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## Identification of Developing and Mature B Cell Populations and Population Dynamics Along the Kidney Gradient of *Oncorhynchus mykiss* (Rainbow trout)

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**Identification of Developing and Mature B Cell Populations and Population Dynamics  
Along the Kidney Gradient of *Oncorhynchus mykiss* (Rainbow trout)**

A thesis submitted in partial fulfillment of the requirement  
for the degree of Bachelors of Science in Biology from  
The College of William and Mary

by

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Williamsburg, VA  
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## Abstract

Rainbow trout are farmed worldwide as a popular food source, and represent a critical stage in the evolutionary history of the immune system. Teleosts lack bone marrow, the site of hematopoiesis in mammals. Instead, the anterior kidney of many fish species has been described as the site of early B cell development. This project sought to expand on a previously published model of the B cell populations in trout immune tissues using flow cytometry analysis of the kidney. The model suggests that a maturation gradient exists along the trout kidney, with the earliest B cell precursors in the anterior kidney, and mature B cells residing in the posterior kidney. If the maturation gradient exists, this project hypothesized that detecting B cell populations in the kidney would reveal an inverse relationship between precursor and mature B cell populations along the kidney gradient. Cell size, cell complexity, and antibodies which detect the transcription factors EBF, Pax5, and Xbp-1, as well as anti-IgM and RAG-1 antibodies were used to define precursor and mature B cell populations throughout the kidney of small and large trout. This project confirmed an inverse relationship between the precursor and mature B cell populations along the kidney gradient in large fish, with specialization of the anterior kidney for early B cell development, and the posterior kidney containing many resting mature B cells. Differences were also discovered between large and small fish mature B cell populations and Xbp-1 expression.

## I. Introduction

Rainbow trout (*Oncorhynchus mykiss*) are a representative species of the Osteichthyes superclass, the bony fish, which is a significant group in the evolutionary history of vertebrates. This superclass is a part of the teleost clade of jawed vertebrates, and tetrapods are its nearest descendents [1]. A full understanding of the teleost immune system is important for several reasons, including the maintenance of healthy populations of aquaculture animals, analysis on the evolution of the system, and discoveries on the immune mechanisms absent from the murine model. Cellular and molecular immunology was developed with humans and mice as the most common representative species. This trend has continued, creating difficulties for the study of lower vertebrates, including fish, because of a lack of molecular tools and reagents, as well as deficient information for comparative studies [2].

### 1.1 The adaptive immune system

The adaptive immune system consists of multiple types of white blood cells, each with specific roles in the immune response. In mammals, lymphocytes are the most common type of white blood cells, and consist of natural killer cells, T lymphocytes (T cells), and B lymphocytes (B cells). B and T cells are responsible for specificity and diversity and are a part of the acquired immune system, while natural killer cells are a part of the innate response and do not recognize specific antigens in the host. The majority of B cells at any specific time are resting mature cells, displaying immunoglobulin (Ig) on their cell surface. The immunoglobulin molecules on an individual B cell and its clones have identical antigen recognition, but there are up to one hundred thousand different clones in an individual, each capable of recognizing unique amino acid sequences. The same is true of the T cell population, which recognizes antigen with its T

cell receptors. This simultaneously creates diversity and specificity in the immune system, critical characteristics of a highly evolved adaptive immune system [2].

Immunoglobulin molecules, also known as antibodies, are glycoproteins which consist of two regions, the variable (V) region, and the constant (C) region and are composed of two heavy chain polypeptides and two light chain polypeptides. The V region is responsible for recognizing antigen structures, and faces away from the B cell when membrane-bound. The constant region only varies among isotypes of immunoglobulin, of which there are five in mammals. This region is embedded in the cell membrane while the cell is a mature resting B cell [2].

When a mature B cell binds to an antigen, indicating the presence of a foreign protein, the cell is activated to begin proliferating and differentiating into the B effector cell, or plasma cell. Plasma cells are characterized by a large endoplasmic reticulum, a lack of membrane-bound immunoglobulin, or antibody, significant production of secreted antibody, and the termination of proliferation. Antibodies secreted by a plasma cell have the same specificity for antigen as the surface Ig molecule of the parent resting cell which initially recognized the foreign amino acid sequence. When antibodies are released from the plasma cell, they play an integral role in the mediation of an infection [2, 3].

Circulating antibody binds to an amino acid sequence of antigens, the so-called B cell epitope, and promotes their phagocytosis by opsonization. For example, a bacterial cell displays particular proteins on the entirety of its surface, and numerous secreted antibodies specific for these proteins will attach to the surface. Phagocytic cells, including macrophages, have a receptor for a part of the constant region on antibody, and the binding of multiple antibody molecules to this receptor induces phagocytosis of the entire antigen, including the antibody molecules. The complex is therefore removed from the system of the host, mitigating its damage

potential, and is destroyed by several processes within the phagocyte. Other functions of circulating antibody molecules include the activation of the complement system and induction of cytotoxicity by natural killer cells [2].

## 1.2 B cell development and transcriptional regulation

Blood cells arise from pluripotent hematopoietic stem cells through hematopoiesis. This process creates two types of progenitors, lymphoid and myeloid, which are committed to these lineages and can no longer self-renew. Common myeloid progenitors give way to red blood cells, antigen presenting cells, phagocytes, and several other types of cells involved in multiple immune processes. The common lymphoid progenitor (CLP) can differentiate into B, T, or NK cells [2].

The mature B cell arises through a complex developmental program, beginning with the common lymphoid progenitor. In mammals, this process occurs exclusively in the bone marrow after birth. The stages of B cell development have been defined by several groups over time, but many of the details of stage-specific functions and epigenetic changes are still unknown. In the process of becoming mature B cells, CLP's first differentiate into pro-B cells. These cells are large, and express CD43, a surface molecule. They are also undergoing the first immunoglobulin gene rearrangement of the heavy chain locus. After this gene rearrangement, pro-B cells become pre-B cells. During the pre-B cell stage, cells proliferate, rearrange their light chain genes, and decrease in size. This process creates immature B cells, which are no longer proliferating and display a fully functional Ig molecule of the  $\mu$  isotype, IgM. The last change is the display of an additional immunoglobulin isotype, IgD [4].



This developmental process is carefully regulated by transcription factors. E2A and EBF (early B cell factor) are two of the earliest B cell-specific transcription factors expressed during B cell development. As determined by creating single or double heterozygous mice, the two proteins work cooperatively to regulate expression of other transcription factors, such as Pax5, and other proteins such as RAG-1 and -2 and Ig $\alpha$  [5, 6]. O’Riordan et al completed EMSA analysis to confirm an EBF binding site on the Pax5 promoter. Another group also found an EBF binding site on the promoter of the Ig $\alpha$  gene, indicating EBF’s direct control of these gene products [5]. CLPs heterozygous for either E2A or EBF, or double heterozygous, become late pro-B and pre-B cells at a significantly decreased level, suggesting the requirement for both of these factors in the successful progression to the pre-B cell stage [6]. Mature B cells are completely absent in EBF  $-/-$  mice [7]. Hagman et al performed RNA analysis to reveal the presence of EBF transcripts in murine B cell lines. Transcripts were present in all proB cell lines, preB cell lines, and low levels in some mature B cell lines [8]. This data, along with the decreased ability of CLP’s to continue developing in the absence of EBF [6] indicates that EBF begins to be expressed at the CLP stage, and is downregulated at the mature stage.

As mentioned, RAG-1 and RAG-2 (recombination-activating genes) are indirectly under the control of EBF through one of its target transcription factors, FoxO1 [5]. RAG-1 and 2 work together to rearrange the heavy chain locus by recognizing recombination signal sequences and cleaving the DNA [2]. Expression of RAG-1 and 2 is reduced by signaling from the pre-B cell receptor, which already has a rearranged heavy chain, but expression increases again during rearrangement of the light chain just prior to the immature B cell stage [4, 9]. A population of B cells in secondary immune tissues, such as the spleen germinal centers, has been suggested to imitate the immature B cell phenotype after immunization. Han et al confirmed that this included

expression of RAG-1 and RAG-2 using immunohistological staining of germinal centers 16 days after immunization of mice. Hypermutation of the immunoglobulin gene has been well documented, but this process does not involve RAG-1 or RAG-2. Han suggests that the expression of these proteins could be reactivated as a byproduct of a process to remove autoreactive cells after hypermutation. The proteins could also mediate heavy chain or light chain secondary rearrangements, a novel theory at the time [10].

Pax5 is a well-studied transcription factor, and is expressed in the B cell lineage of all vertebrates. The protein is specific to B cells, and its expression constitutes commitment to the lineage [11]. Mice deficient in Pax5 accumulate B cells at the early pre-B cell stage, indicating a requirement for the protein in order to progress beyond this stage [5]. Northern blot analysis of multiple B cell lines also showed an absence of Pax5 transcripts in the pro-B cell stage, but a peak at the pre-B cell stage which is maintained in mature B cell lines [12]. Expression of the pre-B light chain, RAG-1 and RAG-2, as well as EBF and E2A are unaffected in Pax5 knockout mice in the pre-B cell stage, suggesting that all of these proteins act upstream of Pax5 [13]. Pax5 must be downregulated during activation of the mature B cell in order to differentiate and become an antibody-secreting plasma cell [14]. It has been proposed that this effect derives from the relationship between Pax5 and the transcription factor Xbp-1 (X-box binding protein 1). Reimold performed EMSA analysis of the Xbp-1 promoter and detected a complex with the same expression pattern as Pax5, which could be detected by Pax5 antibodies, suggesting a direct relationship between the two transcription factors. In addition, Xbp-1 is expressed in a pattern converse to that of Pax5 [12]. This relationship occurs through the repression of Pax5 by the transcription factor Blimp-1, an initial target gene following activation. Repression of Pax5 then

allows Xbp-1 to be derepressed, and its expression continues to increase in the plasmablast and plasma cell stage [15].

Secretory cells, such as plasma cells, have been documented to use the unfolded protein response (UPR) pathway to deal with the stress of secreting large amounts of protein. One of the three main proteins in the UPR of all cells is IRE1. The main activity of IRE1 is splicing of the Xbp-1 mRNA. The link between the UPR and B cell activity is the high expression of Xbp-1 in plasma cells, most likely because it upregulates genes necessary for protein folding [3]. RT-PCR studies on murine bone marrow B cell fractions (early proB through mature stages) reveal a distinct relationship between Pax5 and Xbp-1 expression in humans. The pre-pro-B cell stage had high levels of Xbp-1 and low levels of Pax5, but through the pre-B cell stage, levels of Xbp-1 dropped concomitantly with a rise in Pax5 RNA. It was also observed that Pax5 RNA was absent in plasma cells, but Xbp-1 RNA was abundant [12]. In an independent study, Brunsing et al created a reporter gene for IRE1 activity on Xbp-1 in hematopoietic stem cells. Pro-B cells and pre-B cells expressed the green fluorescent protein (GFP) reporter above the levels observed in control populations. Expression was low in mature B cells [16]. Together, these observations indicate an expected pattern of Xbp-1 expression which includes high levels at the pro and pre-B cell stage, low or no expression in immature and mature B cells, and increased expression after activation to the plasma cell stage.

### 1.3 Teleost immune system

Expression patterns of EBF, RAG-1, Pax5, and Xbp-1 are well studied and established in the mammalian model. These markers, along with several others, have been used to determine lower vertebrate immune function, thanks to highly conserved (DNA binding) domains. The

most critical difference between the mammalian immune system and lower vertebrate systems is the lack of bone marrow in early species including modern fish. In teleosts, the anterior kidney has been described as the bone marrow equivalent, or the primary immune organ, due to the presence of cells which express RAG-1, Pax5, and other lymphopoietic markers [17, 18]. The rainbow trout kidney extends along the backbone of the animal, from the back of the cranial cavity, referred to as the anterior kidney, to the end of the main body cavity, known as the posterior kidney. Patches of hematopoiesis can be detected in the kidney area at 3 weeks post fertilization, along with the first appearance of lymphocytes [19]. Some hematopoiesis may also occur in the posterior portion of the kidney, but at lower levels because of more renal function in this area [20].

The spleen of teleosts plays a significant role as the secondary lymphoid organ, with populations of mature B cells detected there in all stages of adult life [1]. The kidney may also have some secondary immune function, with small populations of mature B cells and plasma cells [21]. The renal system of the kidney and the filtration of blood in the spleen serve to trap antigen circulating in the blood [22]. In a study to determine bacterial clearance, the trout kidney was found to be responsible for 70% of antigen capture [23]. Antigen capture is associated with secondary lymphoid organs because detection of antigen is required to activate mature lymphocytes, which are located in secondary organs.

The immune system of fish is still not well understood, and multiple species have been used as representatives of this group. Zebrafish is often used in genetic studies of other systems, but other common species used include carp, salmon, and trout. Embryonic hematopoiesis differs within the group, with zebrafish having one exclusive site in the intraembryonic ICM, and trout having at least two hematopoietic sites at this stage, the ICM and the yolk sac blood islands [19].

More distant species of modern fish have even more variety in their sites of hematopoiesis which may include the gonads or meninges [25]. These similarities and differences between species may become more clear as more comparative studies are conducted. A disadvantage of using smaller fish, such as the zebrafish, is that they may only provide about one million cells from the kidney of each individual [25]. In defense of the zebrafish model, Traver notes that cells from individuals can be pooled to overcome this difficulty. However, variation among individuals is common when studying fish. Larger animal species, such as the rainbow trout, contain many more cells so that individual differences can be easily observed. Alternate sites of lymphatic function and other irregularities among species require the continued study of multiple species in this clade so that generalizations do not overlook important distinctions.

