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Defining terminally differentiating B cell populations in rainbow trout immune tissues using the transcription factor XbpI

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Abstract

The nature of antibody-secreting cells in the rainbow trout is poorly defined. Here we describe a flow cytometric approach to help differentiate between four major trout B cell subsets present during terminal B cell differentiation: resting B cells, activated B cells, plasmablasts, and plasma cells. To aid in the identification of B cell subsets, the LPS-inducible transcription factor XbpI-S was used as a marker. An antibody specific to the stable form of inducible transcription factor X-box protein I (XbpI) was generated, which detects XbpI-S protein expression for species within the *Oncorhynchus* genus, including rainbow trout. Combinatorial expression patterns, or B cell signatures, were established using antibodies to XbpI-S, Pax5, and IgM in combination with a proliferation marker. We show that XbpI-S induction in trout splenic B cells increases throughout a 10-day *in vitro* LPS induction period and that increased XbpI-S expression correlates with increased HCmu expression in the cell. PBLs displayed a lower level of XbpI-S induction during this incubation period, compared to spleen. We conclude that trout B cells follow a highly conserved B cell activation pathway, albeit slower than what has been observed in mammalian species. The use of XbpI-S as an activation marker for trout humoral immune activation promises to be useful for future *in vivo* studies, and can be applied to a broad range of teleost species.

INTRODUCTION

The humoral immune system of rainbow trout shares important characteristics with that of mammalian species, including a highly diverse antibody repertoire and the presence of a spleen [1]. During embryonic development, both trout and mammalian spleen harbor hematopoietic cells [2], while after birth, the spleen functions as a secondary immune organ where mature lymphocytes are activated by antigen and differentiate into antibody secreting cells (ASCs; [1, 3–6]). Typical germinal centers are absent in teleosts, but instead, melano-macrophage centers have been identified as potential candidates for germinal centers in fish [7]. In support of this hypothesis, Magor et. al reported that in catfish, areas surrounding melano-macrophage centers express immunoglobulin mutator activation-induced cytidine (AID), a marker for somatic hypermutation, and contain Ig-expressing cells [8].

The various stages of B cell differentiation and terminal differentiation are not well-defined in the teleost spleen. Although some important progress has been made [reviewed in 9],

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[§]Abbreviations: ASC: antibody-secreting cell. XBPI-S: X-box protein I-Stable.

including the use of Percoll gradients [5] and hydroxyurea [4] to identify proliferating cells, so far it has not been possible to unequivocally identify distinct terminally differentiating B cell populations in teleosts. Such populations likely include activated B cells, plasmablasts, and (short-lived and long-lived) plasma cells [5, 7–9]. Activated B cells presumably begin producing relatively small amounts of secreted IgM, and, like resting B cells, express membrane IgM and the transcription factor Pax5. Further down the terminal differentiation pathway are plasmablasts, which are larger, proliferating, membrane IgM-positive B cells that produce more secreted IgM. Finally, plasma cells secrete the highest amount of IgM, lack both membrane IgM and Pax5, and cease to proliferate [4, 5, 8, 9]. Short-lived plasma cells are formed in the spleen, while long-lived plasma cells reside in mammalian bone marrow, or the trout equivalent, the anterior kidney [4, 9].

Using density gradients, our group initially provided evidence [5] that the majority of trout B cells isolated from spleen and blood are mature B cells, while *in vitro* LPS-stimulation induces ASCs. Subsequently, we developed an approach to identify and quantify the frequency of IgM-secreting cells in LPS-induced trout spleen and PBL cultures using flow cytometry [6]. This approach was based on expression patterns of secreted IgM and Pax5 in individual B cells. We showed that on day 7 after LPS activation, spleen cultures consisted mainly of secreted IgM+, Pax5+ cells, while PBL cultures produced significantly fewer of such cells [6]. However, in that study it could not be determined whether the secreted IgM+ cells were activated B cells, plasmablasts, or plasma cells.

The first goal of this study was to develop an approach to differentiate between the major trout B cell subsets present during terminal B cell differentiation, namely resting mature B cells, activated B cells, plasmablasts, and plasma cells. Secondly, we wished to use this approach to elucidate the patterns of LPS-activated ASCs *in vitro*, in spleen and PBL. As part of these goals, an important new marker was developed here, namely the transcription factor X-box protein I (XbpI). The *XbpI* gene is a key regulator of the unfolded protein response (UPR) [10–12] and *XbpI* transcripts have been described for human, mice, zebrafish, *Xenopus*, and rainbow trout [reviewed in 13]. The *XbpI* gene is unusual, because in response to stimulation, 26 ribonucleotides are removed from an unstable form of the XbpI transcript (named Xbp-U), which results in a frame-shift and translation of a more stable transcription factor (XbpI-S) with a different C-terminus (Figure 1A), as shown for mouse, human, *Xenopus*, and zebrafish, [10–12]. Furthermore, while resting, mature B cells only make the XbpI-U form, antigenic stimulation leads to a rapid shift towards making the XbpI-S form. Hence, in all vertebrate species studied so far, terminally differentiating B cells induce expression of XbpI-S [10–14].

