Published in final edited form as:

Dev Comp Immunol. 2008 ; 32(12): 1482–1496. doi:10.1016/j.dci.2008.06.008.

Molecular and cellular analysis of B cell populations in the rainbow trout using Pax5 and immunoglobulin markers

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Abstract

To date, the trout B cell is poorly defined, as many essential molecular markers are not yet available for this species. In mammalian systems, the transcription factor Pax5, expressed from pre-B through plasmablast stages, provides an important marker for B cell differentiation. In a previous study we showed that Pax5 is expressed in the trout. Here we identify trout B cell populations that vary in expression of Pax5, membrane and secreted Ig. Immune tissues were separated based on concentration of surface IgM, and analyzed by qPCR and flow cytometry. Results suggest that spleen and PBL contain mostly resting B cells which lack secreted Ig. While the great majority of splenic B cells become strongly activated upon LPS stimulation, PBLs do not. Additionally, anterior kidney contains both developing B and Ig-secreting B cell populations, but few resting, mature B cells. Lastly, posterior kidney contains multiple B cell populations in various states of activation. We conclude that trout immune tissues contain multiple, developmentally diverse and tissue-specific B cell populations as defined by their relative expression of Pax5, surface IgM, and secreted IgM.

Keywords

Pax5; B lymphocyte subsets; B cell development; B cell activation; flow cytometry; antibody-secreting cell

INTRODUCTION

Teleosts provide an interesting animal model for the immunologist, as they do not possess bone marrow or lymph node tissue (1). Teleosts including zebrafish and trout use the anterior kidney as a main site for B lymphogenesis throughout life (2–4). In zebrafish, the dorsal aorta is intimately associated with the kidney and together these tissues have been referred to as “whole-kidney marrow” (5). In trout, the anterior kidney is separate from the dorsal aorta. The trout anterior kidney has no renal function (it has no nephrons) and is made up of lymphoid tissue, interdigitated with adrenal-like tissue, while the posterior kidney possesses both renal and immune tissue (1). In a recent paper we introduced the hypothesis that the posterior kidney provides an environment capable of inducing B cell activation and differentiation into plasma cells (6).

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A striking characteristic of the anterior kidney in trout is the presence of antibody-secreting cells, even in the absence of antigenic challenge. This was first reported in the dab (*Limanda limanda*), and such cells were named "constitutive" antibody-secreting cells (ASCs; 7). Since, we have confirmed the presence of ASCs in the anterior kidney of the rainbow trout (6,8). ASCs may represent plasmablasts or plasma cells that are presumably harbored in the kidney in the absence of antigenic stimulation, as has been observed in mammalian bone marrow (9–11). Alternatively, such ASCs may represent populations of B1-like lymphocytes, which are typically characterized by low-level IgM secretion in the absence of infection or immunization (12,13).

The trout spleen functions as a major secondary immune organ, as in mammalian species. Mature B cells are abundant at this site, and Ig-secreting cells have been detected in LPS-activated cultures derived from splenic B cells (1–3,6,8). Trout PBLs contain mature B cell populations, while LPS-activation of such cells generates Ig-secreting cells (6,8). However, *ex vivo* induction of Ig-secreting cells in PBLs appears to be moderate (6), and the majority of LPS-induced PBLs lack plasma cell characteristics (6,8).

One approach to understanding B-cell differentiation is to examine stage-specific expression of B cell specific transcription factors (14–16). This approach has been fruitful in studies of mammalian B cell development (14–16). The molecular structure and function of developmentally expressed transcription factors shows a remarkable level of conservation between species because of the highly conserved nature of DNA binding domains. Hence, use of transcription factors as developmental markers in less well-studied species such as the rainbow trout is an attractive comparative approach, as antibodies to mammalian homologs can be utilized.

As a marker for the proposed studies, we have selected the B cell-specific transcription factor Pax5, which has been extensively studied by several groups including our own (17–22). Pax5 has been identified in both mammalian and non-mammalian species and contains a highly conserved DNA-binding domain named the paired box (17). Pax5 is expressed in mammalian cells of the B cell lineage as well as in adult testis and during early brain development (17). Within the B cell lineage, Pax5 is expressed from the pre-B cell through mature B stages, is downregulated during terminal differentiation and absent at the plasma cell stage (17,23,24).

During mammalian B cell activation, the reduction in Pax5 levels is at least partly caused by induction of the transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp1; 25–28). Blimp1 in turn shifts Ig expression from the membrane to the secreted form, leading to increases in secreted Ig expression in activated B cells with simultaneous reduction of membrane Ig (28). A third transcription factor, X-box-binding protein, Xbp-1, becomes de-repressed as a result of Pax5 downregulation, and has been shown to be necessary, together with Blimp1, to drive differentiation towards the plasma cell stage (29). The ratio of membrane to secreted Ig is a useful marker for activation: mature B cells have high ratios, activated B cells and plasmablasts lower ratios, and plasma cells lack membrane Ig while secreting the highest amounts of secreted Ig (30,31). In a previous paper we show that both Pax5 and Blimp1 proteins are detectable in trout B cells and plasmablasts, based on western blot analysis and mobility shift assays (6).

Immune tissues often contain complex and temporally diverse mixtures of immune cells, and as such, whole tissue analysis gives limited information on developmental and activation states of B cells. Hence, we developed a method that allows for cell enrichment through physical separation of trout B cell subsets based on their relative concentration of surface IgM. We hypothesized that Pax5 levels would differ in each subset. Mature B cells were expected to have high levels of both membrane Ig and Pax5, activated B cells and plasmablasts lower levels

of both markers, and plasma cells should lack both membrane-bound IgM and Pax5 (30,31). To enrich for such potential subsets, we used positive selection with anti-IgM antibodies and magnetic beads and measured Pax5, membrane IgM (memIgM), and secreted IgM (secIgM) transcripts in each fraction using quantitative PCR. We show that this approach selects for IgM⁺ cells with different levels of memIgM expression, and that in turn this is positively correlated with Pax5 expression.

To dissect the presence and tissue distribution of distinct B cell subsets further, we next developed a method that allowed for analysis of single cells using flow cytometric analysis. Our approach used three B cell markers: Pax5, total IgM, and secIgM. Data presented here show that these markers can help distinguish between resting B, activated B cells or plasmablasts, and plasma cells in trout immune tissues. This is the first report describing the activation stages of individual trout B cells using these markers.

MATERIALS AND METHODS

Animals and facilities

Rainbow trout (200–500 grams) were gifts from Dr. Steve Kaattari (Virginia Institute of Marine Science). Fish were maintained in 100 gallon-tanks with a recirculating system employing biologically-filtered well water. Fresh water exchange was approximately 10% per day. Water temperature was maintained at 12°C. Fish were fed dry floating pellets (Aqua Max Grower 600, Purina).

Cell lines

Murine B cell line 2PK3 (mature B) and plasmacytoma line MPC11 (plasma cell) were purchased through the ATCC. Cells were grown according to ATCC guidelines.

Tissue collection

Fresh cells were collected from blood, kidney, or spleen. Two sections of the kidney were isolated, anterior, and posterior kidney. Anterior kidney (AK) here is equivalent to K1, and posterior kidney (PK) to K5, as described previously (6). Blood was collected in heparinized tubes. Kidney and spleen tissues were collected in 5 mls sterile 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) and single cell suspensions 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).

To remove erythrocytes from tissues and blood, cells were resuspended in HBSS and layered onto Histopaque 1077 cushions (Sigma Aldrich) and spun at 500g at 4°C for 30–45 minutes. White blood cells were removed and washed in 50 mls of HBSS for cells to be cultured or 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 for all other purposes.

