell markers of a given immune organ or tissue. The molecular B cell markers used here are highly conserved among vertebrate species, hence will provide essential tools for analyses in poorly defined vertebrate species. B cell signatures can serve to monitor B lymphopoiesis and disease progression, and should provide important tools to assess effects of environmental pollutants on immune development and function in teleost species.

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Figure 1. Expression levels of trout RAG-1 and EBF proteins using western blot analysis
For RAG-1 analysis, 1×10^6 cells per sample were loaded. For EBF analysis, 100 μ g per sample of protein lysate was loaded. Immune cells were freshly isolated from trout blood (PBL), spleen (SPL), and anterior kidney (K1). Molecular weight markers are shown on the right.

Figure 2. One-color flow cytometric analysis of trout anterior kidney and mouse bone marrow
A. Dot plots indicating the major cell populations in each tissue, and the gated lymphoid population based on size (FSC) and complexity (SSC). **B and C.** Contour graphs showing relationship between cell size (FSC) and expression of molecular B cell markers immunoglobulin mu (HC mu), Pax5, EBF, or RAG-1. A control antibody, an Alexa555-conjugated goat anti-rabbit IgG (C555), is shown in the right panel for each species. Percentages are mean values (see Table II). Arrows indicate two populations based on cell size: low FSC and high FSC. **B.** Trout K1 contour graphs (N=5). **C.** Mouse BM contour graphs (N=5).

Figure 3. Two-color flow cytometric analyses of mouse bone marrow cells using known mouse B cell markers in combination with molecular B cell markers

A. Contour graphs, using mouse cell surface marker B220 in combination with EBF, RAG, and Pax5. **B.** Contour graphs, using mouse cell surface marker CD43 in combination with HCmu, CD11b, and Pax5. Numbers are mean percent gated cells with SE in parentheses (N=3).

Figure 4. Qualitative comparison of mouse and trout expression patterns by two-color flow cytometry

Shown are contour graphs using combinations of two molecular B cell markers: EBF1 and RAG, Hcmu and Pax5, and EBF1 and Pax5. The relevant populations are boxed and labeled. Arrow indicates a Pax5⁺/mu⁻ population of cells (see text). Control antibodies C555 and C647 are shown on the right. **A.** Contours using mouse BM cells, **B.** Contours using trout K1 cells.

Figure 5. Frequency and distribution of late developing and IgM-secreting plasma cells in the trout kidney

Pax5 and HCmu antibodies were used in two-color flow cytometry. **A.** Contour graph of K1 to illustrate the relevant cell populations. Right graphs (B and C): Frequency of cells (as percent of total lymphoid cells) on the Y-axis; distribution in kidney segments K1–K5. Means and SE are shown. **B.** IgM+ plasma cells (N=5). **C.** Late developing B cells (N=5).

A. **B.**

Figure 6. Frequency and distribution of early developing B cells in the trout kidney

Expression patterns of EBF1 or RAG1 expressing cells, using flow cytometry. **A.** Using EBF and RAG1 as markers. Percent EBF+/RAG1+ cells in each segment is shown on the Y-axis; means and SE are indicated (N=3). **B.** Using RAG1 and HCmu as markers. Distribution of RAG1+/HCmu- cells. Means and SE as shown (N=4).

Figure 7.
Model illustrating the predicted distribution of B cell populations in the trout kidney.

Table I

	<div> <div>← <i>Early dev. B</i> →</div> <div>← <i>Late dev. B</i> →</div> <div>← <i>IgM-secreting</i> →</div> </div>					
	CLP	Pro-B	Pre-BI, large pre-BII	small pre-BII, (im)mat. B	Plasmablast, pre-PC	PC
Pax5	–	+	+	+	+	–
cytoHCmu	–	–	+	+	++	++
RAG1	+	+	+	–	–	–
EBF1	+	+	low	low	–	–
B220	–	+	+	+	+	–
CD43	+	+	–	–	–	+
FSC	high.	high	high	low	high	high

Table II

	Parameters	MOUSE BM	TROUT k1	Lymphoid cell stages

	<div>Parameters</div> <div>EBF1+/Pax5+</div> <div>RAG1+/FSC high</div> <div>RAG1+/EBF1+</div>	<div>MOUSE BM</div> <div>1.1 (1.2)</div> <div>22.2 (3.2)</div> <div>20.8 (3.4)</div>	<div>TROUT k1</div> <div>0.9 (1.0)</div> <div>9.1 (2.2)</div> <div>10.0 (1.4)</div>	<div>Lymphoid cell stages</div> <div>pro-B, pre-BI, large pre-BII</div> <div>CLP, pro-B/T, (pre-B/T)</div> <div>CLP, pro-B, pre-BI+II</div>
<div>Early dev. B</div>

Late dev. B		Parameters mu+/Pax5+	MOUSE BM 11.7 (1.6)	TROUT k1 10.1 (1.5)	Lymphoid cell stages Mu+ small pre-BII, (im) mat B
		HC mu+/FSC low	10.5 (1.1)	10.8 (1.5)	Mu+ small pre-BII, (im) mat B
IgM-secreting		Pax5+/FSC low	13.4 (1.2)	18.2 (2.4)	Small pre-BII, (im)mat B
		mu++/Pax5+	0.5 (0.3)	0.94 (0.1)	PB, pre-PC
		mu++/Pax5- HC mu++/FSC high	0.16 (0.1) 0.66 (0.2)	1.3 (0.5) 2.2 (0.4)	PC PB,pre-PC, PC