Our lab uses rainbow trout as a model organism to study the development and activity of immune cells in both primary and secondary lymphoid organs. There are many reasons to include the teleost clade in an examination of the evolution of the immune system. The appearance of the jaw in evolutionary history coordinates with the development of an adaptive immune system, so teleosts represent one of the earliest clades to have a highly functioning system [1]. In addition, mammalian immunology is far from being completely understood, and much remains to be discovered about the embryonic stage of development. Fish provide a system in which *in vivo* studies of the ontogeny of immune organs can be studied at this stage. Also, gene expression and forward genetics studies in teleosts could uncover novel proteins in the highly complex regulatory system of B cell development and activation [25]. Such information could then be extended to comparative studies, and prove useful for the detection and treatment of human disease. Finally, rainbow trout are farmed worldwide as a food source. In 2007, almost \$3 billion worth of rainbow trout were produced worldwide. A variety of diseases are common in

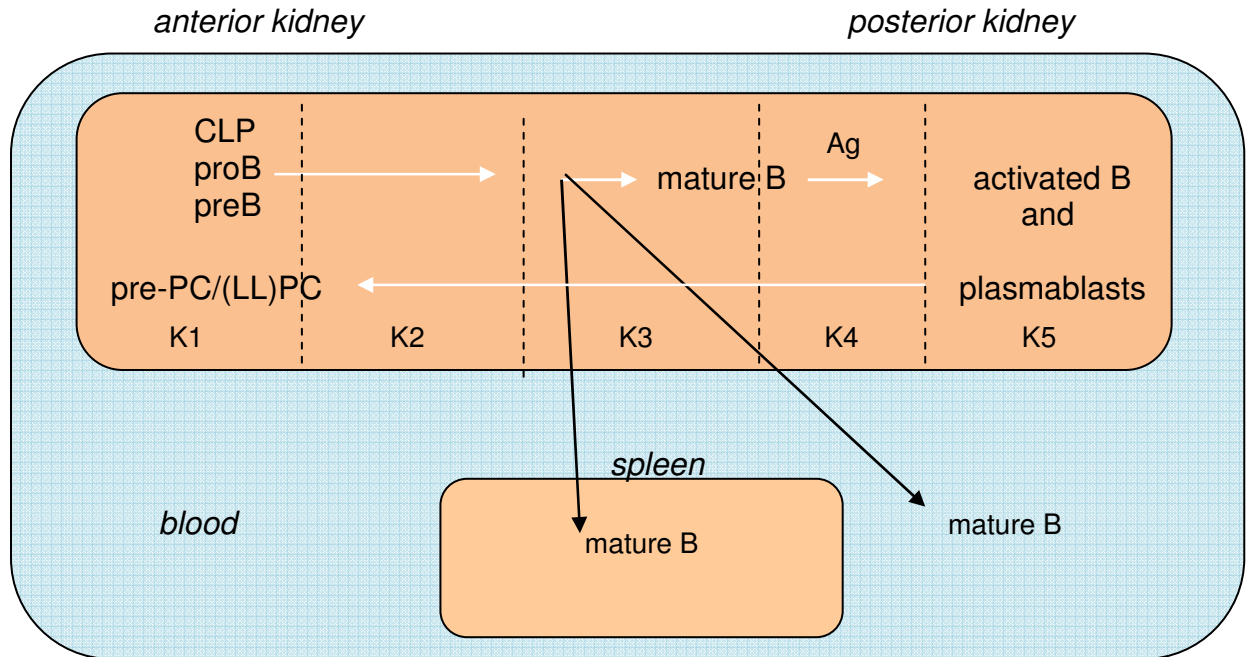
these farm populations, and efforts to limit loss of individuals include basic prevention such as sanitation, chemical bath treatments, and antibiotics in food [26]. More advanced prevention, such as vaccination, and better treatment options could be developed with increased understanding of the immune function of this species. Minimizing chemical treatments is important for eliminating water contamination and creating healthier animals for consumption.

#### 1.4 Previous work

Recent studies of the rainbow trout have included the identification of RAG-1 as a protein for the identification of precursor B cell populations. Kattaari and Hansen performed northern blots on the immune organs of rainbow trout, and found RAG-1 mRNA in the thymus of both juvenile and adult trout. They also report findings of lower levels in the kidney. The group separated the lymphocytes based on the presence of membrane-bound Ig. A much stronger signal was observed in thymocytes without this surface protein, an indication that levels of RAG-1 are higher in precursor B lymphocytes. This is consistent with the expression and function of RAG-1 in mammalian primary lymphoid organs. The sequence of the RAG-1 mRNA was at least 89% similar in the conserved region (aa 417-1042) to other RAG-1 genes. Based on expression and high conservation of base pairs, Kaattari and Hansen suggest that this protein has identical function in all vertebrates [27]. Based on this finding, our lab utilizes RAG-1 as a marker for pro and preB cells in the B cell development process. We use an  $\alpha$ RAG-1 antibody developed to detect part of the conserved region of the ring finger domain of mammalian RAG-1 on rainbow trout tissue samples.

Previous work in our lab defined B cell populations in the kidney, spleen, and peripheral blood lymphocytes (PBL) of rainbow trout using a density gradient. Cells were isolated from

these three tissues and divided into three fractions: P70, P60, and P50, based on their cell density. Comparisons to human and mice samples, along with Western blot analysis for stage-specific markers, led to the hypothesis that the P50 layer contained plasma cells, the P60 layer contained activated B cells and plasmablasts, and the P70 layer contained resting B cells. Further analysis of Ig secretion and proliferation in the cells at each layer confirmed these designations. The P70 population in the anterior kidney had more membrane Ig than secreted Ig, as detected by PCR. However, the cells in this P70 layer had high proliferation measurements, unlike the cells in this layer in the spleen or blood. The expected cells in the P70 layer, resting B cells, do not proliferate until activation, suggesting that this layer in the kidney is heterogeneous and could contain earlier developing cells or other dividing lymphocytes. A trend was observed that the proportion of cells in the P50 layer increased from the anterior to posterior kidney. This coordinated with a decrease in relative abundance of P70 cells. As the P70 cells were predicted to be mainly resting B cells, these cells were cultured in vitro for 7 days with lipopolysaccharide (LPS) stimulation to observe shifts in the abundance of each density group. In the spleen and posterior kidney, there was a shift to large populations of P50 cells, or plasma cells, as well as an increase in the P60 population. However, in the anterior kidney, very few cells became P50 cells, but a large increase in P60 cells was observed. This supports the previous indication that the P70 cells originally identified in the kidney are mostly developing B cells. Cells in this layer could include precursor cells in the process of maturing, which are therefore not able to respond to LPS. Taking these results into account, Zwollo created a model for the B cell populations in the major immune organs of the rainbow trout. This model is shown in (Figure 1), with variations in



**Figure 1.** Model proposed by Zwollo et al with modifications in accordance with this project [18]. The anterior kidney is believed to be the primary lymphoid organ, with populations of common lymphoid progenitors and early precursors. As these cells develop, this model hypothesizes that they occupy the middle section of the kidney, and that a pool of mature resting B cells accumulates in the posterior kidney. Other mature B cells exit the kidney and circulate in the bloodstream or reside in the spleen. The spleen and posterior kidney are believed to act as secondary lymphoid organs where mature B cells are exposed to antigen and activate. These activated cells circulate, and may eventually return to the anterior kidney as long-lived (LL) plasma cells.



accordance with the current project. At the time, the model proposed that B cells develop and mature in the anterior kidney, and then migrate to the posterior kidney and spleen which serve as secondary lymphoid sites for antigen presentation. Also, some activated cells return to the anterior kidney where they may become plasma cells, as has been observed in mammalian bone marrow [21].

Later work in the lab continued to define B cell populations in the trout immune organs using alternative techniques. A magnetic bead system was used to separate populations of cells from a sample into fractions with high levels, intermediate levels, low levels, or a lack of membrane-bound IgM on each cell. *In vitro* stimulation with LPS was used to determine activation potential in the four tissue samples (anterior kidney, posterior kidney, spleen, and PBL). There was not a significant change in the relative proportion of cells in each fraction of the anterior kidney or PBL after stimulation. Posterior kidney had an increase in cells with low to intermediate levels of IgM, representative of the activated or plasmablast stage. The spleen displayed the greatest change, with a large increase in the intermediate IgM fraction, and smaller increases in high and low IgM cell fractions. These results indicate that the posterior kidney and spleen may contain resting B cells capable of becoming plasmablasts and eventually plasma cells upon stimulation *in vitro*. PCR analysis and flow cytometry were then used to identify expression of certain genes, in particular Pax5, in each fraction. As predicted, the high IgM fraction correlated with the highest Pax5 expression in all tissues. Pax5 expression decreased in this fraction after stimulation with LPS, supporting the hypothesis that the resting B cells, which express high levels of Pax5, are activated following *in vitro* stimulation. Unstimulated cells in the anterior kidney displayed relatively low levels of Pax5 in the high and intermediate IgM fractions, which may represent preB cells, immature B cells, or activated B cells. Because the

anterior kidney has been described as the site of lymphoid development, the majority of cells at this site are expected to have low or no membrane-bound IgM [18].

### 1.5 Current study

The previous work in our lab, as well as genetic studies of transcription factors which regulate B cell maturation and activation, led to the development of this project. Previous work in this model system has identified a lack of resting mature B cells, small populations of proliferating and secreting cells, and cells expressing RAG-1 in the anterior kidney. The posterior kidney has been shown to contain populations of cells capable of being activated by in vitro stimulation, as well as a larger proportion of cells in the plasma cell fraction. Since the development of a model in 2005, our lab has continued to clarify the abundance of B cell populations and their functions. The heterogeneity of B cell populations in the anterior kidney of the rainbow trout and the distinction between the anterior and posterior kidney function required further investigation. Therefore, this project sought to expand upon the previously published model by defining B cell subpopulations in the kidney of the rainbow trout by flow cytometry. The main approach of this project was application of defined protein expression patterns in mammals to the teleost system.

Based on mammalian models of primary and secondary lymphoid organ cell populations, as well as previous results in our lab, several hypotheses were tested in this project. First, it is expected that similar cell populations will be observed in both juvenile and adult fish, because the lymphopoietic organs are fully developed a few weeks post hatch. The anterior kidney will consist of many precursor cells, very few resting cells, and possibly some antibody secreting cells. In addition, the previously observed differences between the anterior and posterior kidney

suggest that changes in cell populations will be detected along the anterior to posterior kidney gradient. Posterior tissue sections are expected to contain larger populations of mature resting B cells, activated B cells, and smaller populations of precursor cells than the anterior sections. Relative abundance of cell populations expressing IgM is expected to differ significantly between the sections, as expression of this protein and cell size change throughout B cell maturation and activation. Inverse relationships are also expected along the gradient between the abundance of precursor cells and the abundance of resting or activated B cells. Cell size, cell complexity, and antibodies which detect EBF, RAG-1, Xbp-1, Pax5, and IgM will be used to define B cell populations in five kidney sections of both juvenile and adult rainbow trout.

## II. Methods

### 2.1. Cell lines

The murine plasmacytoma line MPC11 and the murine B cell lymphoma line A20 were purchased through the American Type Culture Collection (ATCC) and grown according to ATCC guidelines. The Abelson murine leukemia virus-transformed cell line PD31 was a gift from Dr. Stephen Desiderio at Johns Hopkins University. The cells were maintained at a concentration of  $0.1-2 \times 10^6$  cells/ml in Mouse Complete Medium (MCM) consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Inc.), 2 mM glutamine, 50 units/ml penicillin, 50  $\mu$ g/ml streptomycin, and 50  $\mu$ M beta-mercaptoethanol.

### 2.2. Rainbow trout and facilities

Out-bred rainbow trout (*Oncorhynchus mykiss*) (200-500 grams) were purchased from Casta Line Trout Farms (Goshen, VA). Additionally, small (<10 inches) and large (>10 inches) fish were a gift from Steve Kaattari (VIMS). 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.

### 2.3. Trout tissue collection

Trout were anesthetized with ethyl-3-Aminobenzoate methanesulfate salt (Sigma) in accordance with our IACUC protocol. Blood (2-5 ml) was drawn from the caudal vein and placed into heparinized tubes. The entire kidney was divided into 5 sections, K1-K5, consisting of 7 vertebrae each, starting from the posterior kidney [21]. Cells were collected and placed into

Petri dishes filled with 2 ml HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , and 20 mM Hepes at pH 7.05). The tissue was cut into small pieces, then repeatedly aspirated with a 10 ml syringe and then filtered through a 40 nm nylon cell strainer (Falcon, BD Biosciences) to create a single cell suspension for each tissue in a 50 ml Falcon tube. The cells were centrifuged at 1300 rpm at 4°C for 10 minutes and the supernatant was removed. The cells were resuspended in 10 ml HBSS and centrifuged again for 10 min at 1300 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 10 ml HBSS. In a separate 50 ml Falcon tube, 10 ml of Histopaque (1077, Sigma Aldrich) was added. The cell suspension was carefully layered onto the Histopaque and the cells were centrifuged for 30 min at 2100 rpm at 4°C. The supernatant, including the cell layer, was removed and transferred to a new 50 ml Falcon tube. The remaining volume was discarded. The cell suspension volume was increased to 45 ml with HBSS and the cells were centrifuged for 10 min at 1300 rpm at 4°C. The supernatant was removed and the cells were resuspended in 10 ml HBSS. The cells were counted using a hemacytometer (Fisher Scientific) and cell counts were determined for each tissue. Cells were centrifuged for 10 min at 1300 rpm at 4°C and the supernatant was removed.

#### 2.4 Nuclear extract preparation

Cells were resuspended in 10 ml PBS (1.9 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 8.1 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 137 mM NaCl, and 2.6 mM KCl, pH 7.4), then centrifuged for 5 min at 1000rpm at 4°C. The supernatant was removed, and cells were resuspended in 1ml PBS. The cells were then transferred to an Eppendorf tube (Fisher Scientific) and centrifuged for 1 min at 10,000rpm at 4°C. The supernatant was removed, and the cells were resuspended in (.5ml per  $2 \times 10^7$  cells) lysis buffer (10mM Hepes pH7.9 +10mM KCl + 0.1mM EDTA + 0.4% Igepal + 1mM DTT + .5mM

PMSF + 1x protease inhibitor cocktail). Cells were incubated on ice for 15 minutes, and then centrifuged for 1 minute at 10,000rpm at 4°C. The nuclear fraction remained in the pellet, and (.25ml per  $2 \times 10^7$  cells) lysis buffer was added. The samples were then centrifuged for 30 seconds at 10,000rpm at 4°C, and the supernatant was removed. The pellets were then resuspended in (.25ml per  $2 \times 10^7$  cells) extraction buffer (20mM Hepes pH 7.9 + 0.4 M NaCl + 1mM EDTA). Samples were then incubated on a shaking vortex for 20 min at 4°C. The samples were then centrifuged for 10 min at 10,000rpm at 4°C. The supernatant was aliquoted and flash frozen at -80°C.