Generation of trout cDNA libraries and screenings

Single cell trout suspensions were obtained as described above from freshly isolated anterior kidney tissue, PBLs, or spleen, and used for the generation of trout cDNA libraries according to manufacturer's instructions using a ZAP-cDNA synthesis kit and ZAP cDNA Gigapack III Gold cloning kit (Stratagene). One anterior kidney library and one combined PBL/spleen library was made. The libraries were screened using dATP- α -³²P (GE Healthcare)-labeled full-length mouse *Pax-5a* cDNA sequence (specificity > 1 × (10)⁸ cpm/μg; ref. 21). DNA was labeled using a random-primed labeling kit (Roche). Positive plaques were analyzed in secondary screenings and a tertiary screening was performed to obtain single positives. Positive pBluescript phagemids were produced using ExAssist helper phage in SOLR bacterial strain

(Stratagene) and resulting pBluescript plasmids were grown in XL1-blue. Plasmid DNA was purified using a Qiagen miniprep (Qiagen Inc) and DNA sequencing performed at the Iowa State University DNA Sequence Facility. A 5'/3' RACE kit (Roche) was used according to manufacturer's instructions to obtain additional sequence.

Positive selection using magnetic beads

Histopaque-purified cells were resuspended in trout complete medium (TCM) medium (6) at 4°C. IgM⁺ cells were isolated using the EasySep magnetic bead separation system (Stem Cell technology Inc). First, cells were washed in EasySep medium (PBS + 2% FBS + 1 mM EDTA) and resuspended in EasySep medium to $(10)^8$ cells/ml. Positive selection was performed according to the manufacturer's instructions (Stemcell Technology Inc.) using the trout IgM-specific monoclonal antibody Warr's 1–14 (32) at a final concentration of 1 µg/ml with the following modifications. Cells were initially exposed to the EasySep magnet for 2 min. Non-selected cells were then poured into a fresh tube and re-exposed to the magnet for another 4 min. Non-selected cells were then re-exposed for 8 min. Non-selected cells from the 8 min incubation were labeled the Rest fraction. Great care was taken to prevent capping of the cells by keeping cells on ice throughout the procedure. Experiments were repeated a total of 5 times. Cells from each fraction were counted and either frozen in $5 \times (10)^5$ cell aliquots for RT-PCR or western blot analysis, or fixed in 10^7 cell aliquots in paraformaldehyde (see below) for flow cytometric analysis.

Membrane IgM levels for each fraction were measured via flow cytometry as follows: cells were washed twice in 1 ml Easy-Sep medium, resuspended at 10^7 cells per ml in Easy-Sep medium containing a goat-anti-mouse IgG-PE antibody (BD Biosciences) at 1 µg/ml and 5% normal goat serum (BD Biosciences), and incubated for 45 min at 4°C. Next, cells were washed twice in 1 ml Easy Sep medium and analyzed by flow cytometry. 50,000 events were acquired per sample using a BD FACSAArray (BD Biosciences) unless noted otherwise. Duplicate samples were analyzed for each experiment. Experiments were repeated a minimum of three times. Histograms were generated using WinMDI 2–8 (J.Trotter 1993–1998) software.

The rabbit polyclonal SecIg antibody (described below, at 1 µg/ml) was used in a standard EasySep approach (according to manufacturer's instructions) with two rounds of 5 min positive selection. The secondary antibody, a biotinylated goat-anti-rabbit IgG (BD Biosciences), was used at 1 µg/ml.

LPS-activation

Freshly isolated cells were cultured in trout culture medium (TCM) as described previously (33) at 18°C and in the presence of blood gas (10% CO₂, 10% O₂, 80% N₂). B lymphocytes were activated using the B-cell mitogen LPS (055:B5 from E.coli; Sigma) at 100 µg/ml. Activated cells in culture were fed every other day with one tenth of the culture volume of a 10x tissue culture cocktail (34) containing 500 µg/ml gentamycin, 10x essential aas, 10x non-essential aas, 70 mM L-glutamine, 70 mg/ml dextrose, 10x nucleosides, and 33% FBS. Cells were collected after 7 days.

Quantitative real-time PCR

RNA was prepared from fresh cells or frozen cell pellets using an RNAeasy kit (Qiagen Inc.) according to manufacturer's instructions. 500 ng of total RNA was used to make cDNA using the iscript kit (BioRad). Quantitative real-time PCR was performed using a SYBR Green PCR kit (BioRad) on a iCycler Multicolor Real-time Detection System (BioRad) under the following conditions: 30 sec at 94°C, 30 sec at 62°C, and 1 min at 72°C. Each RNA sample was measured in duplicate in two independent PCR-reactions, and samples from three different fish were collected for each data point. The primers were as follows: to amplify membrane Ig, tH.S and

tHm.AS were used, as described previously (6). To amplify secreted Ig, primer tH.S was used with primer tHs.AS2 (6). These two primer combinations amplify IgM sequences only. For Pax-5, primer qtPax.F (5'-ACGGAGATCGGATGTTCTCTG-3') was used with primer qtPax-R (5'-GATGCCGCGCTGTAGTAGTAC-3'). For tubulin, primer qtTub.F (5'-CTTCTTGATCTTCCACAGCTTTGG-3') was used with primer qtTub-R (CTGGATAGATGGCAAACCTCAAGC-3'). Expression of individual genes from each sample was normalized to relative expression of trout α -tubulin within the same experiment. Expression levels were calculated as fold-change relative to the highest C_T value for each of the 3 target genes (Pax5, tmemIg, and secIg), which were each set to 1, calculated using the $2^{-\Delta\Delta C_T}$ method (35).

Antibodies

The polyclonal anti-paired domain antibody ED-1 recognizes trout Pax5 and has been described previously (36). The polyclonal rabbit-antiserum SecIg generated for this study is predicted to be specific to trout IgM only (based on immunopeptide sequence). For immunizations, the peptide sequence WLVDDEPVERTSSSC, derived from the trout secreted IgM protein, was conjugated to keyhole limpet hemocyanin, and injected into rabbits. Resulting rabbit serum was protein-A purified. GenScript Corp, Piscataway, New Jersey, carried out peptide synthesis and antibody production of the SecIg antibody. The Warr's 1–14 monoclonal antibody is specific to trout IgM (32). Isotype control antibodies for trout cells were a rabbit IgG named OC-1, which recognizes a sequence on mouse Pax5 but not trout Pax5 (Fig 1A and ref. 21) and a monoclonal mouse IgG1, κ (eBiosciences), conjugated to Alexa 555 or Alexa 647. For flow experiments using mouse cell lines, a goat-anti-rabbit IgG conjugated to Alexa 647 or Alexa 555 (Molecular Probes/Invitrogen) were used as isotype controls. Immunofluorescently labeled antibodies were prepared using Alexa Fluor 555 or Alexa Fluor 647 protein labeling kits according to manufacturer's instructions (Molecular Probes) and frozen aliquots were stored at -80°C .

Western blot analysis

Trout serum samples were prepared for gel electrophoresis by resuspending diluted serum in 2x sample buffer containing 5% 2-BME (37). Proteins were separated by size using denaturing 10% SDS-PAGE gels, as described previously (6). The SecIg antibody was used at 1 $\mu\text{g/ml}$, and a goat-anti-rabbit IgG_HRP (Zymed) was used at 0.5 $\mu\text{g/ml}$. Detection was performed using ECL-Plus kit (Roche).

Fixation and permeabilization of cells for flow cytometry

Fixing and permeabilization of cells was done through modification of a method described by Imber-Marcille et al (38). Cell suspensions in PBS plus 0.02% sodium azide (PBS-SA) were resuspended in a freshly made solution of 1% ice-cold paraformaldehyde (10% stock, EM-grade; Electron Microscopy Sciences) in PBS. Cells were fixed on ice for 15 minutes, and pelleted in a clinical centrifuge. Supernatant was removed and cells resuspended in 1 ml cold PBS-SA, spun as before, and all but one cell pellet volume of the supernatant removed. Cells were then vortexed for 20 seconds or until they were fully in suspension. Next, while vortexing cells, 1 ml ice-cold 80% methanol (kept at -20°C freezer until just before use) was added drop wise. Cells were then incubated at -20°C for a minimum of 16 hours. Cells remained stable at -20°C for up to 6 weeks.