Here we show that combinatorial expression of XbpI-S, Pax5, and IgM, and a cell proliferation marker, provide novel tools that facilitate identification of distinct ASC populations in trout B cells. Furthermore, *in vitro* LPS-induction results in high XbpI-S induction in trout splenic B cells, while in PBL, induced XbpI-S levels are significantly lower.

MATERIALS AND METHODS

Animals and facilities

Outbred rainbow trout (200–500 grams) were gifts from Dr. Steve Kaattari (Virginia Institute of Marine Science). Fish were maintained in a 100-gallon tank with a re-circulating 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) once a day. C57BL/6J mice were between 3–5 months of age, and were obtained from the Jackson Laboratories.

Tissue collection

Rainbow trout were anesthetized with ethyl-3-aminobenzoate methanesulfate salt (Sigma) in accordance with our IACUC protocol. Blood was collected in heparinized tubes and spleen tissues in 5 ml sterile HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM Na₂HPO₄·2H₂O, and 20 mM Hepes at pH 7.05). Single cell spleen suspensions 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). Erythrocytes were removed using Histopaque 1077 cushions (Sigma Aldrich) as described elsewhere [5], and leukocytes were collected in HBSS or PBS (1.9 mM NaH₂PO₄·H₂O, 8.1 mM Na₂HPO₄·7H₂O, 137 mM NaCl, and 2.6 mM KCl, pH 7.4) containing 0.02% sodium azide (PBS-SA) for all other purposes. Spleen tissues from freshly wild-caught *Oncorhynchus nerka*, *Oncorhynchus gorbuscha* and *Oncorhynchus kisutch* were collected by local fishermen in Prince William Sound, Alaska, and samples stored in RNAlater until processing for western blot analyses. C57BL/6J spleen cells were prepared as described elsewhere [15].

LPS-activation

Freshly isolated trout cells were cultured in trout culture medium (TCM) at 18°C and in the presence of blood gas as described previously [5]. Trout cells were activated using lipopolysaccharide (LPS) (055:B5 from E.coli; Sigma) at 100 µg/ml. Trout cells in culture were fed every other day with one tenth of the culture volume of a 10× tissue culture cocktail [5] containing 500 µg/ml gentamycin, 10× essential aas, 10× non-essential aas, 70 mM L-glutamine, 70 mg/ml dextrose, 10× nucleosides, and 33% fetal bovine serum (FBS). Mouse spleen cells were cultured in complete RPMI medium as described previously [15] and activated using LPS (055:B5) at 10 µg/ml. Mouse cells were fed every other day with one culture-volume of complete RPMI. Cells were collected on the indicated days, and processed for flow cytometry or western blot analysis (see below).

RNA isolation and RT-PCR

Total cellular RNA was isolated from freshly isolated or LPS-induced trout spleen cells, using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was prepared using a cDNA kit (BioRad Inc.) according to manufacturer's instructions and using 1 µg RNA. Primers used for PCR analysis were as follows: sense primer tXbpI-F (5'-AGGGCAGGGTGTGGAGTCCAG-3') was predicted to anneal 65 nt. 5' from the 26 ribonucleotide splice gap, based on trout, mouse, *Xenopus* and zebrafish (XbpI-S and XbpI-U) DNA sequence alignment, and anti-sense primer tXbpI-R (5'-GCCTATCCCTATCCATTCTC-3'), predicted to anneal 56 nt 3' from the RNA splice gap (Nucleotide sequence alignment of mouse (Genbank accession No. 13775155), *Xenopus* (Genbank accession No.47575713), trout (Genbank accession No. BX075133), and zebrafish XbpI (Genbank accession No. AF42056 and AF42055, S and U forms respectively) cDNA). The resulting PCR-amplified fragment was expected to be 147 nts. for trout XbpI-U, and 121 nts for trout XbpI-S. Each reaction was denatured at 94°C for 30 sec, annealed at 55°C for 30 sec min, and extended at 72°C for 30 sec. The samples were amplified over 28 cycles. The PCR products were analyzed by gel electrophoresis using 2% agarose gels and detected using ethidium bromide staining.

Antibodies

Using GenScript software, a potentially highly antigenic 31 aa region at the C-terminus of XbpI-S (Figure 1C), was used to synthesize a peptide for immunization of rabbits (Genscript, Inc.). The resulting XbpI-S-specific antibody was generated by Genscript Inc. and named tXbpI-S. The polyclonal rabbit anti-Pax5 antibody (ED-1) has been described previously [16]. The mouse-anti-trout IgM (HCmu; I-14) monoclonal antibody was a gift

from Dr. Greg Warr [17]. For flow cytometric analyses, antibodies were conjugated to Alexa Fluor 555 or Alexa Fluor 647 using protein-labeling kits according to manufacturer's instructions (Molecular Probes). Isotype control antibodies included rabbit IgG (Molecular probes) or mouse IgG (eBiosciences) conjugated to Alexa 555 or Alexa 647. Antibody aliquots were stored in 1% BSA at -20°C .