### 2.5 Whole cell pellet preparation

Freshly collected cells were aliquoted to  $5 \times 10^5$  or  $2 \times 10^6$  cells each. Cells were centrifuged at 10,000rpm for 1min. The supernatant was removed, and pellets were briefly vortexed then quick-frozen at -80°C.

### 2.6 Positive selection by magnetic beads

Histopaque-purified cells were resuspended in trout complete medium (TCM) consisting of RPMI 1640 with 10 mM L-glutamine, 10% fetal calf serum (FCS), 50 g/ml gentamicin, 50 M 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (10 g/ml; Sigma-Aldrich) at 4 °C. IgM<sup>+</sup> 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. One aliquot of cells was saved at this point to serve as an unpurified sample. Positive selection was performed according to the manufacturer's instructions (Stemcell Technology Inc.) using the trout IgM-

specific monoclonal antibody Warr's 1–14 [27] at a final concentration of 1 µg/ml. 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 'IgM--' fraction. Cells from each fraction were counted and frozen in  $5 \times (10)^5$  cell aliquots for Western blot analysis.

### 2.7 Western blot analysis

Whole-cell protein lysates of  $5 \times 10^5$  cells were prepared by resuspending cells in 40ml of a sample buffer (12.5% 4xTris/Cl, pH8.8 + 10% glycerol + 0.0005% bromophenol blue + .01g/mL SDS) containing 5% 2-ME. Nuclear extracts were prepared by adding sample buffer containing 5% 2-ME at a 1:3 volume ratio with the sample volume containing 35ug protein. Proteins were separated by size using denaturing 12% SDS-PAGE gels. Proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore). Membranes were incubated in blocking solution (5% dry milk in PBS) for 1 hour, followed by a 1 hour incubation in blocking solution containing primary antibody (dilutions as indicated in Figure 2). This was followed by three 10 minute washes in PBS. Another 1 h incubation with secondary antibody goat-anti-rabbit IgG-horseradish peroxidase conjugate (1:5000, Zymed) in blocking solution. Membranes were then washed three more times in PBS, and developed using a chemiluminescence kit (ECL, Amersham).

## 2.8 Cell fixation and permeabilization

Freshly isolated cells were centrifuged at 1000 rpm at 4°C for 5 minutes. The supernatant was removed, and the cell pellet was resuspended in 1 ml PBS containing 0.02% NaN<sub>3</sub> and cells were centrifuged again for 5 min at 1000 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 1ml 1% Paraformaldehyde (16% PF solution, E-M grade; Electron Microscopy Sciences) in PBS and incubated on ice for 15 min. Next the cells were centrifuged for 5 min at 1000 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml PBS containing 0.02% NaN<sub>3</sub>. The cells were centrifuged for 5 min at 1000 rpm at 4°C and the supernatant removed. The cell pellet was vortexed for 10 seconds, until the cells were in suspension, and 1 ml of -20°C 80% methanol was added drop-wise to the cells while continuing to vortex. The cells were stored at -20°C for a minimum of 16 hr and remained stable at -20°C for up to 6 weeks.

## 2.9 One- and two-color flow cytometry

Immediately before use, fixed cells were removed from -20°C storage in 80% methanol and counted. 1 ml ice-cold PBS + 0.02% NaN<sub>3</sub> was added to the cell suspension. The cells were centrifuged for 5 min at 1500 rpm at 4°C and the supernatant was removed. Next, the cells were resuspended in 1ml permeabilizing solution (BD perm wash, BD-Biosciences) containing 2% FBS. Cells were centrifuged for 5 min at 1500 rpm at 4°C. The supernatant was removed and 0.5 x 10<sup>5</sup> cells were resuspended in permeabilizing solution + 5% FBS in a volume of 45 µl per sample. The cells were incubated for 15 minutes on a nutator at 4°C. Fluorescent antibodies were diluted to 10x stock solutions at a concentration of 0.15 µg/ml in perm wash+ 5% FBS. From each freshly diluted antibody solution 5 µl was added to the 45 µl cell sample and gently



resuspended. The cells were incubated with the fluorescent antibodies for 90 min at 4°C on a rotating platform, protected from light. After the 90 min incubation, 1 ml perm wash+ 2% FBS was added to each sample. The cells were incubated for 10 min on the nutator, protected from light. After the 10 min incubation, the cells were centrifuged for 3 min at 2000 rpm at 4°C and the supernatant was removed. This wash and 10 min incubation was repeated an additional time. The pellet was resuspended in 200 µl perm wash+ 2% FBS and the cell suspension was transferred to a 96 well round-bottom plate (Fisher). The samples were immediately analyzed by flow cytometry (BD FACSAarray, BD-Biosciences). For each sample, up to 50,000 cells were acquired. From this mixture of cells, a gate was placed around the lymphoid population to exclude red blood cells and debris from analysis. Flow data were analyzed using the WinMDI software, version 2.8.

### 2.10 Antibodies

The Pax-5, RAG-1, EBF, Total IgM (Warrs), and Xbp-1 antibodies (Table 1) were conjugated to Alexa 555 or Alexa 647 using protein-labeling kits (Molecular Probes, Invitrogen). Antibodies were labeled according to manufacturers instructions. Frozen aliquots were stored at -80°C or -20°C. All antibodies except Warrs were stored with 1% BSA.

## Fluorescent Markers Used With Flow Cytometry

Name	Full Name	Source	Target Peptide	Type of Antibody	Role in mammals
Pax-5 (ED-1)	Paired box protein 5	In-house [28]	Trout Pax-5 148aa of paired (DNA binding) domain	Rabbit polyclonal anti-paired domain IgG (ED-1)	B cell lineage commitment factor, regulates maturation [11]
RAG-1 (H-300)	Recombination activating gene 1	Purchased (Santa Cruz Biotechnology Inc.)	RAG-1 aa 744-1043, ring finger domain	Rabbit anti-human polyclonal IgG	Ig and TCR gene rearrangement [2]
Warrs (1-14)	Detects trout immunoglobulin isotype M	Gift from Dr. Steve Kaattari [29]	Trout $\mu$ heavy chain protein	Mouse anti-trout Ig HC monoclonal IgG	Antigen detection
EBF (H-300)	Early B cell factor	Purchased (Santa Cruz Biotechnology Inc.)	EBF aa 1-300	Rabbit anti-human polyclonal IgG	Regulates multiple downstream transcription factors and proteins in early B cell development including Pax5 [5, 6]
t-Xbp-1	Trout Xbox binding protein	In-house (Gen-script)	Trout Xbp-1 SGYERSPPFS DMSS PLCSEGSWDDVFANEL	Rabbit anti-trout polyclonal IgG	Unfolded protein response, expressed in plasma cells for secreted Ig production [3]
C555		Purchased (Santa Cruz Biotechnology Inc.)	Isotype control	Rabbit anti-goat polyclonal IgG	
C647		Purchased (Santa Cruz Biotechnology Inc.)	Isotype control	Rabbit anti-goat polyclonal IgG	

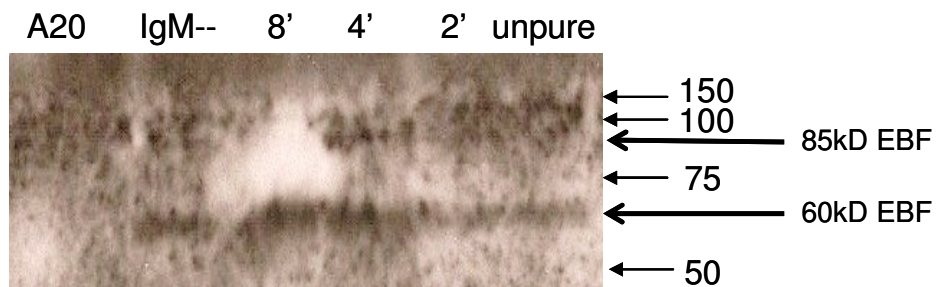
**Table 1.** Fluorescent antibodies used in thesis work. This table presents the antibodies used, their source, full name, target peptide, type of antibody, and role in mammals.

### III. Results

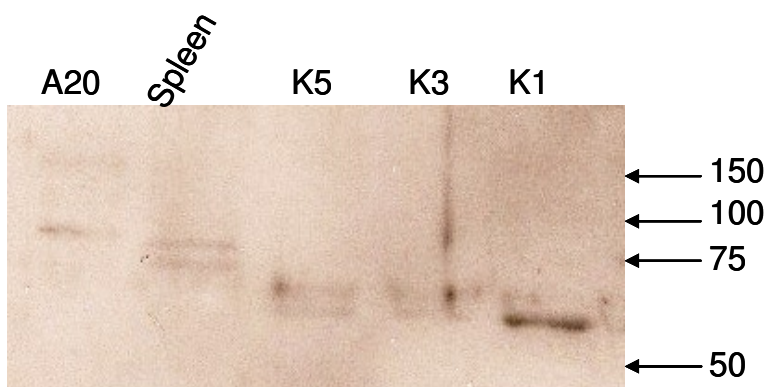
#### 3.1 Western blots

Previous work in this lab, comparative studies, and work by other groups led to the selection of five antibodies for the detection of stage-defining protein expression in the trout kidney. Western blots were performed to test the ability of these antibodies to detect their target protein in the trout system. An 80kD EBF protein can be detected in mammals with the antibody defined in Table 1. Trout posterior kidney (K5) cells were separated into four fractions by magnetic bead selection, and whole cell lysates were made with  $5 \times 10^4$  cells (Figure 2A). The unpure fraction was not selected by magnetic beads. The 2' fraction, 4' fraction, and 8' fraction were exposed to the magnet for the specified time. The 2' fraction contains cells which have high levels of IgM on their surface, mostly immature and mature B cells. The 8' fraction contains cells with low levels of surface IgM, including preB cells and plasmablasts. The IgM- fraction is the portion of the sample that was not selected after an 8' exposure to the magnet, including non-B cells, proB cells, and CLPs. Using the EBF Ab, two protein signals are detectable, one around 85kD in the 4', 8' and IgM- fractions, where proB and preB cells are expected. A20 is a mature B cell line control, and does not express EBF. The size difference could be due to a difference in protein size between the two organisms, for the 85kD protein, or degradation of the protein, for the 60kD protein.

The RAG-1 antibody used in this project was specific for the 130kD mammalian protein. However, the sequence it recognizes is a conserved region, shared with trout RAG-1 [27]. The RAG-1 antibody was tested for its ability to detect RAG-1 in nuclear extracts of spleen, K1, K3, and K5 trout cells (Figure 2B). Again, the A20 mature B cell line was used as a control. Signals can be observed in every sample, but at varying sizes. The murine RAG-1



**Figure 2A.** *EBF Western blot. Trout posterior kidney cells were separated into four fractions by magnetic bead selection, and whole cell lysates were made with  $5 \times 10^4$  cells per sample. The primary antibody was diluted  $1 \mu\text{g}/\text{mL}$  blocking buffer. Stronger signals can be observed in the 8 minute and IgM- fractions, where proB and preB cells are expected. A20 is a mature B cell line, and does not express EBF. The size is smaller than the murine 80kDa size.*



**Figure 2B.** *The RAG-1 antibody was tested for its ability to detect RAG-1 in nuclear extracts of spleen, K1, K3, and K5 trout cells. RAG-1 primary antibody was diluted  $1 \mu\text{g}/\text{mL}$  in blocking buffer. Signals can be observed in every sample, but at varying sizes. The murine RAG-1 peptide is 130kDa, and the trout RAG-1 sequence as described in [27] is approximately the same size.*

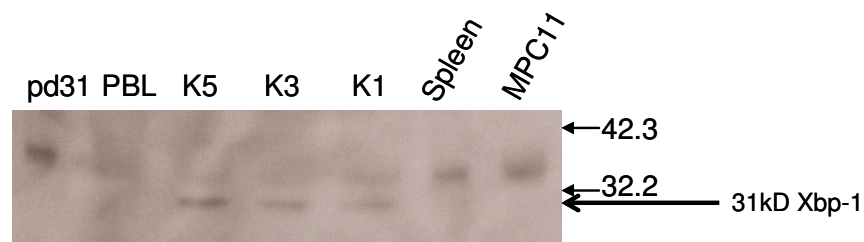
peptide is 130kDa, and the trout RAG-1 sequence as described in [27] is approximately the same size. The smaller RAG-1 protein sizes could be due to degradation of the protein during the extraction or electrophoresis process. More recent work by Maggie Barr has shown that both proteins can be detected at their expected sizes, 80 and 130kD for EBF and RAG-1 respectively (unpublished data).

The antibody to detect Xbp-1 was designed by Dr. Zwollo for a specific C-terminal sequence of the trout peptide (Table 1). Therefore, a Western blot was performed to assess the antibody's ability to detect this protein in nuclear extracts of varying concentrations from K1, K3, K5, spleen, and PBL trout cells (Figure 2C). Signals can be seen in the kidney samples at approximately 31kDa, the size of the peptide in mice. This indicates that the antibody is detecting the Xbp-1 protein, and can be used for flow cytometry analysis of trout samples.

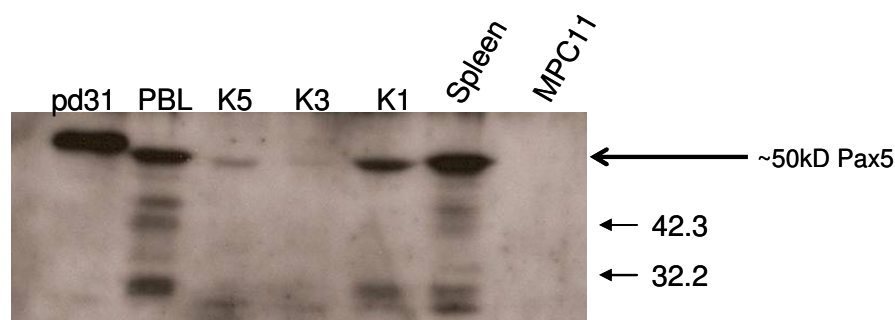
The Pax5 antibody has also been specifically designed to detect the paired domain of the trout Pax5 protein. Whole cell lysates were made from  $5 \times 10^5$  cells of trout K1, K3, K5, spleen and PBL (Figure 2D). The full length murine Pax5 peptide is 53kD, and the trout full length peptide is slightly smaller. Both PBL and SPL have signals at this size. Spleen, K1, and PBL have the strongest signals at approximately 50kDa, similar in size to the signal in the murine pd31 preB cell line, indicating that another factor is affecting the correct position of the peptide in these samples.