Flow cytometric analysis

Cells were removed from -20°C and 1 ml of ice-cold PBS-SA was added. Cells were pelleted and washed in 1 ml of permeabilizing solution (BD perm wash, BD-Biosciences) containing 2% FBS. Cell pellets were resuspended in perm wash + 5% FBS to 10^7 cells/ml and incubated

for 15 minutes on ice with gentle shaking. Fluorescent Abs were freshly diluted to 10x Ab solutions (0.15 mg/ml) in perm wash containing 5% FBS. 5 ul of 10x Ab solution was then added to 45 ul of cell suspension for a final antibody concentration of 0.015 mg/ml. Cells were incubated with the conjugated antibodies at 4°C for 90 min in the dark with shaking. After the Ab incubation, 1 ml of perm wash + 2%FBS was added to each tube and cells incubated in the dark for 10 min, shaking, at 4°C. Cells were pelleted and resuspended in 1 ml of perm wash + 2% FBS, incubated for 10 min with shaking, and spun as before. All supernatant was removed, cells resuspended in 200 ul perm wash containing 2% FCS, and cells transferred to wells in a 96-well polystyrene round bottom plate (Fisher) for immediate analysis. 50,000 events were acquired per sample using a BD FACSArray (BD Biosciences), unless noted otherwise. Duplicate samples were analyzed for each experiment. Experiments were repeated a minimum of three times. Graphs and plots were generated using WinMDI 2–8 (J.Trotter 1993–1998) software.

Immunofluorescence

Following flow cytometry, fluorochrome-stained cells were counterstained using 300 nM DAPI (Sigma #D9542; prepared from 5mg/ml stock in dimethylformamide) in PBS. Cells were incubated for 10 min and a small volume was loaded onto clean microscope slides. Coverslips were mounted using 70% glycerol based-medium containing 2.5% DABCO (1,4-diazabicyclo [2.2.2]octane; Sigma #D2522) anti-fade agent. Coverslips were sealed with nail polish and slides were viewed on an Olympus BX51 fluorescent microscope equipped with a Q Imaging Retiga SRV CCD camera.

RESULTS

Trout Pax5 cDNA sequence

Two trout cDNA libraries (ZAP/cDNA) were constructed using a cDNA library kit: trout anterior kidney (AK) and trout PBL& spleen cDNA library. The libraries were screened with a full-length mouse *Pax5* cDNA probe. Two partial trout *Pax5*-positive clones were obtained after tertiary screenings and were used to rescreen the library to obtain full-length clones. Seven independent *Pax5* clones were isolated after tertiary screenings and cDNA sequences determined by the Iowa State DNA sequence facility. The final trout *Pax5* cDNA sequence has been submitted to GenBank (Accession number EU147491).

Figure 1 shows the predicted trout *Pax5* protein sequence in comparison with the mouse *Pax5* sequence (17). The trout *Pax5* protein has 79% overall similarity with mouse *Pax5*, the majority of differences being outside of the highly conserved paired domain (Figure 1). Three major differences were observed. First, a 25 aa in-frame sequence is absent from the homeodomain homology region of trout *Pax5* (Figure 1). Zebrafish (39) and pufferfish *Pax5* (40) also have this same deletion, while *Xenopus* and humans do not (17,41). Second, a 30 aa in-frame sequence is present in the C terminal transactivation/repression region of the trout, but is absent from mouse *Pax5* (Figure 1). This in-frame addition is also found in zebra fish and puffer fish but not in human or *Xenopus* *Pax5* (17,39–41). Third, two cysteine residues are present in the *Pax5* transactivation domain of trout (Figure 1, boxed aas) but not mouse; one residue is unique to trout (aa 281) whereas the other (aas 355) is shared with pufferfish but absent from zebrafish, mouse, human, or *Xenopus* (17,39–41). The functional significance of these species-specific differences is unclear at this time.

Isolation of B cell subsets based on relative concentration of surface IgM

In order to explore the presence and distribution of IgM-positive B cell subsets in trout immune tissues, we initially developed a method to isolate specific B cell populations based on their concentration of surface Ig. Mammalian mature B cells have the highest levels of membrane

Ig (memIgM) on their cell surface, whereas developing B cells and plasmablasts have lower levels (23,30,31). Plasma cells no longer express memIg (25,26). Given the conserved molecular pathways that direct vertebrate B cell development and activation, we assumed that trout B cell subsets would have a similar pattern of IgM expression.

Trout B cell populations were isolated using the well-characterized monoclonal anti-trout IgM antibody Warr's 1–14 (32) in positive selection experiments using magnetic beads (EasySep). Three B cell subsets were isolated: cells with highest concentration of memIgM move fastest to the magnet (StemCell Technology technical staff, personal communication) and hence, were removed first during the initial 2 minute exposure to the magnet. Non-selected cells were re-exposed for 4 minutes, cells collected, and non-selected cells again exposed for another 8 minutes.

Based on the mammalian system, we hypothesized that the "short exposure" fractions (2 min) contained B cells with the highest levels of memIgM and Pax5, and low levels of secreted IgM (secIgM). In contrast, the 8 min samples would be expected to select for cells with lowest levels of memIgM and Pax5, and the highest levels of secIgM, including activated B cells, plasmablasts or developing B cells, but not plasma cells. Non-selected cells after the 8 min exposure ("Rest" fraction) may contain pro- and pre-B cells, late plasmablasts and/or plasma cells, and all non-B cells. Both fresh, unstimulated tissues (D0), and cells 7 day-post *ex vivo* LPS-activation (D7), were analyzed.

A representative experiment with the frequency of cells in each fraction is shown in Figure 2. For all freshly isolated (unstimulated) tissues, the majority of IgM⁺ cells were in the 2 min fraction, and fewest cells in the 8 min fraction. To verify that each fraction differs in concentration of memIgM, cells were stained immediately after magnetic bead selection, and stained with a goat-anti-mouse-PE antibody followed by flow cytometric analysis. Results confirmed that positive selection using this approach enriches for cell subsets that differ in concentration of surface IgM (Figures 2B and 2C, and data not shown).

Unstimulated spleen had the lowest frequency of 8 min cells, with fewer than 2% of all cells being in this fraction, suggesting that only very few activated B cells reside in the spleen of healthy, non-challenged trout. Seven days after LPS activation, a shift towards more cells being in 4 and 8 min fractions (likely representing more activated B cells) was evident in both spleen and PK, supporting the hypothesis that both sites represent secondary immune organs containing B cells with activation potential.

Real-time PCR analysis

The mRNA levels of Pax5, memIg, and secIg were determined using real-time PCR. Because the contribution of plasma cells or other IgM⁺ B cells in Rest samples is unknown, our subsequent analyses focused only on the 2, 4, and 8 min fractions. Four sites were analyzed: spleen, PBL, AK, and PK.

The location of the Pax5 primers was designed to lie outside of the highly conserved paired domain, to minimize potential cross-reactivity with other Pax gene family members. To specifically target secreted forms of Ig HC RNA, a primer (tH.m.AS) unique to secIg RNA (within the CH4/C-terminal exons) was used. Similarly, a membrane-specific primer was designed to recognize sequence within the TM1/TM2 region, unique to the membrane form of Ig (tHs.AS2). Based on the primer sequences used, only IgM transcripts will be amplified. Figure 3 shows the real-time PCR results for the three target genes, for both freshly isolated (unstimulated) and seven day LPS-stimulated cells (D7).

In agreement with our hypothesis, we observed a consistent decrease in memIg RNA expression going from 2, to 4, to 8 min samples (Figure 3, top panels). All four tissues showed this pattern, for both D0 and D7 time points. This result confirmed that our method of positively selecting B cell subsets was valid.

Pax5 expression has been shown to drop during terminal differentiation of mammalian B cells (23,24). In agreement with this, our real-time PCR data showed that almost all samples expressed the highest levels of Pax5 RNA in the 2 min (highest surface Ig) samples, and the lowest levels in 8 min samples (lowest surface Ig, Fig 3; second row panels). Hence, decreased surface IgM correlates with decreased Pax5 and most likely, increased B cell activation. One exception was the unstimulated spleen, which expressed somewhat similar Pax5 levels in all IgM⁺ fractions. This result will be discussed below. After LPS activation (D7), all tissues showed the highest Pax5 expression in the 2 min samples, and lowest Pax5 expression in the 8 min samples. It should be noted that after LPS-activation, kidney samples showed a significant reduction in Pax5 expression in its 2 min fraction, correlating with the lower memIgM values that were detected for these tissues. Hence, the 2 min fraction of kidney samples had simultaneous, significant decreases in IgM and Pax5 expression after LPS-activation (Fig. 3). This result will be addressed in later experiments.