Fixation, permeabilization, staining , and flow cytometry

Leukocyte suspensions in PBS plus 0.02% sodium azide were fixed and permeabilized as described previously [6]. To detect membrane IgM on B cells, live, unfixed leukocytes were pelleted at 4°C . The cells were washed once in Staining Buffer (PBS+ 2% FBS + 1 mM EDTA) and pellets resuspended in the same buffer. Mouse anti-trout IgM antibody (1–14), conjugated to Alexa555 (Molecular probes Inc.) was then added to the cells at 1 $\mu\text{g}/\text{ml}$. Cells were incubated for 30 min at 4°C protected from light. After the incubation, the cells were washed twice in 1 ml Staining Buffer and fixed and permeabilized as above.

Flow cytometric proliferation assay

To detect cell proliferation, trout or mouse cells were incubated in the presence of 30 μM of the nucleoside analog 5-ethynyl-2'-deoxyuridine (Edu; Click-iT, Invitrogen) during the last 16 (trout) or 2 (mouse) hours in culture, prior to cell collection. Trout cells were collected and fixed immediately (see above). Fixed cells were washed in 1ml perm wash (Invitrogen) containing 2% FBS. Cells were resuspended in Click-iT Reaction buffer (Invitrogen) according to the manufacturer's instructions and incubated for 30 min at 25°C protected from light. The cells were then washed twice in 1ml perm wash+ 2% FBS and resuspended in 200 μl perm wash+ 2% FBS. The cells were then immediately analyzed by flow cytometry (BD FACSAArray, BD-Biosciences).

Western Blot Analysis

Trout cell suspensions were centrifuged in aliquots of 0.5×10^6 cells and pellets were quick-frozen at -80°C . Whole-cell protein lysates were prepared by resuspending cells in 40 μl of a sample buffer containing 5% 2-ME [5], and proteins separated by size using denaturing 12% SDS-PAGE gels. Proteins were 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: ED-1 (Pax5 [16]; 1 $\mu\text{g}/\text{ml}$) or tXbpI-S (described below; 0.5 $\mu\text{g}/\text{ml}$). Four 5 min washes in PBS were then followed by a 1 h incubation with the secondary antibody goat anti-rabbit IgG-HRP conjugate (0.1 $\mu\text{g}/\text{ml}$; 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).

RESULTS

The first goal of this study was to develop an approach to differentiate between the major B cell subsets that are present during trout terminal B cell differentiation, including resting mature B cells, activated B cells, plasmablasts, and plasma cells. We hypothesized that the transcription factor XbpI would provide an excellent marker to measure the terminal differentiation state of trout B cells. Two XbpI protein forms are differentially expressed during vertebrate B cell differentiation, XbpI-U and XbpI-S. In response to B cell stimulation, 26 ribonucleotides are removed from an unstable form of the XbpI transcript (named Xbp-U), which results in a frame-shift and translation of a more stable transcription factor (named XbpI-S) with a different C-terminus [10–12] as shown in Figure 1B. Levels of both forms are low in resting B cells, but XbpI-S is strongly induced upon antigenic stimulation and remains highly expressed during terminal B cell differentiation [10, 11].

The presence of two XbpI forms had already been shown for mouse, human, zebrafish, and *Xenopus* [10–12], but not yet for rainbow trout. Hence we wished to verify that the stable form of XbpI (XbpI-S) is also inducible in trout. An RT-PCR approach was used, and trout-specific sense and anti-sense primers (tXbpI-S-F and tXbpI-S-R) were designed to flank the predicted splice site. Samples from freshly isolated spleen tissues (day 0) or stimulated by LPS for 24 hours, expressed very low to undetectable levels of XbpI-U and XbpI-S (Figure 1A). In contrast, LPS-stimulated “Day 7” splenic cultures (collected on day 7) expressed the induced XbpI-S RNA transcript, as reflected by the presence of an approximately 121 nt PCR-amplified fragment, as shown in Figure 1A. Together, these data support the presence of the XbpI-S transcript in activated, but not resting, trout B cells. XbpI-S antibody design was based on aa sequence that is unique to the XbpI-S form only. Figure 1C compares the 31 aa sequence in this region between species: although the region is highly conserved, several residues differ between trout and other species, with zebrafish XbpI-S being most similar [12].

Expression of XbpI-S in *Oncorhynchus* immune tissues

The tXbpI-S antibody was initially tested on two rainbow trout secondary immune tissues, spleen and PBLs, using western blot analysis. Although XbpI-S is also expressed in the anterior kidney, this tissue was not used in this study, because the presence of many, both developing and terminally differentiated, B cells in this primary immune organ was expected to complicate initial interpretation [15]. As shown in Figure 2A, low levels of an approximately 50 kD protein were detected in both tissues, which corresponds to the reported size for XbpI-S protein [10]. To test whether the tXbpI-S antibody could also detect XbpI-S of other *Oncorhynchus* species, spleen tissues from *O. nerka*, *O. kisutch*, and *O. gorbuscha* were similarly tested by western blot. As shown in Figure 2B, low levels of an approximately 50 kD protein, corresponding to the size of XbpI-S, are detectable in all three *Oncorhynchus* species. As a control, the same protein samples were also tested using the Pax5 antibody; as expected, Pax5 was detectable in all three species (Figure 2B, bottom figure).