### 3.2 Flow cytometry of individual protein expression and cell size

Based on previous research in mammals, a schematic of expected protein expression has been created (Figure 3). The stages of B cell development and activation are drawn on the left, and expression in mice of each protein in this study is given on the right. Dotted lines



**Figure 2C.** *Xbp-1* Western blot. Nuclear extracts of varying concentrations from K1, K3, K5, spleen, and PBL trout cells were used to test the *tXbp-1* antibody. Primary *tXbp-1* antibody was diluted used at  $1\mu\text{g}/\text{mL}$  in blocking buffer. Signals can be seen in the kidney samples at the expected 31kDa size.

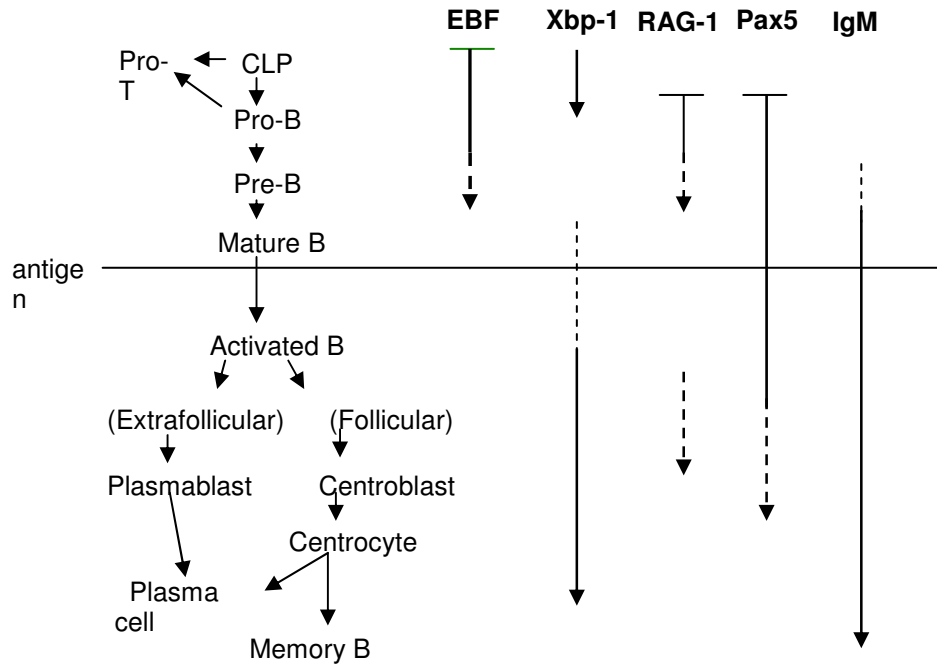


**Figure 2D.** *Pax5* Western blot. Whole cell lysates were made from  $5 \times 10^5$  cells of trout K1, K3, K5, spleen and PBL. Primary ED-1 antibody was diluted  $1\mu\text{g}/\text{mL}$  in blocking buffer. The full length murine Pax5 is 53kD, and both PBL and SPL have signals at this size. Spleen, K1, and PBL have the strongest signals at approximately 60kDa, similar in size to the signal in the murine pd31 preB cell line. The rainbow trout full length Pax5 peptide is slightly smaller than the mouse peptide.

represent stages at which expression is expected to be low. Based on this diagram, populations which are positive for each protein by flow cytometry detection can begin to be defined.

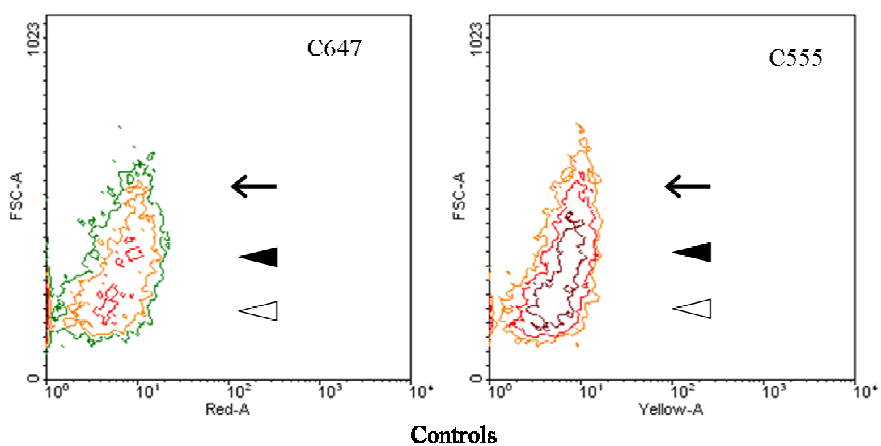
Figure 4 shows the positive populations for each protein, as detected by flow cytometry. K1 cells from a small (<10 inch) and a large fish (>10 inch) were stained with each antibody. The positive population for each protein is gated (and the percentages given are relative to the total lymphocyte population). An (←) arrow indicates “large cells” in the lymphoid population based on FSC values from flow cytometric analysis. The solid arrow indicates the cell size defined as “intermediate” for the purposes of this project. The empty arrow indicates the “small” cell size, based on lower FSC values. There is a significant population of EBF+ cells in both large and small fish (Figure 4A, filled arrow). In both samples, the cells which make up the EBF+ population are in an intermediate size range, relative to the entire EBF- population. The RAG-1+ population (Figure 4B, filled arrow) is also in this intermediate cell size range. In contrast, the majority of IgM+ cells are in the small size range (Figure 4C, open arrow, population ‘a’). There is also a second population population, consisting of larger cells, which express IgM (population ‘b’), and a population of intermediate cells, which express high levels of IgM (especially clear in the large fish sample, population ‘c’). This high IgM-expressing population likely consists of plasmablasts or long-lived plasma cells, which make large quantities of IgM for secretion. Because our experiments are conducted on fixed cells, the IgM antibody stains both membrane and cytoplasmic IgM. Interestingly, very few cells constitute IgM secreting cells in the small fish sample shown. Like IgM, the majority of Pax5+ cells are also small (Figure 4D, open arrow). Some Pax5+

## Expression of transcription factors during mammalian B cell development and activation

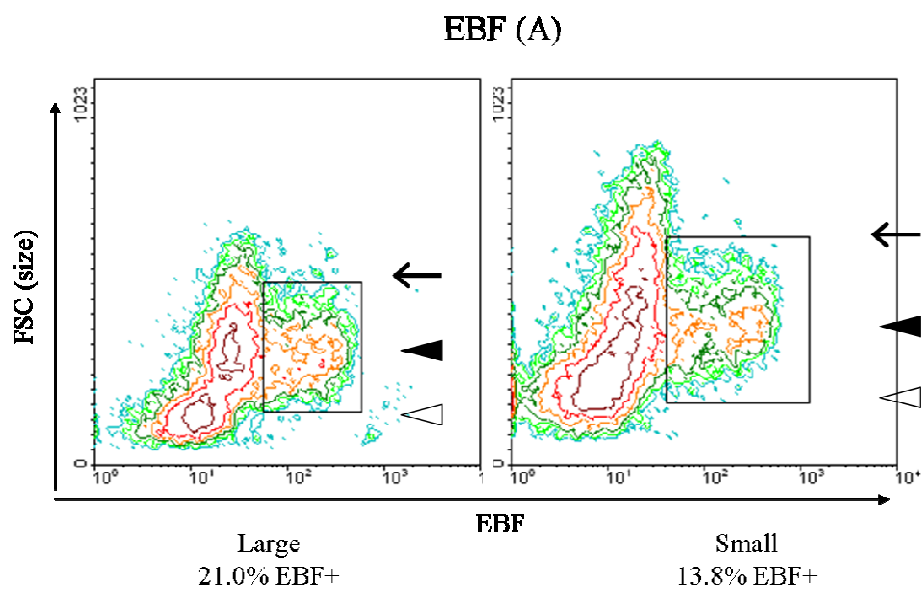


**Figure 3.** This figure is a schematic of the expression of the five proteins being studied in this project during B cell development and activation. The expression patterns shown are based on mammalian studies. Dotted lines represent gradual increases, decreases, or stages which are still being studied.

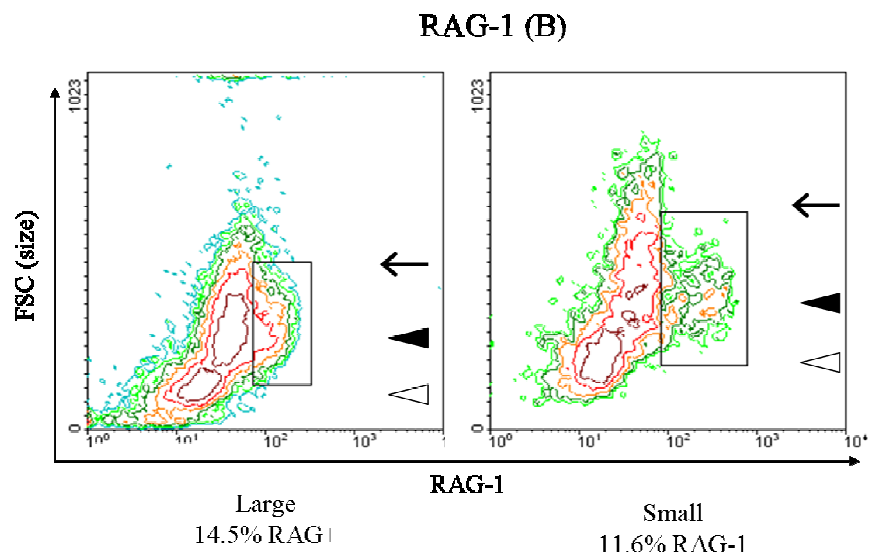




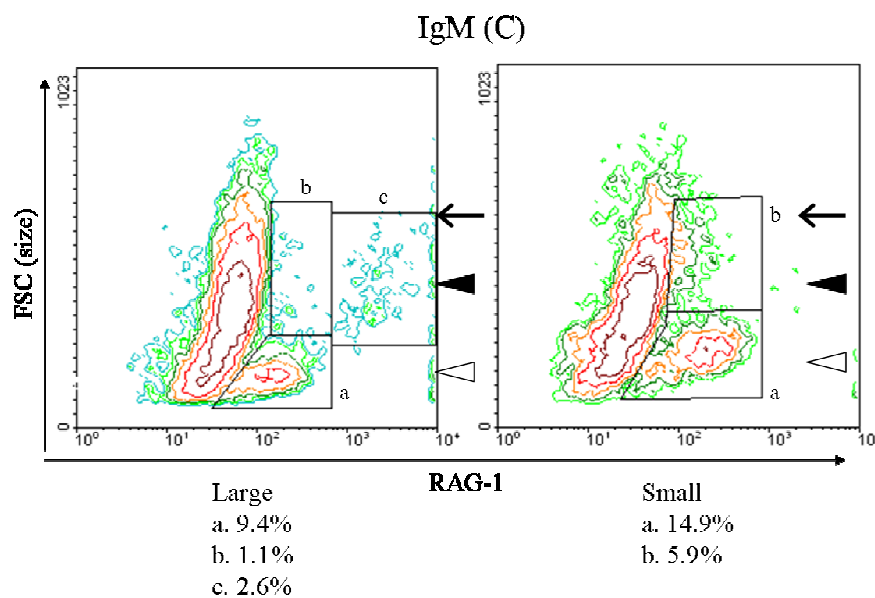
**Figure 4.** *K1 cells from a small and large fish were stained with each antibody. Positive populations are gated, and percent of total lymphocytes is given. Percentages are only representative of the sample shown.*



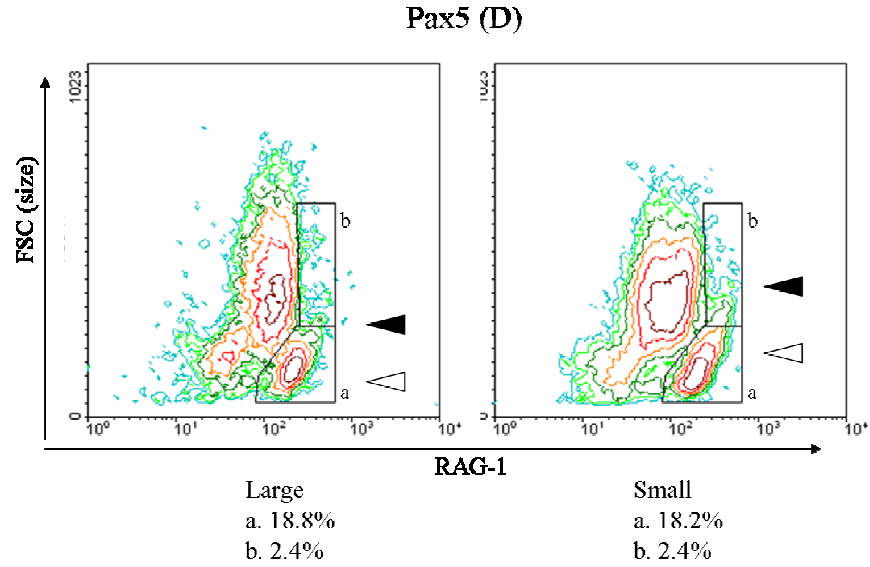
**4A) EBF.** *This antibody detects proteins in cells which are in the intermediate size range.*



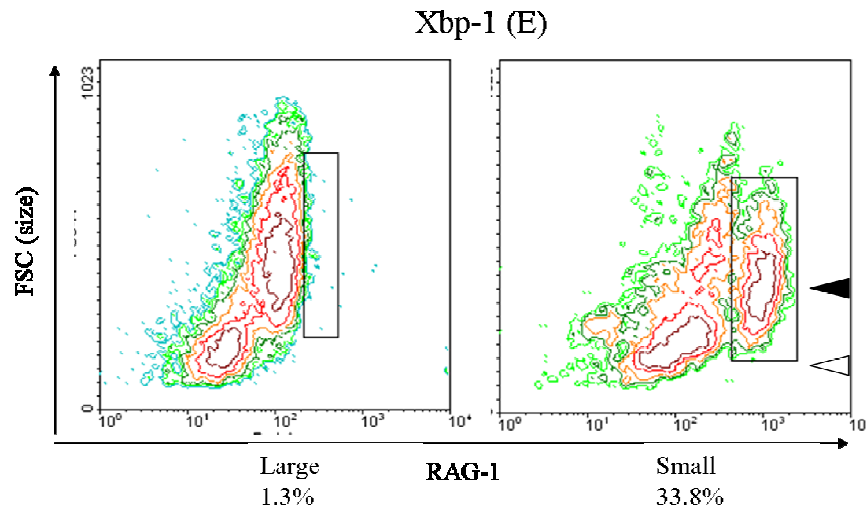
**4B) RAG-1.** This antibody detects proteins in cells which are in the intermediate size range, similar to the EBF+ population.