Somewhat unexpectedly, we observed that almost all the 2 min samples made more secIg RNA transcripts than 8 min samples, independent of stimulation status or tissue. Hence, in most cases, 2 min trout B cells, (which have the highest surface IgM for a particular tissue), also appear to make the highest overall levels of secIg transcripts.

SecIgM levels were almost undetectable for unstimulated PBLs, and very low for spleen (Figure 3, bottom panels). In contrast, unstimulated AK and PK expressed high levels of secIgM transcripts. After LPS activation, 2 min fractions from PBL, spleen and AK, but not PK showed significant increases in the level of secIgM transcripts. In addition, both AK and spleen had a significant increase in their 8 min fraction secIgM levels (Figure 3).

In summary, qPCR data suggest that overall there is a strong correlation between surface IgM and Pax5 expression levels. One exception was the spleen 8 min fraction, which showed high Pax5 and low memIgM. This will be addressed below. The distribution and levels of secIg expression are somewhat unexpected. This can be explained by the presence of multiple B cell subpopulations per fraction, which we describe in more detail below.

Immunodetection of trout secreted IgM

The Warr's antibody (1–14) recognizes both membrane and secreted forms of trout IgM (32). Prior to this study, no antibody specific to the secreted form of IgM was available for trout. Given its importance as a marker for B cell activation, a secreted IgM-specific polyclonal antibody (named "SecIg") that recognizes a sequence in the unique CH4/C-terminal regions (42,43) of secreted IgM only, was generated. The antibody should detect cytoplasmic secreted IgM, serum IgM, and receptor-bound IgM (if such receptors exist). Using western blot analysis, we showed that the SecIg Ab is able to recognize a 72 kD protein in trout serum (Figure 4A), which corresponds to the reported size of the secreted form of trout IgM (42).

The SecIg antibody was also tested using immunofluorescent co-localization experiments with splenic B cells that had been LPS-stimulated for 7 days. Results are shown in Figure 4B. The data support the specificity of the antibody: staining with SecIg produces very strong accumulations of signal inside the cell membrane, presumably staining the Golgi apparatus where the protein is being assembled, and a weak ring of staining along the membrane, presumably where secretion is taking place (Figure 4B, SecIg). Large cells showed bright staining with the SecIg antibody while lacking a Pax5 signal (Figure 4B, see arrow), suggesting

such cells represent plasma cells. Smaller secIg^+ cells had weaker secreted IgM fluorescence and weaker, but positive Pax-5 staining, suggesting they represent activated B cells or plasmablast-like cells (Figure 4B, SecIg and Pax5 panels). A composite consisting of SecIg, Pax5, and DAPI is shown for comparison (Figure 4B, third panel). Isotype controls did not result in any significant staining (Figure 4B, OC-555 and OC-647).

To rule out the possibility that the $\text{SecIg}^+/\text{Pax5}^-$ cells in Figure 4B represented non-B cells with FcR-bound, secreted IgM (rather than being plasma cells), a control experiment was performed in which unfixed 7 day LPS-activated splenic cell suspensions were incubated with the SecIg antibody, followed by a standard (5 min) positive selection step using the EasySep approach. Both selected and non-selected cells were then fixed and permeabilized and stained with anti-total IgM (Warr's) and anti-Pax5 (ED1) antibodies and analyzed by flow cytometry. SecIg-selected cells (e.g., cells that carry FcRs loaded with secreted IgM) would be expected to stain positive for total IgM, but negative for Pax5. The majority of non-SecIg-selected cells (all other cells) would be expected to stain positive for Pax5 (except plasma cells and non-B cells).

Results from this experiment (repeated twice) showed that less than 1% of the LPS-stimulated B cells were selected by the SecIg Ab (data not shown). Positive selection normally results in efficiencies ranging between 95–99% (StemCell Technology Inc.), hence very few of such cells must have been present in our samples. In addition, the great majority of “SecIg selected cells” stained positive with Pax5 (93.7% $\pm$ 3.1), and 91.5% ($\pm$ 2.0) stained positive for total IgM. In contrast, 61.5% ($\pm$ 0.5) of the non-selected cells were IgM^+ using Warr's, and 73% ($\pm$ 2.1) of non-selected cells stained positive for Pax5. Hence, we conclude that the frequency of $\text{FcR}^+/\text{secIgM}^+$ cells is very low (lower than 0.1% of the splenic B cell population) and that the $\text{Pax5}^-/\text{SecIg}^+$ cells observed using immunohistochemistry (Figure 4B) must represent true plasma cells.

One-color flow cytometry using Pax5

Our magnetic bead separation data showed that memIgM RNA levels correlated strongly with Pax5 RNA levels, and that overall levels of memIgM RNA decreased as surface IgM levels decreased. However, some of the data were unexpected, including low but significant secIg expression in memIgM^+ cells, and the low Pax5 levels seen in IgM^+ kidney B cells. Being aware that qPCR approaches have the limitation of measuring RNA levels in pooled cell populations, we proposed that some fractions may contain multiple, distinct B cell populations. To address this possibility, we developed a method to analyze the expression of Pax5 and IgM in individual cells, using flow cytometry.

Initially we tested our method using murine B cell lines with known levels of Pax5. As shown in Figure 5, we were able to specifically stain fixed and permeabilized mouse B cell lines using the anti-Pax5 antibody ED1 conjugated to fluorochrome Alexa-647 (Pax5-647). The Pax5^+ mature B cell line 2PK3 had a strong fluorescence peak that was well-separated from that of the Pax5^- plasmacytoma line MPC11. The difference in fluorescence is approximately 4–5 fold (Figure 5). As a control, we mixed equal numbers of 2PK3 and MPC11 cells and analyzed this sample in parallel (Figure 5, blue line). The mixed sample showed two peaks, each corresponding with one each from the 2PK3 and the MPC11 sample. An isotype control antibody (C-647) was used on the mixed cell sample (Fig 5, dotted line). Data using other Pax5^+ cell lines such as pre-B cell line PD31 and mature B cell line A20 gave similar results (data not shown).

Next we used one-color cytometry with the anti-Pax5 antibody to analyze the fractions from our positive selection experiments. Figure 6 depicts the results of these experiments in the form of contour graphs, showing the relationship between cell size (FSC) and Pax5 staining intensity

for each cell population. Figure 6A shows results using unstimulated PBL and spleen cells. The great majority of 2 and 4 min samples, both in PBL and spleen, are Pax5⁺ cells. PBL and spleen fractions had a similar, Pax5⁺ population (Figure 6A, arrow 1) and each tissue had a distinct, minor population: PBL had a small population of large, Pax5^{high} cells in its 2 min fraction (Figure 6A arrow 2). Spleen had a minor population of small cells (in 2 and 4 min fractions) that expressed a range of low to medium Pax5 (Fig 6A, arrow 3). Furthermore, the majority of 8 min cells from PBL and spleen were Pax5⁻, but this fraction was difficult to analyze by flow cytometry because so few cells were available (only between 2000 and 5,000 cells were counted for the 8 min fraction). Pax5 patterns following LPS stimulation (D7) were similar to those for unstimulated cells, both in PBL and spleen (results not shown).

Figure 6B shows Pax5 data for unstimulated samples from the kidney. AK had one, somewhat heterogeneous population for 2 and 4 min fractions, and very few cells (mostly Pax5⁺) in the 8 min sample. Posterior kidney had at least 3 populations of IgM⁺ cells. Of those, two populations were Pax5⁺ cells in the 2 min fraction of PK, one population with high Pax5 staining (higher than AK) and one with large variation in size, and lower Pax5 staining (Figure 6B, arrows 1 and 2A respectively). A third population, which was also present in 4 and 8 min samples, was Pax5⁻. The 4 min sample of PK had the higher Pax5⁺ expressing population seen at 2 min (Figure 6B, arrow 1) as well as a second population of larger cells with low Pax5 levels (Fig 6B, arrow 2B). The 8 min fraction had very few cells (less than 5000 were counted), the majority of which were Pax5⁻.