Lastly, it was tested whether LPS-induced increases in XbpI-S protein, as shown in murine B cells [14], could also be detected using the tXbpI-S antibody in trout tissues. Freshly isolated trout spleen cells were activated in culture with LPS and cells were collected on days 0–6. Western blot analysis showed a gradual increase in XbpI-S expression within one day of stimulation and increasing significantly through day 6 (Figure 2C). As a control, the same cell lysates were also analyzed by western blot using the Pax5 antibody.

Flow cytometric analysis of XbpI-S protein expression

Next, the use of the tXbpI-S antibody was tested by one-color flow cytometry. The relative intensity of XbpI-S protein expression in individual trout B cells would be expected to increase following *in vitro* LPS activation. Cells collected from LPS-activated cultures were fixed and stained with the tXbpI-S antibody and analyzed by flow cytometry. Figure 3A shows a histogram (gated on all lymphocytes, see insert in Figure 3A) from LPS-activated spleen cells collected on days 0, 2, 4, and 8. Two peaks were observed on Day 0, an XbpI-S-negative peak (Peak 1) and an XbpI-S-staining peak (Peak 2). A third peak emerged by Day 2.

To determine the cell frequencies during LPS activation for each of the 3 cell populations, gates were set for each peak (shown as blue dotted lines in Figure 3A). Frequencies are listed on the right of Figure 3A. On day 2, the XbpI-S positive peak (Peak 2) had moved significantly toward increased XbpI-S staining intensity, although the frequencies of XbpI-negative and positive populations were not significantly different from those of day 0.

(Figure 3A). The increasing XbpI-S staining intensity trend continued on days 4 and 8 (Figure 3A). Two days after LPS-activation, a third population of high XbpI-S expressing cells emerged (Peak 3; XbpI+ cells). Upon LPS stimulation, a *decrease* in the frequency of low XbpI-expressing cells was observed: the frequency of Peak 2 (XbpI-S+/-) cells dropped from 48% on day 0 to 31% on day 8. There was a concomitant *increase* in frequency of high XbpI-S expressing cells (Peak 3, XbpI-S+) from 6% on day 0, to 19% by day 8.

Next, we attempted to determine relative XbpI-S expression values during LPS activation. This was done as follows: mean fluorescent intensity values for non-XbpI-S-expressing cells were obtained by gating the *non*-XbpI-S-staining cell population (using BDFACS ARRAY statistics software files) and values from that gate were defined as the “0% intensity” values. The maximum XbpI-S intensity value for each experiment was determined from the average fluorescent intensity of gated Population 1 (see Figure 4A), as discussed below, and values from that gate defined the “100% intensity” values. The main reason to set the maximum intensity value to 100% was to be able to compare the relative increases in XbpI-S between experiments and between tissues. To obtain the fluorescent intensity values for XbpI-S-expressing cells, *all* XbpI-S-staining cells (all lymphocytes except for the XbpI-S-negative cells) were gated for each sample, and the mean fluorescent intensity of the gated cells was then determined for each day as above.

Figure 3B shows the relative increase in XbpI-S intensity in splenic cells from the gated (XbpI-expressing) populations upon LPS activation. Figure 3C shows the same approach but using trout PBLs. Comparison of spleen and PBL induction patterns revealed two interesting differences: 1) XbpI-S levels in spleen cells increased continuously throughout the 10 day induction period in all 4 experiments. In contrast, XbpI-S expression in PBLs stopped increasing after the cells had been cultured for 4–6 days and remained low throughout the culture period, and 2) the initial increase in XbpI-S levels in PBLs varied considerably more between experiments, compared to spleen.