**4C) IgM.** The majority of IgM+ cells are in the small size range. There is also a population of larger cells which express IgM (b), and a population of intermediate size cells which express high levels of IgM (c) in the large fish sample.



**4D)** *Pax5*. The majority of *Pax5*+ cells are in the small size range.



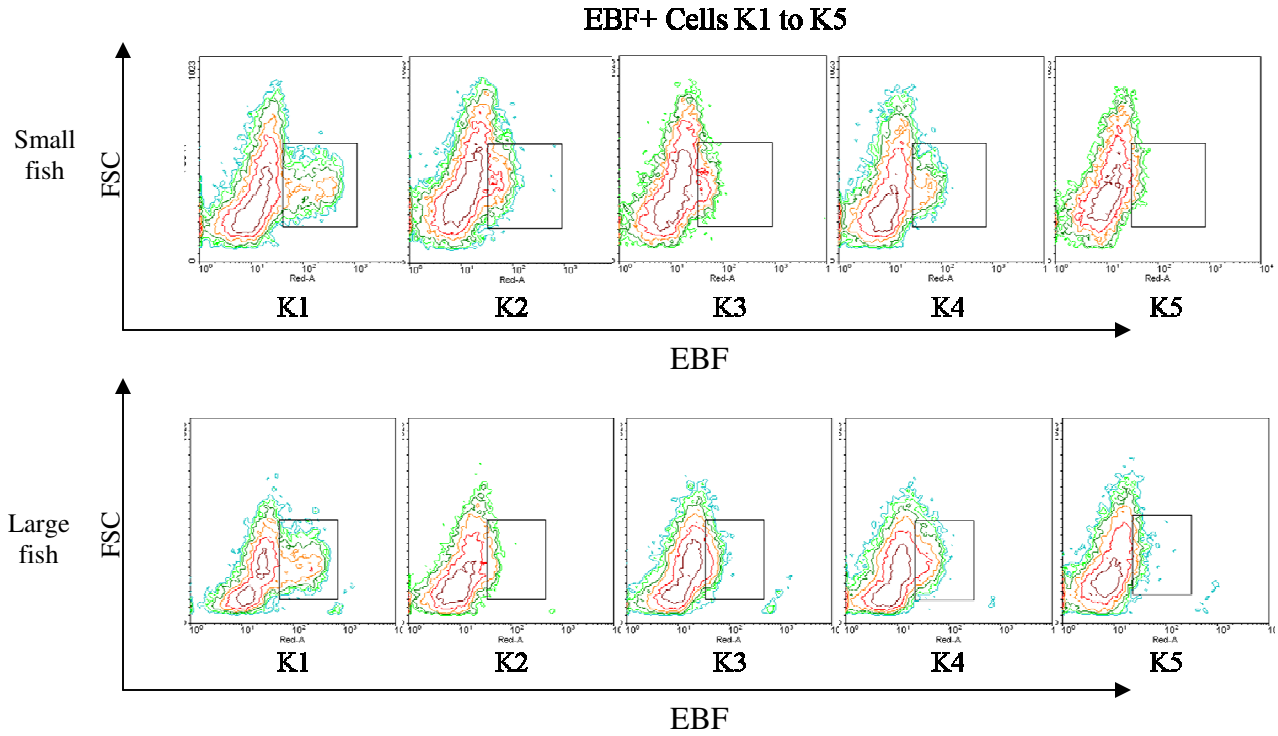
**4E)** The *Xbp-1*+ population is in the intermediate and large FSC range. The large fish did not show the positive population for *Xbp-1* as seen in the small fish

cells are also in the intermediate size range representing preB cells or plasmablasts. A similar pattern is observed in both the small and large fish samples shown. The expression pattern for Xbp-1 shows the most striking difference between small and large fish. As seen in Figure 4E, the large fish sample does not have an Xbp-1+ population, but the small fish sample shows a large population of cells expressing this protein. These cells are intermediate and large, suggesting they are early progenitors.

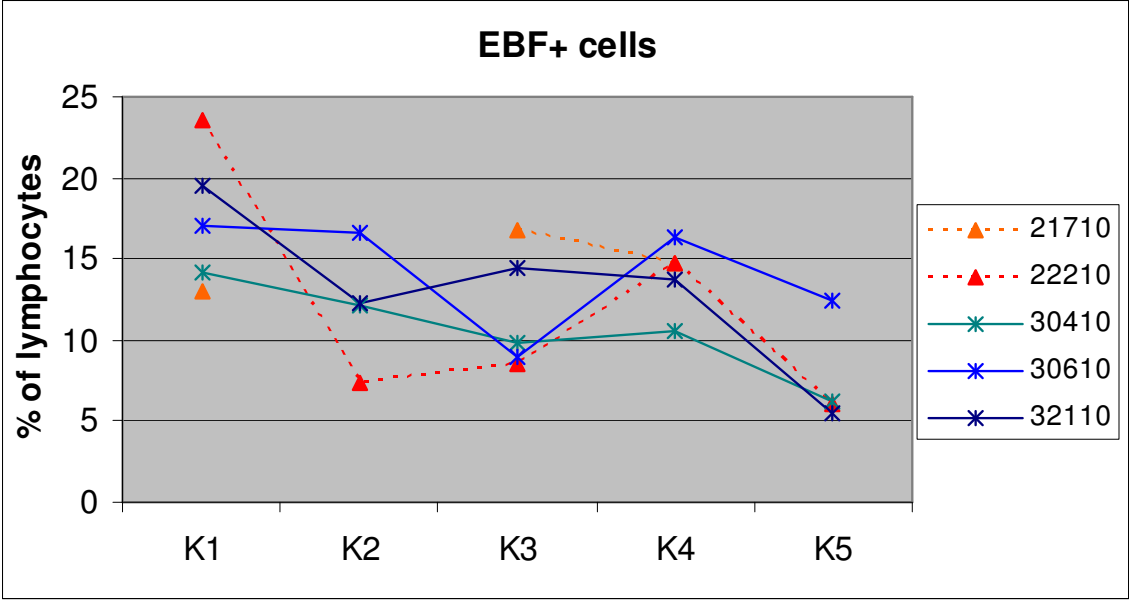
### 3.3 Protein expression along the kidney gradient

Next, the EBF, RAG-1, IgM, Xbp-1, and Pax5 positive populations were explored along the kidney gradient in small and large fish. Examining the percent of total lymphocytes which express the protein in each kidney section can indicate changes in B cell populations along the gradient. An example of the populations along the gradient is displayed in Figure 5. This figure shows the EBF+ population from K1 to K5 in both a small and large fish. There is a visible decrease in the population from K1 to K2 and K3. An increase in K4 can also be observed. This technique was used to find the percent of total lymphocytes which express each protein in the kidney, and these observations are shown in Figure 6.

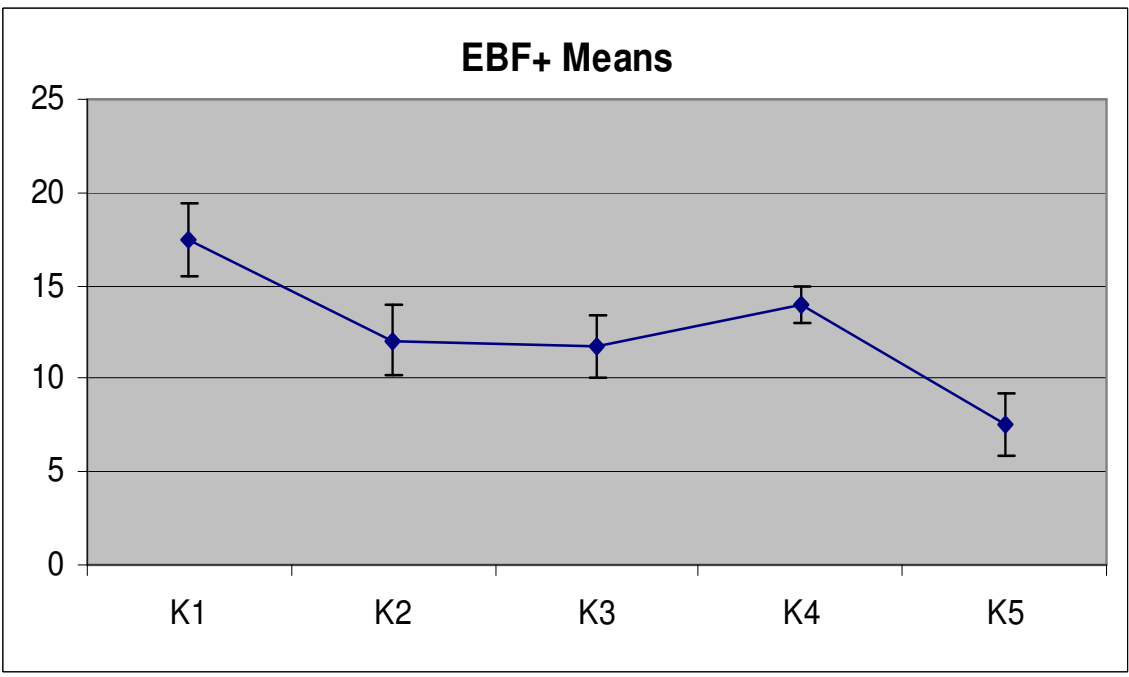
The most common trend observed in the EBF+ population along the kidney gradient is a relative decrease in frequency of EBF+ cells from K1 to K2 and K3, as well as an increase in K4 (Figure 6A). All four experiments display the highest frequency in K1, and a relative decrease in EBF+ cells from K4 to K5. The means and standard errors for this data are displayed in Figure 6B, which shows the general trend as described.



**Figure 5.** This figure shows the EBF+ population from K1 to K5 in both a small and large fish. There is a visible decrease in the population from K1 to K2 and K3. There is also an increase from K3 to K4, but there seem to be very few EBF+ cells in the K5 section of either fish size.



**Figure 6A.** This figure shows the percent of EBF+ cells along the kidney gradient. Dotted lines are large fish, solid lines are small fish. Trends seen in most experiments include a decrease from K1 to K3, and a second peak in K4.

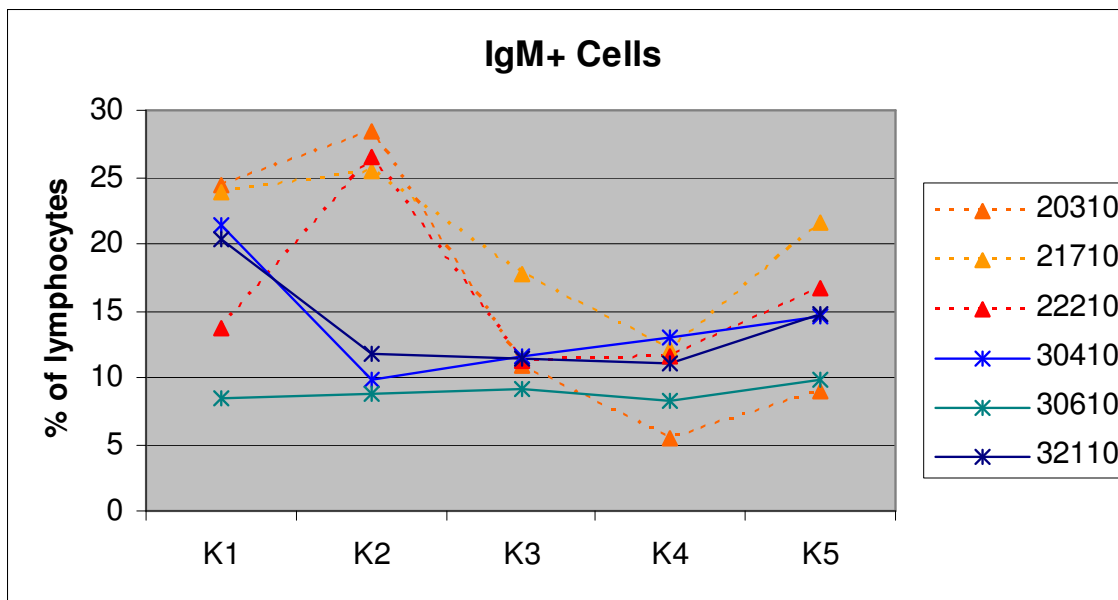


**Figure 6B.** This figure is a graph of the mean percentage of the EBF+ population in all experiments. The changes between K1 and K2 and between K4 and K5 are significant.