Compared to unstimulated samples, LPS-activation of AK and PK showed a dramatic increase in Pax5⁻/Pax5^{low} cells, although a Pax5⁺ population remains (Figure 6C). This correlates well with qPCR data, which revealed a strong drop in Pax5 levels after LPS activation in both tissues. Furthermore, instead of two Pax5⁺ populations as seen in the unstimulated IgM⁺ cells (2 and 4 min), LPS-stimulated PK fractions only had one (Fig 6C). Furthermore, the 8 min PK sample of D7 showed two distinct populations, one Pax5⁺ (Fig 6C, arrow 1), and one Pax5⁻.

One-color flow cytometric analysis of secreted Ig expression

Figure 7 shows flow cytometric analysis using our newly developed SecIg antibody on the fractions from our positive selection experiments. SecIg levels for unstimulated PBL and spleen were very low to undetectable (results not shown). In contrast, LPS stimulation (D7) of these tissues produced varying amounts of secIgM. PBL had one population of cells, most abundant in the 2 min fraction but also present in the 4 min fraction, with moderate levels of secIgM (Figure 7A). However, many PBLs in the 2 and 4 min fractions were secIgM⁻, suggesting that such cells are resting, (IgM⁺) B cells (Fig 7A).

The spleen showed a very different pattern: the majority of cells in 2 and 4 min fractions comprised a heterogeneous population of secIgM⁺ (activated) cells. Significantly, these cells showed a clear correlation between cell size and secIgM level, with larger cells (higher FSC) expressing more secIgM (Figure 7A), suggesting that the larger cells are more activated. This agrees with our immunofluorescence data in Figure 4. The 8 min fraction contained relatively few cells (between 5000–10,000 counted), and most of these were secIgM⁺ (Figure 7A).

Finally, Figure 7B shows the secIgM patterns for unstimulated AK and PK cells. Quantitative PCR suggested that unstimulated kidney cells made high levels of secIgM. Flow cytometric analysis of AK revealed that the majority of 2 min AK cells expressed low-intermediate levels of secIgM, while the 4 min fraction had a diverse population of cells, some of which made intermediate amounts of secIg, and some of which were secIgM⁻. The 8 min samples of the AK had very few cells (3000–5000 counted), but this fraction contained the highest secIgM producers for AK.

Significantly, PK also had abundant, moderately positive secIgM populations in all 3 selected fractions, with the 8 min sample containing cells with the highest levels of secIgM (Figure 7B, arrow 1), but also many secIgM⁻ cells. After LPS stimulation, 2 min fractions from AK showed an increase in secIgM levels, while PK had a shift towards more secIgM⁺ cells in its 8 min fraction (results not shown).

Two-color flow cytometric analysis of trout B cells

It should be emphasized that the positive selection methods used here are useful for revealing the existence of distinct cell populations, but they cannot accurately determine true cell frequencies of B cell populations in isolated tissues. Hence, our next goal was to determine the actual cell frequencies of various B cell populations in unfractionated tissues. Total trout immune cells were analyzed in two-color flow cytometry using combinations of Pax5, secIg, and anti-total IgM (Warr's) antibodies. Both unstimulated and LPS-stimulated cells from spleen and PBL were analyzed.

Table I summarizes these data, showing means and standard deviations for each value (n=3). Double staining for Pax5 and total IgM showed the following for unstimulated tissues: both PBL and spleen D0 contained similar frequencies of double-positive (Pax5⁺/total IgM⁺) cells (43.2 and 40.0% respectively). Following stimulation, this frequency remained unchanged for PBLs (40.0%), while that of spleen cells increased (to 62.8%). Cells that stained positive for both Pax5 and total IgM could either be resting or activated IgM⁺ B cells, or IgM-secreting plasmablasts, suggesting that spleen, but not PBL, has increases in resting or activated (or plasmablast) B cells of the IgM isotype after LPS activation.

Additionally, a population of Pax5⁺/total IgM⁻ cells was detectable in both spleen and PBL. This population was abundant both in unstimulated spleen (16.5%) and PBL cell samples (20.8%), but dropped for both tissues after LPS activation. Cells with the Pax5⁺/total IgM⁻ phenotype may include B cells or plasmablasts that express IgT or IgD (or a variant of IgM which is not recognized by the Warr's antibody). Less likely but formally possible, such cells may also represent developing B cells, which already express Pax5, but not yet IgM.

The frequency of antibody-secreting cells was measured using the SecIg antibody. Spleen showed a major increase in number of antibody-secreting cells after stimulation, as evident from the increased frequency of both secIg⁺/total IgM⁺ and secIg⁺/Pax5⁺ cells. Almost all splenic B cells were double-positive after LPS activation, either using SecIg and Warrs 1-14, or SecIg and Pax5 antibodies (62.9% and 71.0% respectively). However, very few IgM⁺ true plasma cells (secIg⁺/Pax5⁻) were detectable at this time in the spleen (1.9%). It is unclear what the secIgM⁺/total IgM⁻ cell population in D7 spleen (8.4%) represents. These cells did not stain with the Warrs antibody but are secreting IgM, as detected using the SecIg antibody. It is possible that they represent a subpopulation of cells that lack the B cell epitope recognized by the Warrs mAb.

PBLs did not become highly activated by LPS after 7 days, with very few cells becoming secIg⁺. However, a small but significant number of secIgM⁺/Pax5⁻ cells (4.7%), presumably IgM⁺ plasma cells, was detectable. Overall, most PBLs at this point were of the secIgM⁻/total IgM⁺ and secIgM⁻/Pax5⁺ phenotype, representing either resting mature B cells, or in case of secIgM⁻/Pax5⁺, mature or developing B cells.

Flow cytometric patterns for the kidney were much more complex, because of the presence of multiple Pax5 and secIg cell populations, and will be reported as part of a subsequent study.

DISCUSSION

Separation of B cell populations based on concentration of surface IgM reveals the presence of distinct B cell subsets in trout immune tissues

During mammalian terminal B cell differentiation, Pax5 expression is reduced as cells move towards the final differentiation stages (17,23,24) and is absent in plasma cells. This decrease is at least partly caused by induction of the transcriptional repressor Blimp1 during B cell activation (25–28). Blimp1 also shifts Ig expression from the membrane to the secreted form, leading to increases in secreted Ig expression in activated B cells with simultaneous reduction of membrane Ig (28). Hence, while mammalian mature B cells express high levels of membrane Ig, activated B cells and plasmablasts express lower levels; plasma cells lack membrane Ig altogether (30,31).

We used this information to explore the existence of distinct B cell populations in the rainbow trout. Expression patterns of membrane IgM, secreted IgM, and Pax5 were determined in physically separated B cell populations based on their differences in surface IgM. Both quantitative PCR analyses as well as flow cytometric analyses suggested that the positive selection approach is valid; we were able to enrich for B cell subsets with significantly different levels of memIgM. Fractions with the highest amounts of surface IgM per cell typically had the highest level of both memIgM and Pax5 expression. Furthermore, decreases in surface IgM expression correlated with decreased memIgM and Pax5 expression. Hence, trout B cells with high surface IgM most likely represent mature B cells, while cells with lower surface IgM represent cells that have moved further towards terminal differentiation, becoming Pax5^{low}, activated B cells or plasmablasts. However, for a hematopoietic tissue such as the AK, lower IgM levels could also indicate the presence of precursor B cells.

One-color flow cytometric analysis using Pax5, total IgM, and secIgM-specific antibodies revealed more complex patterns than detectable through qPCR, including the presence of multiple subpopulations with varying levels of expression for Pax5 and secIgM. Hence, flow cytometric analysis was necessary to obtain a more accurate picture in terms of when and where B cells are present and whether they are in resting or activated states. Below we discuss the main conclusions from this study.