Identification of plasma cell and plasmablast populations

To facilitate distinguishing between resting mature B, activated B, plasmablasts, and plasma cells, the tXbpI-S antibody was used in combination with the anti-HCmu antibody in two-color flow cytometry. Because fixed and permeabilized cells were used, both cytoplasmic and membrane IgM were stained, hence, the advantage of this approach was that the *total* amount of IgM per cell could be measured. In a recent study focusing on trout kidney, we referred to this as (total) HCmu [15]. As shown in Figure 4A, 3 distinct B cell populations could be identified in LPS-activated spleen cultures (shown is day 4), labeled Populations 1, 2, and 3. Population 1 most likely included the plasma cells, because these cells express the highest amounts of both XbpI and HCmu. A fourth population was comprised of double negative cells (DN), and stained with neither XbpI-S nor HCmu antibodies (Figure 4A). As a negative control, isotype control antibodies C555 and C647 [15] were used as shown in Figure 4B. To confirm that Population 1 represented plasma cells, the Pax5 marker was used together with the HCmu marker. Plasma cells lack Pax5, but make very high amounts of IgM, as shown previously in trout kidney K1 [15]. Figures 4C (day 0 cells) and 4D (day 4 cells) illustrate that Population 1 includes two subpopulations: a Pax5-lacking, and a Pax5-expressing population. To distinguish between these two phenotypes, Pax5-lacking plasma cells will be referred to as mature plasma cells (MPCs), while Pax5 expressing cells will be referred to as “transitional plasma cells” (TPCs). This will be discussed further below. Figure 4E shows the cell size (FSC) for each of the 3 gated populations in Figure 4A, and confirms that Population 3 cells (resting B cells) were smallest (lowest FSC), Population 1 cells (plasma cells) largest (high FSC), and Population 2 cells intermediate in size. Double-negative cells are shown for comparison (Figure 4 E; bottom).

Next, the identity of plasmablasts was investigated. Plasmablasts, which are per definition dividing cells, were identified using Edu, a nucleoside analog that is incorporated into the DNA of dividing cells, in combination with B cell markers IgM or Pax5. Figure 5A shows two Hcmu⁺ populations: Edu⁺ (proliferating) and Edu⁻ (non-proliferating). The results suggest that plasmablasts do *not* make the highest amounts of IgM, and vice versa, that cells with the highest amounts of IgM [T/M-PCs] do *not* divide. Figure 5B shows that all proliferating cells in culture are Pax5⁺ B cells, and Figure 5C shows that proliferating cells have high, but not the highest, levels of XbpI-S. Lastly, the anti-IgM antibody was used to stain fresh (unfixed cells) in combination with Edu, and this showed that plasmablasts still express membrane IgM (Figure 5D). Based on this phenotype, plasmablasts must be Population 2 type cells (Figure 4A). Figure 5E verifies the size of (Edu⁺) plasmablasts, which are relative large cells (with high FSC) as shown for LPS-activated splenic cells on days 2, 4, and 8.

Together, using four different B cell differentiation markers, we can unequivocally distinguish plasmablasts from (T/M)-plasma cells: plasmablasts express lower levels of XbpI-S and Hcmu per cell, are Pax5⁺ and membrane IgM⁺ cells, and are dividing cells. Plasmablast, TPC and MPC phenotypes are summarized in Table I.

Use of the XbpI-S marker to distinguish between resting and activated B cells

One-color flow cytometry showed that, as expected, non-activated B cells had low levels of XbpI-S (Peak 2 D0 cells in Figure 3A). Using two-color flow cytometry, we could define resting B cells as having the phenotype XbpI-S⁺/⁻/Hcmu⁺, and as such, they fall into Population 3 type cells (Figure 4A). Population 3 cells, however, could also include IgD/IgT-expressing (resting) B cells, but this was not determined in this study. In contrast to resting B cells, activated B cells have increased levels of both XbpI-S and Hcmu (Figure 3, and compare Figure 4C and 4D). Hence, activated B cells have the phenotype XbpI-S⁺/Hcmu⁺⁺ (Population 2 in Figure 4A). Neither resting nor activated B cells proliferate, and as such neither incorporated Edu. The phenotype of resting and activated B cells is summarized in Table I.

Increases in total IgM and XbpI expression are linked

Next, the B cell activation dynamics of B cell cultures after LPS-induction *in vitro* were investigated. First, changes in expression of Hcmu were determined in individual cells during a 10-day period, for both spleen and PBL cells. Expression of total Hcmu was determined similar as described for Figure 3, by gating all Hcmu expressing cells, and then measuring the mean fluorescent intensities of the cells within these gates (using BDFACS ARRAY software). The maximum fluorescence intensity was defined through the mean fluorescent intensity of gated Population 1 cells for each experiment. As expected, average Hcmu expression per cell increased in B cells after LPS induction (Figure 6A) both in spleen and PBL cultures, confirming that upon activation, B cells make increasingly more IgM. Next it was tested if Hcmu induction was linked to XbpI-S induction during LPS activation. XbpI-S intensity values were determined as described above. Indeed, as shown in the scatter plot in Figure 6B (combining data from both spleen and PBL), increased expression of Hcmu in a cell correlated with increased XbpI-S levels in that cell. However, during the 10 day time period, Hcmu expression lagged behind XbpI-S expression. Importantly, Hcmu levels did not exceed 25% (+/- 3.1%) of their maximum (Population1, or plasma cell level) value, while XbpI-S expression increased to close to its maximum value. Based on these results, we speculate that the maximum amount of IgM secretion in plasmablasts is approximately 25% of the level in plasma cells.