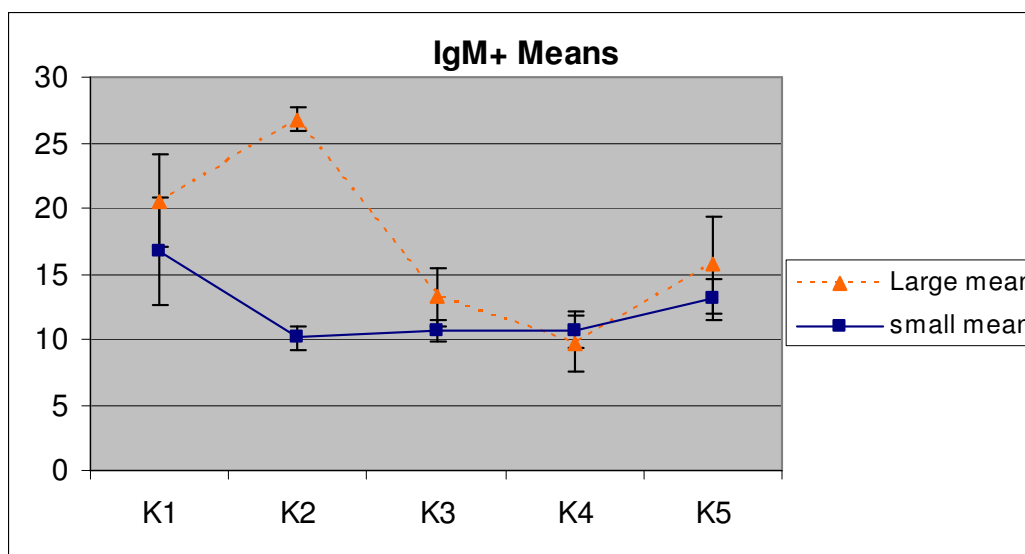
Figure 6C shows the percent of IgM+ cells in each of the five kidney sections. This population includes all IgM+ cells, including the 'b' and 'c' populations described in Figure 4C. Similar patterns can be seen within the small and large fish experiments. The large fish all show an increase in the percent of IgM+ cells from K1 to K2, followed by a decrease from K2 to K4, and another increase from K4 to K5. The small fish have less variation between kidney sections, but two of the experiments show a decrease in percent from K1 to K2 and a slight increase from K2 to K5. The means for the large and small fish are shown in Figure 6D. The most significant difference between the two fish groups is observed in the change between K1 and K2. In these sections, the large fish have an increase in the percent of IgM+ cells, and the small fish have a decrease in the percent of IgM+ cells.

The Pax5+ population in the kidney is shown in Figure 6E. The three large fish experiments all show a similar pattern: there is an increase in the percent of Pax5+ cells from K1 to K2, and a decrease from K2 to K4. One of the small fish samples also has this pattern. The other two small fish samples show a decrease from K1 to K4, and a slight increase from K4 to K5. The means of these experiments are shown in Figure 6F. This figure highlights the significance of the relative decrease in Pax5+ cells from K2 to K3, and the increase from K4 to K5.

Because of the lack of expression of Xbp-1 in large fish, as shown in Figure 4E, only small fish samples were studied for changes in the Xbp-1+ population down the kidney gradient. The Xbp-1+ population in three small fish experiments is shown in Figure 6G, and all three show a similar trend along the gradient. There is a decrease in percent of Xbp-1+ cells from K1 to K2, and all three show a second peak in K3.

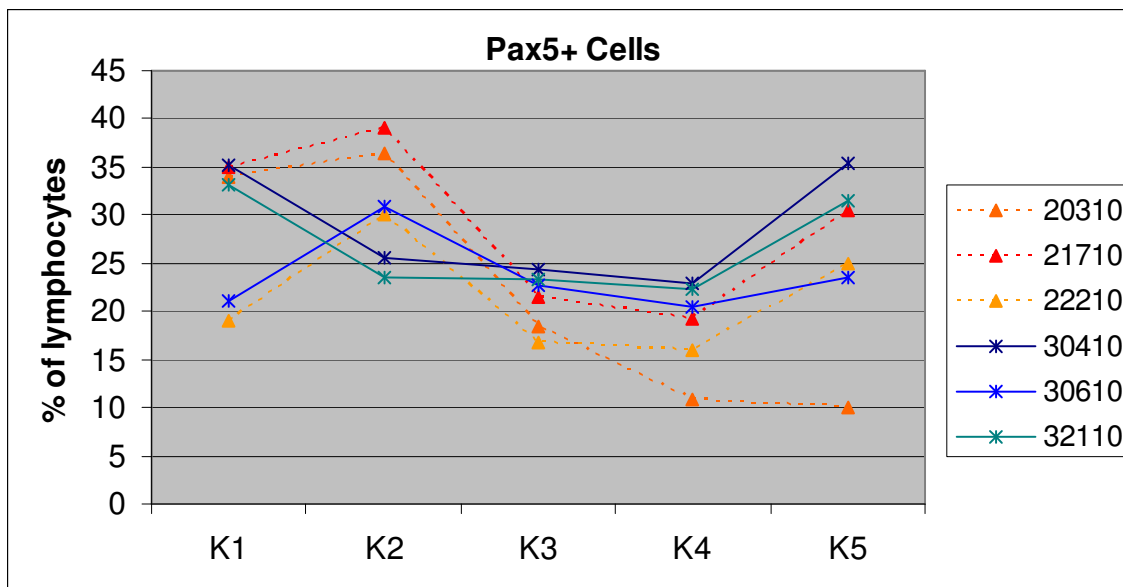


**Figure 6C.** This figure shows the percent of IgM+ cells along the kidney gradient. Dotted lines are large fish, solid lines are small fish. Similar patterns can be seen within the small and large fish experiments. The large fish all show an increase in the percent of IgM+ cells from K1 to K2, followed by a decrease from K2 to K4, and another increase from K4 to K5. The small fish have less variation between kidney sections, but two of the experiments show a decrease in percent from K1 to K2 and a slight increase from K2 to K5.

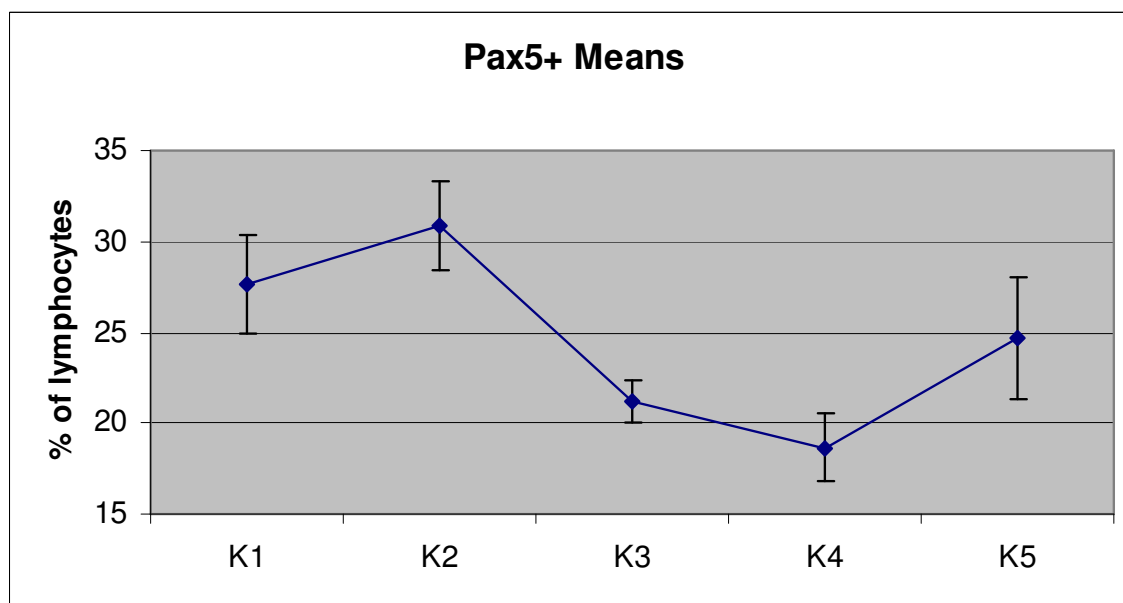


**Fig 6D.** This figure shows the mean percentage of IgM+ cells along the kidney gradient. Dotted line is the mean of the large fish experiments, solid line is the mean of the small fish experiments. Standard error bars are shown. The most significant difference is observed between K1 and K2. In this section, the large fish have an increase in IgM+ cells, and the small fish have a decrease in IgM+ cells.

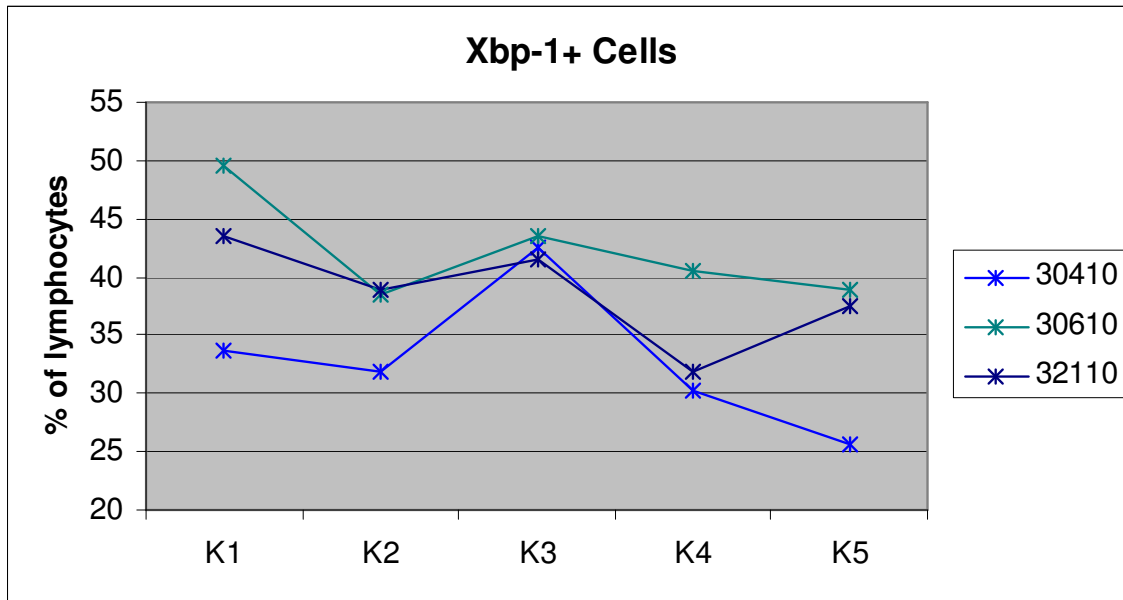




**Figure 6E.** This figure shows the percent of total lymphocytes that are Pax5+. Dotted lines are large fish, solid lines are small fish. The three large fish experiments all show a similar pattern. There is an increase in percentage of Pax5+ cells from K1 to K2, and a decrease from K2 to K4. One of the small fish samples also has this trend. The other two small fish samples show a decrease from K1 to K4, and a slight increase from K4 to K5.



**Figure 6F.** Mean percentage of Pax5+ cells for each kidney section. This figure highlights the significance of the relative decrease in Pax5+ cells from K2 to K3, and the increase from K4 to K5.



**Figure 6G.** This figure shows the percent of total lymphocytes that are Xbp-1+. All three experiments are small fish samples, and a similar trend can be observed in all of them. There is a decrease in percent of Xbp-1+ cells from K1 to K2, and all three show a second peak in K3.

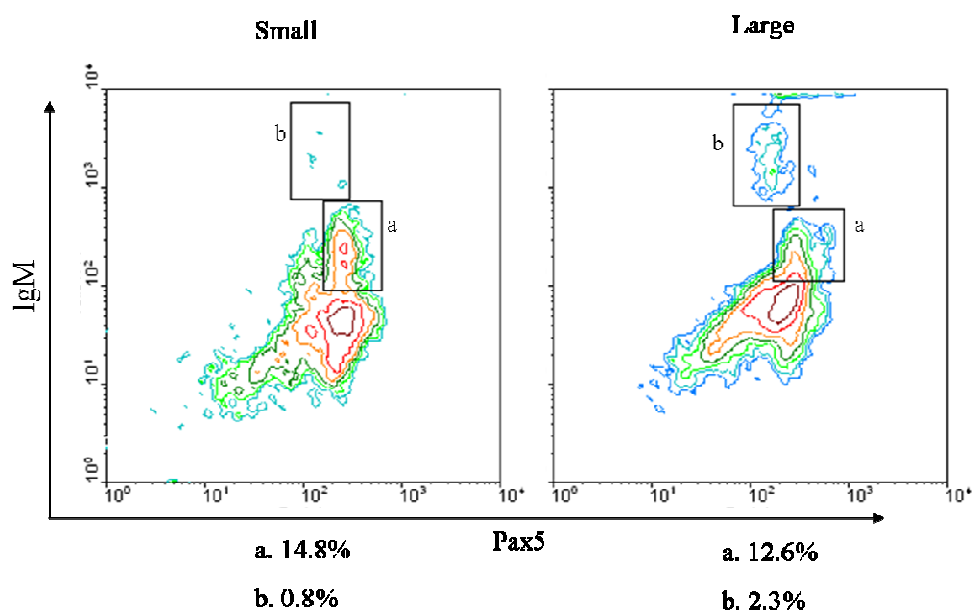
### 3.4 Defining B cell stages with two-color staining

The expression patterns of these five proteins have been extensively studied in the mammalian system of B cell development. Using this information, the expected expression profile for each major B cell development stage has been defined in Table 2. Two-color flow cytometry can show the expression of two proteins in each cell. Populations of cells which express neither, one, or both of the proteins can be defined as distinct types of cells by the definitions in Table 2.