Unstimulated splenic B cells are in resting state, whereas LPS is a strong inducer of splenic B cell activation *ex vivo*

Both flow cytometric analysis and qPCR suggest that freshly isolated splenic B cells are almost entirely in the resting state. qPCR data revealed very low secIgM levels in all fractions of IgM⁺ unstimulated spleen cells, while flow cytometric analyses confirmed a lack of activated, IgM-secreting cells in this tissue. We conclude that the spleen must be a site where mature B cells home to and might be stored. It is unclear if the trout spleen possesses memory B cells, which presumably have the Pax5⁺/secIgM⁻/memIg⁺ phenotype. We speculate that such cells, if they exist, most likely would be stored in the spleen, as they are in mammalian species (reviewed in ref 44), but this needs further investigation.

LPS activation is most dramatic in the spleen, as measured in culture 7 days after LPS activation. qPCR data support this activation, as reflected by an increase in secIgM transcripts. More specifically, LPS-stimulated B cells with the lowest levels of surface IgM (8 min) made the fewest memIgM and Pax5 transcript levels, and showed the most significant increase in secreted IgM expression after LPS activation.

Flow cytometric results from unfractionated spleen and PBL cells detected a significant Pax5⁺/total IgM⁻ population at D0, which was greatly reduced after LPS-stimulation. The origin of this population is unknown. Are these cells activated upon stimulation, or do they die

in culture? Are they IgT/IgD-positive cells? Or, alternatively but somewhat less likely, are they developing B cells? Future studies will need to address these questions.

Our LPS-activation data from spleen are in agreement with an earlier study from our lab (6), using Percoll-enriched ACSs: freshly isolated spleen had very few IgM-secreting cells, as measured using ELISPOT analysis. Such cells were found both in 50% Percoll layers (representing late plasmablasts and plasma cells) as well as the 60% layers (which include activated B cells and early plasmablasts). Furthermore, Percoll analysis of purified resting splenic B cells (70% Percoll B cells) which had been LPS-activated in culture for 7 days showed that almost all resting B cells had become activated, being either in the 60% or 50% layers (6).

Our data consistently indicate that trout splenic B cells do not fully differentiate into plasma cells during the 7-day culture period after LPS stimulation. Both qPCR and flow cytometric data support this finding: the majority of IgM⁺ cells secrete IgM, while the majority of IgM-secreting cells express Pax5, suggesting that such cells are not (yet) plasma cells. Hence, the B cell response in trout splenic B cells is much slower as compared to mouse spleen cells, where *ex vivo* activation with much lower doses of LPS (10 µg/ml in mouse versus 100 µg/ml in trout) leads to a much earlier formation of plasma cells, with approximately 30% of the cells being plasma cells in culture after 4 days (29). This may, at least in part, be related to the much lower temperature at which trout B cells are cultured as compared to mouse B cells (18°C versus 37°C). It is unknown at this time if, when, and how frequent trout splenic B cells fully differentiate into plasma cells. Future studies can now begin to address this question. This will be particularly important for vaccination studies, as plasma cells secrete much higher amounts of antibody as compared to plasmablasts (28), hence providing better immune protection.

LPS-activation of blood-derived B cells is slow

Quantitative PCR data show that freshly isolated PBLs lack secIg transcripts, while LPS causes only a very moderate increase in secIg RNA expression. Cytometric analysis of positively selected fractions indicate that, compared to spleen, PBL cells do not make as much secIg per cell, and that the majority of secIgM⁺ cells are also surface IgM⁺. Additionally, two-color cytometric analysis shows that the frequency of activated B cells is low both in unstimulated and stimulated samples. Together, these data suggest that PBLs are slow to activate in culture. It cannot be ruled out that many PBLs may undergo apoptosis in culture before full activation is achieved. In support of this hypothesis, we observed that many PBLs die during the initial days in culture, but that a population of Pax5⁺ B cells emerges over time to replace such cells (Zwollo and Rosata, unpublished observations).

Why is there such a difference in activation patterns between PBLs and splenic B cells? This may be caused by intrinsically different populations of B cells found in blood, which do not survive LPS-culturing, or may reflect the lack of proper "support cells" or cytokines in PBL cultures necessary for survival or efficient activation. Future studies using *in vivo* LPS immunizations as opposed to the *ex vivo* studies described here, will address this issue.

Anterior kidney contains developing B cells as well as Ig-secreting cells, but few resting, mature B cells

Trout AK is a complex and poorly understood immune tissue. This tissue is considered the main site for lymphopoiesis in the trout while also housing ASCs (6–8). Both qPCR and one-color flow cytometric analysis of positively selected (memIgM⁺) fractions indicate that AK has an unusually low expression of Pax5 in 2 min fractions of both unstimulated and dramatically, LPS-stimulated cells, as compared to other tissues. It should be kept in mind that the majority of AK cells likely is memIgM⁺, and that memIgM⁺ cells probably represent

activated B cells which already have lowered their Pax5 expression en route to terminal differentiation. One-color flow-cytometry supports this hypothesis, as 2 min fractions from AK consist of one population of Pax^{intermediate} which has only secIg^{low} cells. In contrast to PBL and spleen, no Pax5^{high}/secIgM⁻ cell population was detectable in the 2 min fraction of AK. Such cells would represent mature, resting B cells. This is agreement with immunofluorescence studies, which show a lack of Pax5⁺/total IgM⁺ cells in unstimulated AK tissue sections (Zwollo and Haines, unpublished observations).

Overall, AK appears to possess a variety of B cell populations, some of which are memIgM⁺ and some of which are not. With so many distinct B cell populations present in the AK, it is almost easier to ask which cell populations are absent. There is at least one: no population was detected with the resting mature B cell phenotype (surface IgM^{high}/Pax5^{high}/secIg⁻, see above), suggesting that the AK harbors a variety of developing B cells and Ig-secreting B cell populations, but very few resting, mature B cells. We speculate that as trout B cells become mature, they leave the AK to either PK or the blood, and eventually home to the spleen. This pathway was proposed in our recent model described elsewhere (6).

Posterior kidney contains multiple B cell populations, each with distinct Pax5 and secIg levels

The positive selection data also revealed the unique nature of PK as an immune site. Interestingly, qPCR data suggested that unstimulated PK cells had the highest levels of secIg RNA of all four tissues, suggesting that PK contains the most activated B cell subset in the trout. Flow cytometric analysis using Pax5 staining showed that PK contains multiple B cell populations: 3–4 populations are detectable in the 2 min fraction, 3 in the 4 min fraction, and 2 populations at the 8 min fraction. SecIg patterns show that both secIg⁺ and secIg⁻ populations are present in most fractions. Another surprising finding from the qPCR experiments was that LPS-activated PK B cells had the steepest drop in Pax5 transcripts after LPS activation. Use of flow cytometry explains such patterns: after LPS-activation, there is a shift towards more Pax5⁻ populations, most significantly for the 2min fraction, which reduces overall Pax5 levels for positively selected fractions.

We conclude that in the PK, qPCR is of limited use in quantitatively measuring B cell activation states, because of the presence of multiple, unknown B cell subsets at this site. Additionally, our data show that PK is a complex, secondary immune tissue, that contains multiple populations of Ig-secreting B cells, but likely also contains resting mature B cells and possibly plasma cells. Hence, this study supports a model where the PK of the trout both harbors and generates IgM-secreting cells, and that IgM-secreting cells reside in non-infected, healthy animals, possibly providing long-term protection against pathogens.

Identification of antibody-secreting cells in the trout: plasmablasts, plasma cells, or B-1 cells?

Earlier studies (6,8) had shown that the trout spleen and AK contain populations of antibody-secreting cells (ACS) that secrete IgM in the absence of infection or immunization, yet are resistant to hydroxyurea, (a DNA replication inhibitor; ref. 8). At the time, it was proposed that such cells were (long-lived) plasma cells. A second ASC that has been detected in trout immune tissues is the continuously dividing plasmablast (8). A third type of ACS in the trout might be the B-1 cell (12,13). With our newly developed flow cytometric approach capable of single-cell analysis, we can now expand studies on the complex B cell populations that are present in immune tissues of the rainbow trout. Once additional developmental, activation, and proliferation markers have been tested, future studies should be able to reveal the frequency and distribution of such ASC populations in native animals and during antigenic challenge.

Increased understanding of the diverse forms of ASCs will undoubtedly be beneficial for future vaccine design and monitoring of fish health.