***In vitro* plasma cell generation**

Next it was explored if and when plasma cell generation occurs during LPS-activation. Using XbpI-S and HCmu markers on activated spleen cultures, we found that during the 10-day activation period, there was a time-dependent decrease in the frequency of IgM⁺, resting B cells (HCmu⁺/XbpI-S⁺; Population 3), and a gradual increase in the frequency of T/M-PC cells (HCmu⁺⁺⁺/XbpI-S⁺ or Population 1), as shown in Figure 7A. Furthermore, the frequency of activated B cells/plasmablasts (HCmu⁺⁺/XbpI-S⁺; Population 2) increased rapidly early on, then decreased after day 4, presumably as a result of further differentiation of such cells into (T/M) PCs. Results for PBL were much more variable between experiments, but in contrast to the spleen, the frequency of IgM⁺, resting B cells (Population 3) did not change during the 10-day period (data not shown). To determine how many of the plasma cells were MPCs, the frequency of Pax5⁺/HCmu⁺⁺⁺ cells was measured during the 10-day period. Consistently, the data showed that both spleen and PBLs generated only very few MPCs, with frequencies of less than 0.5% (data not shown). Hence, the majority of PCs (HCmu⁺⁺⁺/XbpI-S⁺ cells) detected throughout this 10-day activation period were TPCs.

Next, patterns for plasmablast generation (Pax5⁺/Edu⁺ cells) were determined in spleen cultures during the 10-day period. The frequency of Pax5⁺ proliferating cells increased upon LPS-activation, with maximum proliferation on days 4–6 and then decreasing (Figure 7B). Repeating the experiment using membrane IgM or HCmu in combination with Edu markers gave a similar pattern (data not shown). When plasmablast patterns were determined in cultured trout PBLs, there was a large amount of variation in the level of proliferation between experiments. On the average, the maximum level of cell proliferation was 0.5% (+/– 1.9%) during the 10-day period (data not shown). We conclude that trout spleen provided a much more reliable source to test B cell proliferation capacity, at least *in vitro*, as compared to trout PBL.

As a control for the proliferation assay, the proliferation experiments were repeated in a highly studied animal model, the mouse. For this experiment, spleen cells isolated from C57BL/6 mice were induced *in vitro* with LPS and collected on days 0–5. Fixed and permeabilized mouse cells were then analyzed by flow cytometry using the same methods as for trout, and the same Pax5 antibody. Flow cytometric results (Figure 7C) showed that mouse splenic B cells with phenotype Edu⁺/Pax5⁺ proliferated earlier (with a peak on day 2) and much more vigorously (more than 40% of the cells on day 2) than trout splenic B cells. This difference was particularly striking considering that mouse B cells were exposed to only one-tenth the amount of LPS (10 µg/ml instead of 100 µg/ml used for trout B cell activation), and Edu was only present in cultures for 2 hours (compared to 16 hrs for trout).

DISCUSSION

This study aimed to identify distinct populations of terminally differentiating B cells in the rainbow trout, using the inducible transcription factor XbpI-S. As we show here, XbpI-S expression is rapidly induced in trout B cells, within 24 hours after LPS-stimulation, and, at least in splenic cultures, keeps increasing for 6–8 days. Our flow cytometry results also suggest that maximum production of HCmu in individual cells does not occur until 8–10 days after induction, and this may explain the low frequency of MPCs. Importantly, increases in XbpI-S expression correlated positively with total levels of IgM. Similar studies using the murine B cell line CH12, have shown that XbpI-S protein is readily detectable within 24 hours of LPS induction, and remains at this level for at least 48 hours [14]. XbpI-S induction in CH12 B cells also correlates with increases in HCmu protein: low levels of HCmu are detectable for the first 12 hours, then increase exponentially, with the most rapid synthesis rate between 24 and 48 hours post-induction [14]. It should be mentioned here that

the studies in CH12 B cells were done using western blot, which does not allow measuring HCmu expression in *individual cells*, so this issue remains somewhat unresolved.

As was observed in the mouse cell line, the initial increase of trout XbpI-S expression was more rapid than that of HCmu. In both species, activated B cells did not start making significant amounts of HCmu until they expressed fairly high XbpI-S levels. This is perhaps not too surprising, as XbpI-S is a master regulator of terminal B cell differentiation, a transcription factor that controls a number of “terminal differentiation” genes, and is necessary for induction of the unfolded protein response, which leads to the ability to synthesize and secrete large amounts of IgM [10–14]. Hence, we propose that activated trout B cells need to accumulate a minimum or “threshold level” of XbpI-S protein to enable further differentiation into a plasmablast and ultimately, a plasma cell.

Interestingly, the induction of XbpI-S differed greatly between the two trout immune tissues studied here: PBL and spleen. While spleen cultures appear to support cells’ increased XbpI-S expression throughout the ten-day culture period, in PBL cultures the induction is significantly slower. More strikingly, the levels of XbpI-S in PBL B cells reach a plateau after about 4–6 days. The intermediate level of XbpI-S expression that is reached by the time PBLs hit this plateau, suggests that the terminal differentiation pathway of PBL B cells is blocked at a specific activation stage. This is also in agreement with the observation that compared to spleen, significantly fewer plasmablasts are formed in LPS-stimulated PBLs. This supports our threshold model: trout B cells from LPS-activated PBL cultures cannot generate high enough levels of XbpI-S necessary to drive terminal differentiation from the activated B cell stage into the plasmablast stage, at least during the first 8 days of LPS-induction.