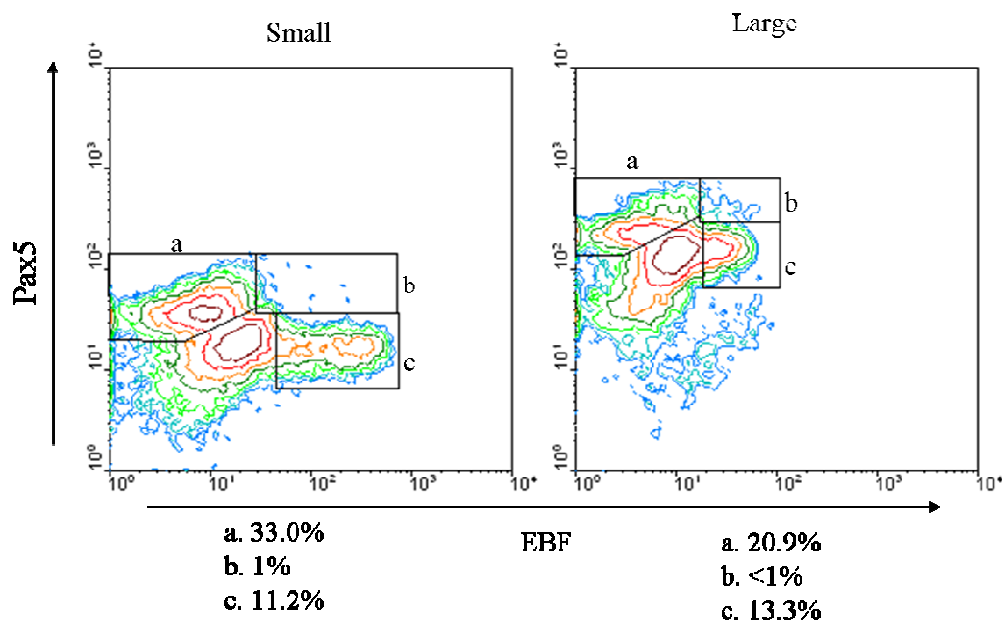
Figure 7 displays the B cell populations defined in this project by the expression of two proteins. The samples shown are K1 cells from a small and a large fish, and the percentages given are only representative of the sample shown. First, cells were costained for Pax5 and IgM (Figure 7A). The Pax5+/IgM+ population (a) is the preB, immature B, or mature B cell population, as defined in Table 2. The high IgM and low Pax5 population (b) are plasmablasts or plasma cells, as described in the introduction. Figure 7B shows results using the EBF and Pax5 antibodies. The Pax5+/EBF- population (a) is the preB, immature B, or mature B population, as described in Table 2. The Pax5+/EBF+ population (b) contains cells transitioning from the proB cell stage to the preB cell stage, and is usually a very small population. The Pax5-/EBF+ population (c) are CLPs and proB cells. The increased intensities shown for Pax5 in the large fish sample (Figure 7B, right panel) is not a change in expression, but is instead due to the use of the Pax5 antibody at the 647nm wavelength (instead of 555nm) as was used in the small fish sample. Alexa 647 has a stronger fluorescent intensity relative to Alexa 555. As in Figure 7B, in Figure 7C, EBF and IgM are shown to lack coexpression in lymphoid cells. The EBF+/IgM- population (a) is the CLP or proB

	<b>CLP</b>	<b>pro B</b>	<b>pre B</b>	<b>Immature B</b>	<b>Mature B</b>
<b>Cell Size</b>	Intermediate	Intermediate	Small	Small	Small
<b>Pax5</b>	--	low	+	+	+
<b><math>\mu</math> HC</b>	--	--	+	+	+
<b>RAG-1</b>	low	+	low	--	--
<b>EBF</b>	+	+	--	--	--
<b>tXbp-1</b>	+	+	--	--	low

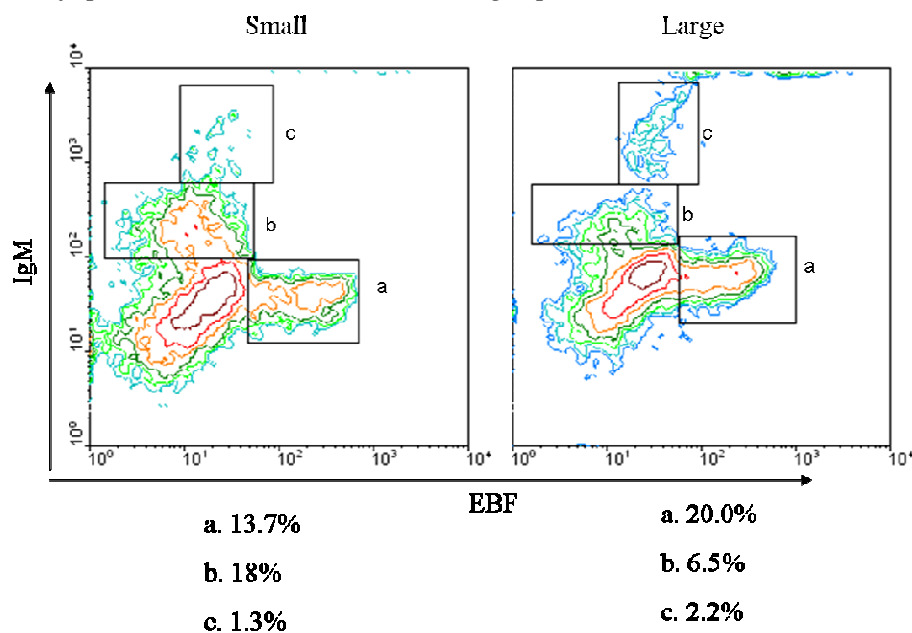
**Table 2.** This table summarizes the expected expression pattern of the five proteins and cell size during B cell development.



**Figure 7.** K1 cells from a small and a large fish were stained with combinations of two antibodies. Stage-defining populations are gated. **A)** Pax5 and IgM costain. The Pax5+/IgM+ population (a) is the preB, immature B, or mature B cell population. The high IgM and low Pax5 population (b) are plasmablasts or plasma cells. Percentages are of the total lymphocyte population, and only represent data from the fish shown here.



**Figure 7B)** *EBF* and *Pax5* do not costain. The *Pax5*+/*EBF*- population (a) is the preB, immature B, or mature B population. The *Pax5*+/*EBF*+ population (b) contains cells transitioning from the proB cell stage to the preB cell stage. The *Pax5*-/*EBF*+ population (c) are CLPs and proB cells. Percentages are of the total lymphocyte population, and only represent data from these samples. Note: left panel: *Pax5*~555, *EBF*~647; right panel: *EBF*~555, *Pax5*~647.



**Figure 7C)** *EBF* and *IgM* do not costain. The *EBF*+/*IgM*- population (a) is the CLP or proB population. *EBF*-/*IgM*+ population (b) consists of immature, or mature B cells. The very high *IgM* population (c) which also expresses low *EBF* could be plasmablasts or plasma cells. The percentages shown only represent the data from this sample.

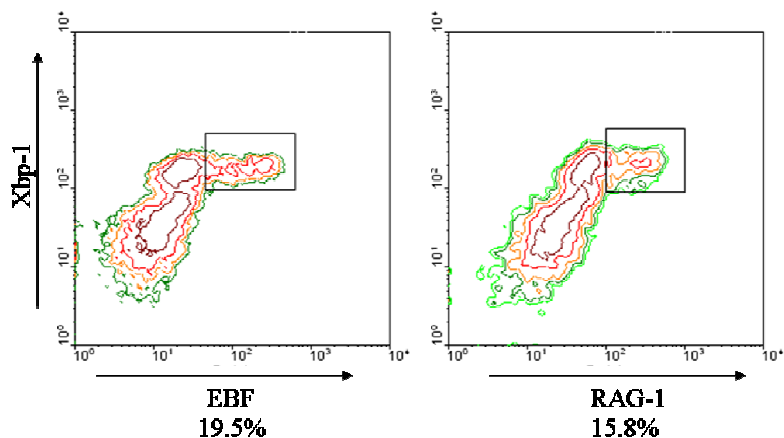
population. EBF-/IgM+ population (b) consists of immature or mature B cells. The very high IgM population (c) (which lacks EBF) could be plasmablasts or plasma cells. This population was also observed in Figure 7A, population 'b'. Figure 7D and E show the coexpression of Xbp-1 with EBF and RAG-1 in a small fish K1 sample. As previously explained, the lack of Xbp-1 expression in large fish samples eliminated the study of this population in large fish for the duration of the project. All of the EBF+ and RAG-1+ cells are also Xbp-1+. The EBF+/Xbp-1+ population consists of CLPs and proB cells, as defined in Table 2. The RAG-1+/Xbp-1+ population includes proB and preB cells.

### 3.5 B cell populations along the kidney gradient

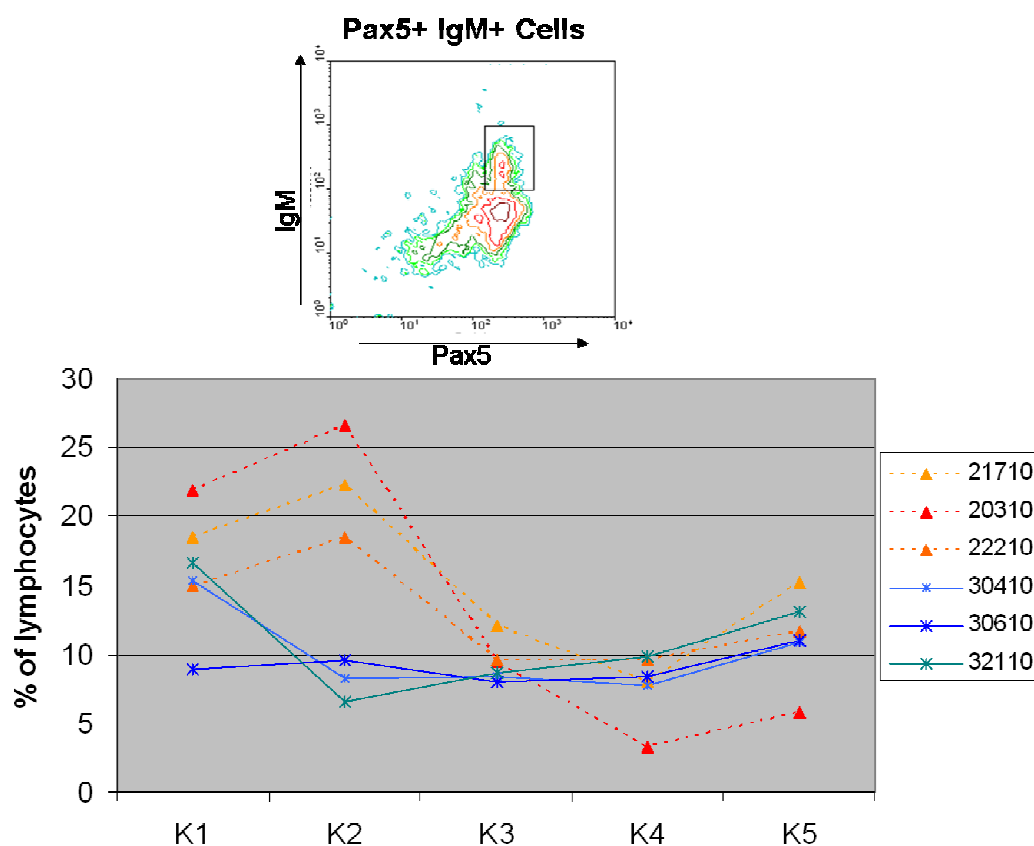
As shown in Figure 7, B cell populations were defined and gated based the presence of lack of specific coexpression using several combinations of two markers, using IgM, EBF, Pax5, RAG-1, or Xbp-1. In order to observe changes in B cell populations along the kidney gradient, the percent of lymphocytes in each population was determined in each kidney section for both small and large fish.

Figure 8 shows the preB, immatureB, or mature B population in each of the experiments, as defined in Figure 7A. These cells are small and positive for both Pax5 and IgM. The small fish do not show a significant trend along the gradient, but all of the large fish show a peak at K2, with a significant decrease at K3 and K4, followed by a slight increase at K5.

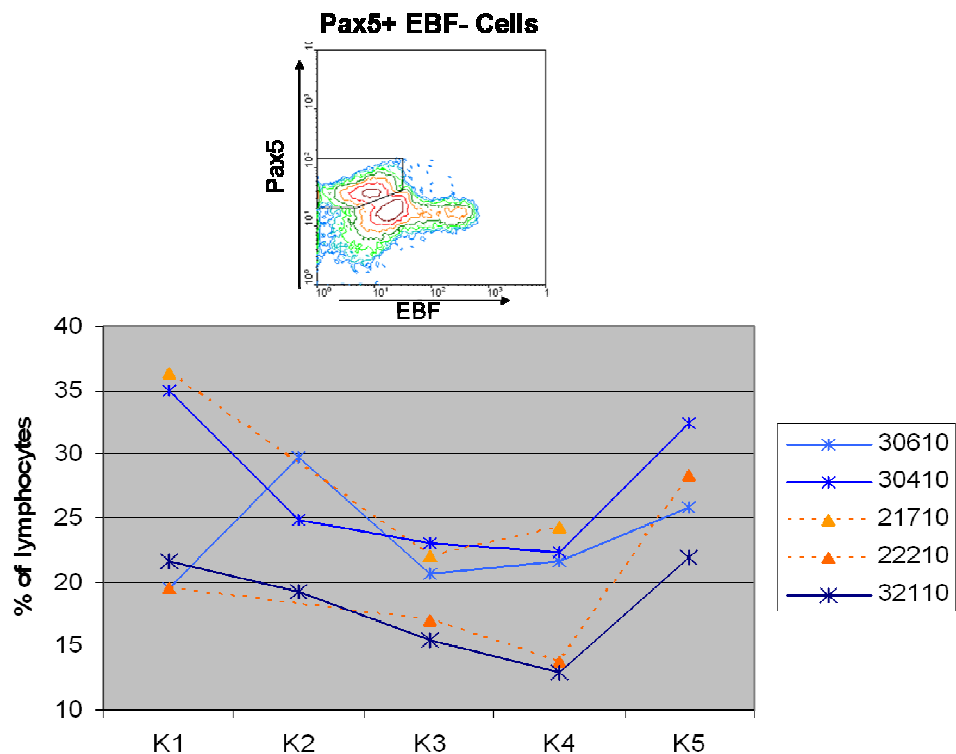
EBF-/Pax5+ populations are shown in Figure 9. These cells are preB, immatureB, or mature B cells. Unlike the Pax5+ IgM+ population in Figure 8, this population could include all Ig+ B cell populations (including IgD/IgT). Most of the experiments show a decrease from



**Figure 7D)** *K1* cells from small fish were stained with *Xbp-1* and *EBF* or *RAG-1* antibodies. The *Xbp-1*+/*EBF*+ population is the *CLP* or *proB* population. The *Xbp-1*+/*RAG-1*+ population is the *proB* or *preB* population.