ACKNOWLEDGEMENTS

We thank Drs. Steve Kaattari and Erin Bromage for critical reading of the manuscript and for providing us with rainbow trout. We are grateful to Dr. Erin Bromage for help with design of the trout tank, and thank Tom Meier for building the trout tank system.

This research was funded through NIH-AREA grant # R15AI070249-01 and an award through the Jeffress Memorial Trust.

REFERENCES

1. Zapata, AG.; Cooper, EL. The Immune System. Comparative Histophysiology. Chichester, UK: John Wiley and Sons; 1990.
2. Kaattari SL, Irwin MJ. Salmonid spleen and kidney harbor populations of lymphocytes with different B cell repertoires. *Dev. Comp. Immunol* 1985;9:433–444. [PubMed: 3899761]
3. Irwin MJ, Kaattari SL. Salmoineid B lymphocytes demonstrate organ-dependent functional heterogeneity. *Vet. Immunol. Immunopath* 1986;12:39–45.
4. Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, Handin RI, Herbomel P. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity* 2006;25:963–975. [PubMed: 17157041]
5. Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon L. Transplantation and in vivo imaging of multi-lineage engraftment in zebrafish bloodless mutants. *Nature Immunology* 2003;4:1238–1246. [PubMed: 14608381]
6. Zwollo P, Cole S, Bromage E, Kaattari SL. B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. *J. Immunol* 2005;174:6608–6616. [PubMed: 15905499]
7. Davidson GA, Lin S-H, Secombes CJ, Ellis AE. Detection of specific and “constitutive” antibody secreting cells in the gills, head kidney, and peripheral blood leucocytes of dab (*Limanda limanda*). *Vet. Immunol. Immunopath* 1997;58:363–374.
8. Bromage ES, Kaattari IM, Zwollo P, Kaattari S. Plasmablast and plasma cell production and distribution in trout immune tissues. *J. Immunol* 2004;173:7317–7323. [PubMed: 15585855]
9. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-lived antibody response after acute viral infection. *J. Virol* 1995;69:1895–1902. [PubMed: 7853531]
10. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral Immunity Due to Long-Lived Plasma Cells. *Immunity* 1998;8:363–372. [PubMed: 9529153]
11. Manz RA, Thiel A, Radbruch A. Lifetime of Plasma Cells in the Bone Marrow. *Nature* 1997;388:133–134. [PubMed: 9217150]
12. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol* 2002;20:253–300. [PubMed: 11861604]
13. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The “Ly-1 B” cell population in normal immunodeficient, and autoimmune mice. *J. Exp. Med* 1983;157:202–218. [PubMed: 6600267]
14. Hagman J, Grosschedl R. Regulation of gene expression at early steps of B-cell differentiation. *Current Opinion in Immunol* 1994;6:222–230.
15. Dorshkind K. Transcriptional control points during lymphopoiesis. *Cell* 1994;79:751–753. [PubMed: 8001114]
16. Orkin SH. Transcription factors and hematopoietic development. *J. Biol. Chem* 1995;270:4955–4958. [PubMed: 7890597]
17. Adams B, Dörfler P, Aguzzi A, Kosmik Z, Urbánek P, Maurer-Fogy I, Busslinger M. Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev* 1992;6:1589–1607. [PubMed: 1516825]

18. Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 1994;79:901–912. [PubMed: 8001127]
19. Rolink AG, Schaniel C, Busslinger M, Nutt SL, Melchers F. Fidelity and infidelity in commitment to B-lymphocyte lineage development. *Immunol Rev* 2000;175:104–111. [PubMed: 10933595]
20. Nutt SL, Morrison AM, Dorfler P, Rolink A, Busslinger M. Identification of BSAP (Pax-5) target genes in early B-cell development by loss and gain-of-function experiments. *EMBO J* 1998;17:2319–2333. [PubMed: 9545244]
21. Zwollo P, Arrieta H, Ede K, Molinder K, Desiderio S, Pollock R. The Pax-5 gene is alternatively spliced during B-cell development. *J Biol. Chem* 1997;272:10160–10168. [PubMed: 9092562]
22. Lowen M, Scott G, Zwollo P. Functional analyses of two alternative isoforms of the transcription factor Pax-5. *J. Biol. Chem* 2001;276:42565–42574. [PubMed: 11535600]
23. Lee SC, Bottaro A, Insel RA. Activation of terminal differentiation by inhibition of histone acetylation. *Mol. Immunol* 2003;39:923–932. [PubMed: 12695118]
24. Henderson A, Calame K. Transcriptional regulation during B cell development. *Annu. Rev. Immunol* 1998;16:163–200. [PubMed: 9597128]
25. Angelin-Duclos C, Cattoretti G, Lin K, Calame K. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *J. Immunol* 2000;165:5462–5471. [PubMed: 11067898]
26. Shaffer AL, Lin K, Kuo T, Yu X, Hurt EM, Rosenwald A, Giltman JM, Yang L, Zhao H, Calame K, Staudt LM. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 2002;17:51–62. [PubMed: 12150891]
27. Savitsky D, Calame K. B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. *J. Exp. Med* 2006;203:2305–2314. [PubMed: 16954370]
28. Lin K-I, Angelin-Duclos C, Kuo TC, Calame K. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to IgM secreting plasma cells. *Mol. Cell. Bio* 2002;22:4771–4780. [PubMed: 12052884]
29. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, et al. XBPI, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* 2004;21:81–93. [PubMed: 15345222]
30. Tarte K, Zhan F, De Vos J, Klein B, Shaughnessy J Jr. Gene expression profiling of plasma cells and plasmablasts: toward a better understanding of the late stages of B-cell differentiation. *Blood* 2003;102:592–600. [PubMed: 12663452]
31. O'Connor B, Cascalho PM, Noelle RJ. Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. *J Exp Med* 2002;195:737–745. [PubMed: 11901199]
32. DeLuca D, Wilson M, Warr GW. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. *Eur J Immunol* 1983;13:546–551. [PubMed: 6347695]
33. Yui MA, Kaattari SL. *Vibrio anguillarum* antigen stimulates mitogenesis and polyclonal activation of salmonid lymphocytes. *Dev. Comp. Immunol* 1987;11:539–549. [PubMed: 3315760]
34. Tittle T, Rittenberg MB. Expression of IgG memory response in vitro to thymus-dependent and thymus-independent antigens. *Cell. Immunol* 1978;35:180–188. [PubMed: 563774]
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *N Methods* 2001;25:402–408.
36. Zwollo P, Rao S, Wallin JJ, Gackstetter ER, Koshland ME. The transcription factor NF-B/p50 interacts with the blk gene during B cell activation. *J. Biol. Chem* 1998;166:2617–2626.
37. Coligan, J.; Kruisbeek, A.; Margulies, D.; Shevach, E.; Strober, W., editors. *Current Protocols in Immunology*. Section 8.4.4. J. Wiley and Sons, Inc.;
38. Imbert-Marcille B-M, Robillard N, Poirier A-S, Coste-Burel M, Cantarovich D, Milpied N, Billaudel S. Development of a method for direct quantification of cytomegalovirus antigenemia by flow cytometry. *J. Clin. Microb* 1997;35:2665–2669.
39. Pfeffer PL, Gerster T, Lun K, Brand M, Busslinger M. Characterization of three novel members of the zebrafish Pax2/5/8 family: dependency of Pax5 and Pax8 expression on the Pax2.1 (noi) function. *Development* 1998;125:3063–3074. [PubMed: 9671580]

40. Ootani M, Miyadai T, Hiroishi S. Identification of genes encoding critical factors regulating B-cell terminal differentiation in torafugu (*Takifugu rubripes*). *Comp. Biochem. Physiol. Part D Genomics Proteomics* 2006;1:109–114.
41. Heller N, Brandli AW. *Xenopus Pax-2/5/8* orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. *Dev. Genet* 1999;24:208–219. [PubMed: 10322629]
42. Lee MA, Bengten E, Daggdelt A, Rytting AS, Pilstrom L. Characterization of rainbow trout cDNAs encoding a secreted and membranebound Ig heavy chain and the genomic intron upstream of the first constant region. *Mol. Immunol* 1993;30:641–648. [PubMed: 8487781]
43. Andersson E, Matsunaga T. Complete cDNA sequence of a rainbow trout IgM gene and evolution of vertebrate IgM constant regions. *Immunogenetics* 1993;38:243–250. [PubMed: 8319974]
44. Honjo, Tasuku; Alt, Frederick W.; Neuberger, Micheal, editors. *Molecular Biology of B cells*. Elsevier Academic Press; 2004. Chapter 14; p. 203-221.