Comparison of proliferating B cells (Pax5+/Edu+) between trout and mouse show that the latter proliferate significantly earlier (between days 2–4, compared to days 4–6 in trout). This is particularly striking, considering that trout B cells were exposed to up to 10 times the concentration of LPS compared to mouse B cells. Another striking species difference concerned the frequency of proliferating splenic B cells in response to LPS. At their maximum, mouse splenic cultures had almost 40 times more proliferating B cells compared to trout. Mouse plasmablasts undergo anywhere between 5–10 cell cycles before they differentiate into plasma cells, depending on their cytokine environment [19]. Furthermore, it has been reported by others that by day 4 post-LPS-stimulation, 30% of mouse splenic B cell cultures have left the cell-cycle and consist of plasma cells [20]. In contrast, as shown here, after 10 days, less than 4% of the trout spleen cultures contained plasma cells. We hypothesize that, at least *in vitro*, trout plasmablasts’ undergo fewer cycles of cell division, and/or progression through each cell-cycle is slower compared to mouse plasmablasts. Future studies should focus on the effects of cytokines on trout plasmablast cell-cycle progression, including the relationship between cytokine profiles and the number of cell cycles. It is likely that trout B cells are less sensitive to LPS activation, and/or that such cells cannot be fully activated unless in the presence of specific cytokines.

Our studies revealed a type of ASC with a phenotype in-between a plasmablast and a Pax5– plasma cell; this (Pax5+/–) cell was named a “transitional-plasma cell” (TPC). To distinguish the TPC from the Pax5– plasma cell, we renamed the latter as being a “mature plasma cell” (MPC). As suggested by our results, the majority of high IgM-secreting cells in this study were TPCs, with only very few MPCs, both in spleen and in blood. At this time, it is unknown whether TPCs play significant roles during *in vivo* responses, a topic that will be investigated in future studies. We hypothesize that the transitional presence of the transcription factor Pax5 in TPCs, allows a certain amount of flexibility in the TPCs response to its environment, which is lost upon the cell becoming a MPC. Previous studies

in human ASCs have identified similar populations of IgM-secreting cells that still express Pax5, including human “tonsil plasma cells” [21] and a type of antigen-induced plasmablast-intermediate [22]. A small population of TPCs was recently identified by our group [15] in the anterior kidney (or K1) of the trout (in that study, such cells were referred to as pre-PCs). Based on Pax5 expression patterns in trout [15], anterior kidney (K1) presumably houses both MPCs and TPCs, but has significantly more MPCs compared to spleen [15]. This is in agreement with the current hypothesis that anterior kidney (K1), but not spleen, provides a major site for (long-lived) plasma cells, which carry the MPC phenotype [4, 5, 9]

The use of XbpI-S as an activation marker for trout humoral immune activation promises to be useful for a broad range of fish species; it is particularly exciting that the newly generated anti-tXbpI-S antibody can detect XbpI-S protein expression in species within the *Oncorhynchus* genus, including *O. mykiss*, *O. nerka*, *O. kisutch*, *O. gorbuscha*, (and *O. clarkii*; Zwollo, unpublished data). Together with the anti-Pax5 antibody, tXbpI-S now provides fish immunologists with a new molecular tool to determine the spleen B cell signature of fish *in vivo*, either during maturation, in response of immunization, or as a result of infectious agents.

- > XbpI-S induction in trout splenic B cells increases during *in vitro* LPS induction.
- > Increased XbpI-S expression correlates with increased HCmu expression in trout.
- > Trout blood displays a lower level of XbpI-S induction compared to trout spleen.
- > Trout B activation is slower than what has been observed in mammalian species.
- > XbpI-S should provide a B-activation marker for a broad range of teleost species.

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Figure 1. Trout XbpI-S transcripts and XbpI-S antibody design

A. Photograph of ethidium bromide-stained, 2.5% agarose gel containing 10 µl PCR-product from D0 (0), D1 (1), and D7 (7). Molecular weight markers (MW), in bps, are indicated on the right. Locations of the expected 121 nt (XbpI-S) and predicted 147 nt (XbpI-U) fragments are indicated by lined and dotted arrows respectively. The asterisk indicates the position of PCR primers. **B.** Schematic for the Unspliced form of XbpI (XbpI-U; light grey box) and the Spliced form (XbpI-S), which differ at their C-terminal protein sequence (dark grey area) [13]. Position of the tXbpI peptide used for immunization is indicated. **C.** Comparison XbpI-S protein sequence in the region corresponding to aa 334–364 of mouse XbpI-S (Genbank AF4431921). For trout, predicted protein sequence based on Genbank cDNA sequence (BX075133) and genomic trout XbpI DNA sequence were used (** Dr. Palti, National Center for Cold and Cool Water Aquaculture; unpublished data).

Figure 2. Western blot analysis on *Oncorhynchus* immune tissues using tXbpI-S antibody
A. XbpI-S expression in freshly isolated rainbow trout PBLs and SPL (spleen) cells (5×10^5 cells/lane). MWM are shown on the right in kD. **B.** Staining of spleen tissue (30 μ g protein/lane) from 3 other *Oncorhynchus* species: *Oncorhynchus nerka* (ON), *Oncorhynchus kisutch* (OK), and *Oncorhynchus gorboscha* (OG). **C.** Rainbow trout spleen immune cells cultured in the presence of LPS and collected on day 0 (D0) – day 6 (D6). Cell lysates of 5×10^5 splenic cells/lane. MWM (in kD) are indicated on the right.

Figure 3. One-color flow cytometry and detection of XbpI-S expression in *in vitro* LPS-activated spleen and PBL cells

A. Left: histogram showing increased intensity of XbpI-S staining (using tXbpI-S conjugated to Alexa 555) upon LPS-stimulation. Insert: dot-plot to show the gated lymphocyte population. Grey solid line: isotype control; black solid line, D0; black dotted line, D2; black dot-striped line, D4; black bold line, D8. Arrows indicate the intensity peak of the XbpI-S stained populations for each day. Cell populations that did not stain with tXbpI-S mark the 0% intensity peak. 100% indicates the maximum fluorescent intensity for XbpI-S (mean intensity value of Population 1 in Figure 4A). Blue dotted lines indicate gates for determining cell frequency of Peak 1 (XbpI-S-), Peak 2(XbpI-S+/-), and Peak 3 (XbpI-S+ cells). Right panel: list of cell frequencies (in percent) for each peak and for each day. **B.** Relative fluorescent intensity (in percent) in spleen B cells; results from 4 independent experiments with 3 fish pooled in each. **C.** As B. but for PBL, 4 independent experiments using 3 fish each.

Figure 4. Use of two-color flow cytometry to identify populations of terminally differentiating trout B cells

Spleen cultures were activated *in vitro* with LPS. **A.** Contour graph of Day 4 cells after staining with anti-IgM (total HCmu) and tXbpI-S antibodies; three distinct staining (Population 1–3) and one non-staining (double-negative; DN) cell population. **B.** Control (Day 4) sample stained with C555 and C647 isotype control antibodies. **C.** Detection of resting B cells and MPCs in Day 0 cells using anti-IgM (total HCmu) in combination with anti-Pax5 antibody. **D.** Detection of activated B cells, TPCs and MPCs in Day 4 cells using the same antibodies as in C. **E.** FSC/SSC profiles for each of the gated Populations 1–3 and DN cells.

Figure 5. Detecting trout plasmablasts (PB)

Trout splenic *in vitro* LPS-activated cultures (day 4). T/M-PCs are also indicated. **A.** Using anti-IgM (HCmu) with Edu. **B.** Using anti-Pax5 with Edu. **C.** Using anti-tXbp1-S with Edu, and **D.** Using IgM pre-staining to detect membrane-IgM-positive proliferating cells, with Edu. **E.** FSC/Edu profiles for LPS-activated splenic B cells on days 2, 4, and 8 (D2, D4, and D8). D0 cells did not receive the Edu reagent.

Figure 6. Relationship between HCmu and XbpI-S expression during LPS-activation of trout B cells

Activation period: Day 0–10 after LPS activation. Mean intensity fluorescent value for each sample was determined using FACSArray software (see text). **A.** Relative HCmu intensity for days 0, 2, 4, 6, and 10 post LPS activation. Means and SE (N=4). **B.** Scatter plot showing the relationship between total HCmu intensity and XbpI-S intensity, using days 0, 2, 4, 6, 8, and 10 fluorescent intensity values for both spleen and PBL. Values on X- and Y-axes indicate percent fluorescent intensity relative to their maximum value.

Figure 7. Dynamics of trout terminal B cell differentiation in spleen

Cell frequencies were measured on specific days after LPS-activation *in vitro*. Days after LPS activation shown on the X-axis. **A.** Cell staining with tXbpI-S and anti-IgM (HCmu). Mean percent of each cell type and SE (N=4). Diamonds: frequencies of resting mature B cells (Population 3; XbpI-S+/-/HCmu+), squares: activated B cells and plasmablasts (Population 2; XbpI+/HCmu++) and triangles: frequency of (M/T)-plasma cells (Population 1; XbpI-S+/HCmu+++). **B.** Trout spleen, frequency of plasmablasts (Edu+/Pax5+ cells). Mean and SE (N=4). **C.** Staining as in **B**, but for mouse spleen, mean and SE (N=4).

Table I

Combinatorial expression of B cell markers during terminal B cell differentiation.

	Xbpl-S	HCmu	Mem IgM	Pax5	EdU
Resting B	+/-	+	+	+	-
Activated B	+	++	+	+	-
Plasmablast	+	++	+	+	+
T-plasma cell	+	+++	-	+/-	-
M-plasma cell	+	+++	-	-	-