Figure 1.

Trout Pax-5 predicted protein structure based on cDNA sequence (Genbank#EU147491). Dotted box: paired domain; diagonally striped box: octamer sequence; checkered boxed: homeodomain homology region; vertically striped box: transactivation domain. Open rectangles: positions of cysteine residues. Underlined aa sequence in mouse, but not trout Pax5: peptide recognized by isotype control OC-1.

A.

B

C.

Figure 2.

Magnetic bead separation of B cell subsets using the anti-IgM mAb 1–14 (Warrs). **A.** Relative cell numbers in 2 min, 4 min, 8 min, and Rest fractions after positive selection. Antibody-labeled cells were initially exposed to the magnet for 2 min. Non-selected cells were re-exposed to the magnet for another 4 min. Non-selected cells were then re-exposed for 8 min. Non-selected cells from the 8 min incubation were named the Rest fraction, and may contain T cells, red blood cells, thrombocytes, granulocytes, B cell precursors, and plasma cells. Dark squares, 2 min fractions; intermediate gray squares, 4 min fractions; white squares, 8 min fractions; light grey, Rest fractions. Mean values from three independent experiments. **B and C.** Histograms of flow cytometric analysis to measure membrane IgM concentration on fractions

after selection; LPS-activated PBLs on day 7 (**B.**) and LPS-activated spleen cells on day 7 (**C.**). Between 10,000 and 30,000 cells were acquired. B cell populations: 2: 2 min, 4: 4 min; 8: 8 min; R: rest fraction. B cell fractions were stained with a goat-anti-mouse IgG conjugated with PE, hence reflecting the amount of surface IgM per cell for each subset.

Figure 3.

Real-time PCR analysis on trout B cell fractions after positive selection with anti-IgM mAb 1–14 (Warrs). Expression of three target genes is shown: membrane Ig (memIg), Pax5, and secreted Ig (secIg). Expression is shown for four trout immune tissues: spleen (SPL), PBL, anterior kidney (AK), and posterior kidney (PK). Day 0 (D0) data are shown in graphs on the left, Day 7 (D7) on the right. The numbers 2, 4, and 8 represent the number of minutes cells were exposed to the magnet. Numbers shown on the Y-axis indicate fold change relative to the highest C_T value for each target gene. Values are the average of two duplicates in two independent PCR reactions $\pm$ S.D. from each fish, and from a total of three fish. Values were normalized using trout-specific α -tubulin sequence. Asterisks indicate a significant difference from the corresponding Day 0 sample ($P < 0.05$).

Figure 4.

Testing the specificity of the SecIg polyclonal antibody. **A.** Western blot using the SecIg antibody. Trout serum, 0, 0.1 or 1 ul of a 1:10 serum dilution. Protein size (in kD) shown on the right. **B.** Immunofluorescent staining using fixed and permeabilized trout spleen cells on D7. Top panel from left to right: SecIg, Pax-5, and composite with SecIg, Pax5, and DAPI. Lower panel: isotype control rabbit polyclonal OC-555 and OC-647. Arrow indicates an $\text{secIg}^+/\text{Pax5}^-$ plasma cell. Scale bar = 20 μm .

Figure 5.

One-color flow cytometry using polyclonal anti-Pax5 antibody ED-1 conjugated to Alexa-647 (Pax5-647) on fixed and permeabilized cells. Results from two mouse B cell lines: MPC11, Pax5⁻ plasmacytoma; 2PK3, Pax-5⁺ mature B cell line. Red filled: 2PK3; black line: MPC11. Blue line: cell suspension containing a 1:1 mixture of 2PK3 and MPC11 cells. Dotted line: cell suspension of mixed 2PK3 and MPC11 cell sample stained with isotype control goat-anti-rabbit IgG antibody labeled with Alexa 647.

A. D0 spleen and PBL; Pax5

B. D0 kidney; Pax5

C. D7 Kidney; Pax5

Figure 6.

Contour blots of one-color flow cytometry using Pax5-647 on positively selected, fixed and permeabilized, cell fractions. Contour lines are shown as log algorithms with intervals of 50%. Fractions (2 min, 4 min, and 8 min) are shown on the right. Isotype control OC-1 conjugated to Alexa 647 is shown for each 2 min fraction on the bottom two panels. For arrows refer to the text. Results from one representative experiment. Experiments were repeated twice. **A.** D0, spleen and PBL. **B.** D0, AK and PK. **C.** D7, AK and PK.

A. D7 spleen and PBL; secIg

B. D0 kidney; sec Ig **Figure 7.**

One-color flow cytometry using SecIg-Alexa 555 on positively selected, fixed and permeabilized, cell fractions. Contour lines are shown as log algorithms with intervals of 50%. Fractions (2 min, 4 min, and 8 min) are shown on the right. Isotype control OC-1 conjugated to Alexa 555 is shown for each 2 min fraction on the bottom two panels. For arrows refer to the text. Results from one representative experiment. Experiments were repeated twice. **A.** D7, PBL and spleen. **B.** D0, AK and PK.

Table 1

Pax5 / total IgM	+/+	+/-	-/+	-/-
	<i>resting IgM+ B activated IgM+ B IgM+ plasmablast</i>	<i>pre-B resting IgM- B activated IgM- B IgM- plasmablast</i>	<i>IgM+ plasma cell</i>	<i>IgM- plasma cell non-B</i>
PBL D0	43.2 (+/- 3.0)	20.8 (+/- 3.8)	0.4 (+/- 0.2)	35.5 (+/- 4.1)
PBL D7	40.7 (+/- 4.4)	9.3 (+/- 2.5)	1.0 (+/- 0.7)	49.0 +/- 5.6)
SPL D0	40.0 (+/- 3.7)	16.5 (+/- 0.8)	1.75 (+/- 0.5)	42.0 (+/- 3.6)
SPL D7	62.8 (+/- 1.4)	4.9 (+/- 1.2)	0.5 (+/- 0.2)	31.7 (+/- 2.1)
secIgM / total IgM	+/+	+/-	-/+	-/-
	<i>IgM+ activated B IgM+ plasmablast IgM+ plasma cell</i>	<i>Warrs- plasma cell?</i>	<i>resting IgM+ B</i>	<i>any IgM- B non-B</i>
PBL D0	0.3 (+/- 0.2)	1.8 (+/- 1.0)	43.6 (+/- 4.5)	54.3 (+/- 3.2)
PBL D7	0.7 (+/- 0.8)	3.7 (+/- 0.7)	42.1 (+/- 4.4)	53.5 (+/- 6.0)
SPL D0	1.5 (+/- 0.4)	1.1 (+/- 0.4)	40.1 (+/- 1.2)	56.6 (+/- 2.1)
SPL D7	62.9 (+/- 1.3)	8.4 (+/- 0.2)	0.2 (+/- 0.1)	28.5 (+/- 1.9)
secIgM / Pax5	+/+	+/-	-/+	-/-
	<i>activated IgM+ B IgM+ plasmablast</i>	<i>IgM+ plasma cell</i>	<i>pre-B any resting B</i>	<i>non-B</i>
PBL D0	4.4 (+/- 0.2)	1.5 (+/- 1.3)	61.7 (+/- 5.7)	32.4 (+/- 4.2)
PBL D7	1.3 (+/- 0.1)	4.7 (+/- 0.1)	42.6 (+/- 4.0)	51.2 (+/- 4.3)
SPL D0	2.1 (+/- 0.1)	2.1 (+/- 0.4)	51.3 (+/- 2.0)	44.4 (+/- 2.8)
SPL D7	71.0 (+/- 2.2)	1.9 (+/- 0.4)	0.5 (+/- 0.1)	26.6 (+/- 1.2)