**Figure 8.** This chart shows the *preB*, *immatureB*, or *mature B* population in each of the experiments, as defined in Figure 6A. Dotted lines are large fish, solid lines are small fish. The small fish do not show a significant trend from *K1* to *K5*, but all of the large fish show a spike at *K2*, with a significant decrease at *K3* and *K4*, followed by a slight increase at *K5*.



**Figure 9.** This figure shows the percentage of lymphocytes which are Pax5+ and EBF-. Dotted lines are large fish, solid lines are small fish. These cells are preB, immatureB, or mature B cells. Unlike the Pax5+ IgM+ population, this could include cells which are mature B cells but have a different Ig isotype. Most of the experiments show a decrease from K1 to K4, with an increase in K5.

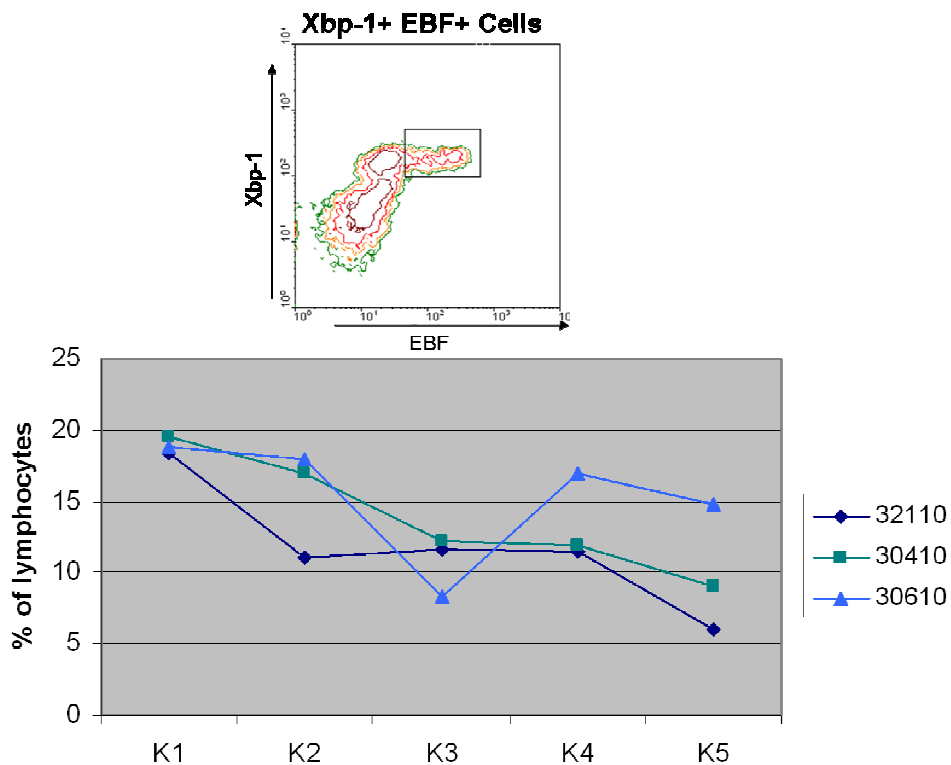


K1 to K4, with an increase in K5. Although this population should mostly consist of the same cells as the population in Figure 8, the small and large fish do not have different trends, and there is no increase in the percent from K1 to K2.

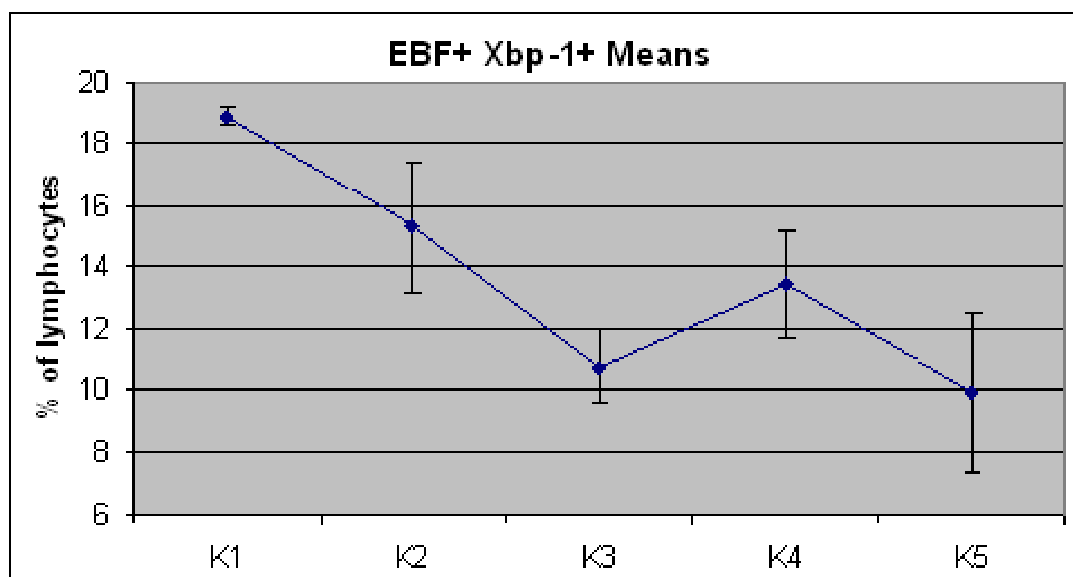
Figure 10 is a chart of populations of lymphocytes that are EBF+ and Xbp-1+. These cells are CLP, proB, or preB cells, as shown in Figure 7D. Only small fish were analyzed for this population, as their kidney tissues show expression of Xbp-1. All three independent experiments show a trend of a decrease from K1 to K3, and another decrease from K4 to K5.

Another population observed on the kidney gradient was the EBF+/IgM- population (Figure 11A). Interestingly, this population shows a similar trend in small and big fish. The means and standard errors for these experiments are shown in Figure 11B. Overall, there is a decrease in the relative population size from K1 to K3. A peak is noticeable at K4 in several of the experiments. All four experiments with data for K5 show a decrease from K4 to K5. This trend is very similar to the pattern observed in the EBF+/Xbp-1+ population. However, this population shows a peak in percentage in K4, while the EBF+/Xbp-1+ population is more subtle at this section.

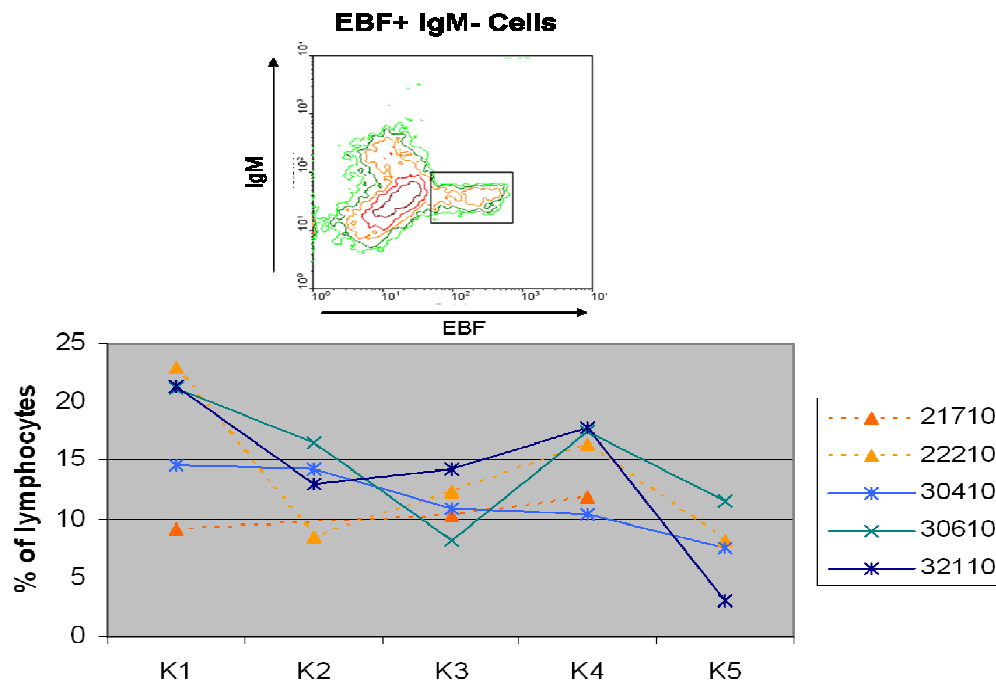
The RAG-1+/Xbp-1+ population was also analyzed along the kidney gradient (Figure 12). It is expected to be similar to the EBF+/Xbp-1+ population, although it may exclude CLPs. This population includes mostly proB and preB cells. All three experiments display a decrease in percentage from K1 to K2, and an increase in K4 and K5. However, there is variation in trends among the experiments from K2 to K4.



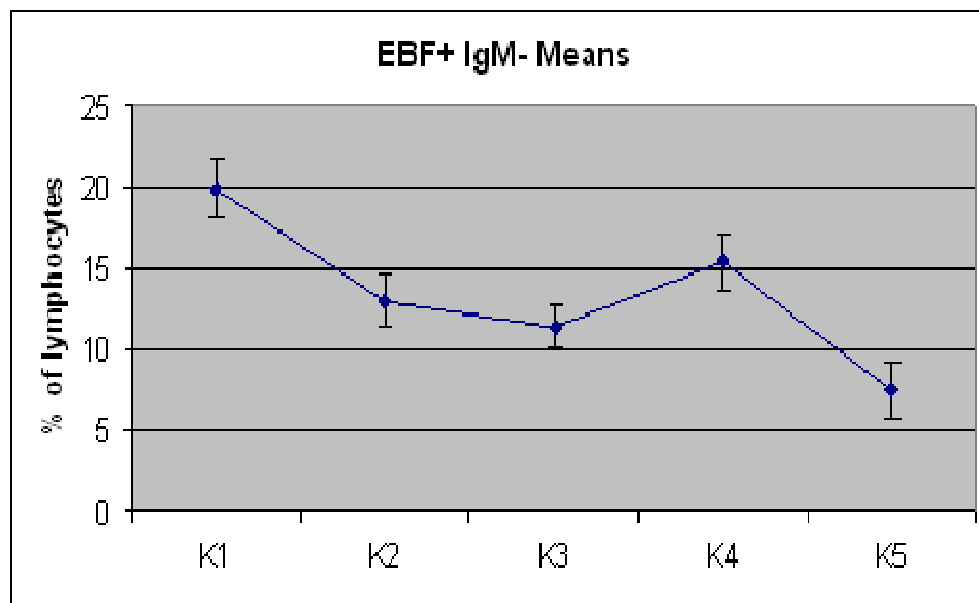
**Figure 10A.** This graph shows the percent of lymphocytes that are EBF+ and Xbp-1+. These cells are CLP, proB, or preB. Experiments shown are all with small fish samples. All three experiments show a trend of a decrease from K1 to K3, and another decrease from K4 to K5.



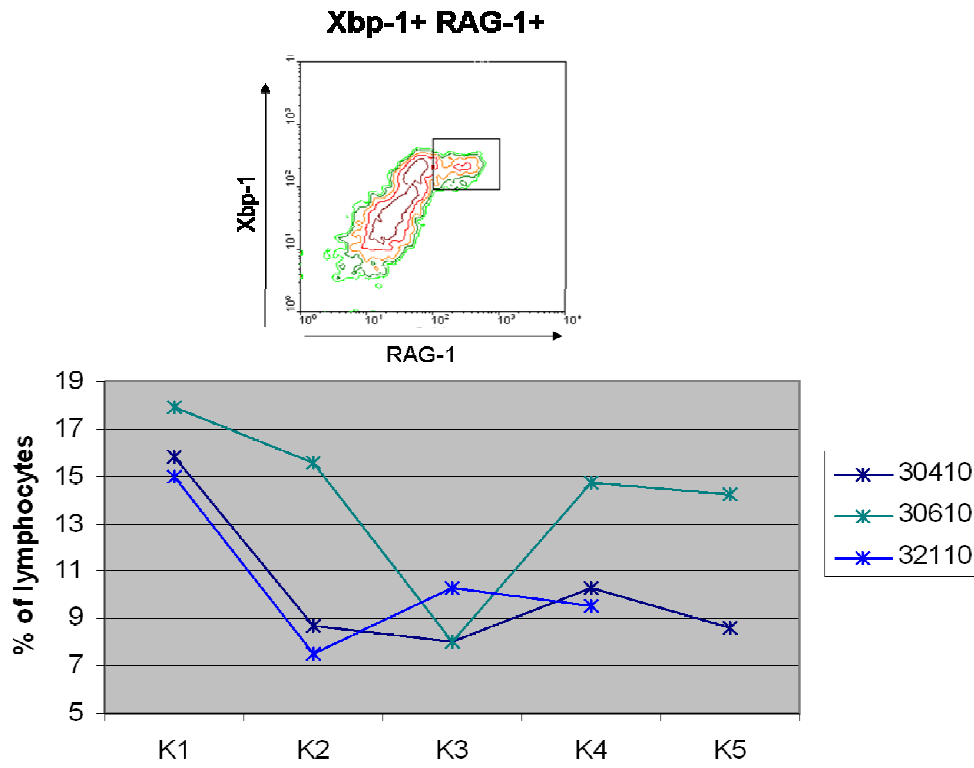
**Figure 10B.** This is a graph of the mean percent of the EBF+ Xbp-1+ population in all experiments. The standard errors indicate that the decrease from K1 to K2 and from K3 to K4 are significant. K1 and K2 have significantly larger percentages than K5.



**Figure 11A.** This figure charts the percent of lymphocytes which are EBF+ and Warrs-. Dotted lines are large fish, solid lines are small fish. Interestingly, this population shows a similar trend in small and big fish. Overall, there is a decrease in the relative population size from K1 to K3. A peak is noticeable at K4 in several of the experiments. All four experiments with data for K5 show a decrease from K4 to K5.



**Figure 11B)** This is a graph of the mean percent of the EBF+/IgM- population in all experiments. The standard errors indicate that the decrease from K1 to K2 and from K4 to K5 are significant, as well as the increase from K3 to K4.



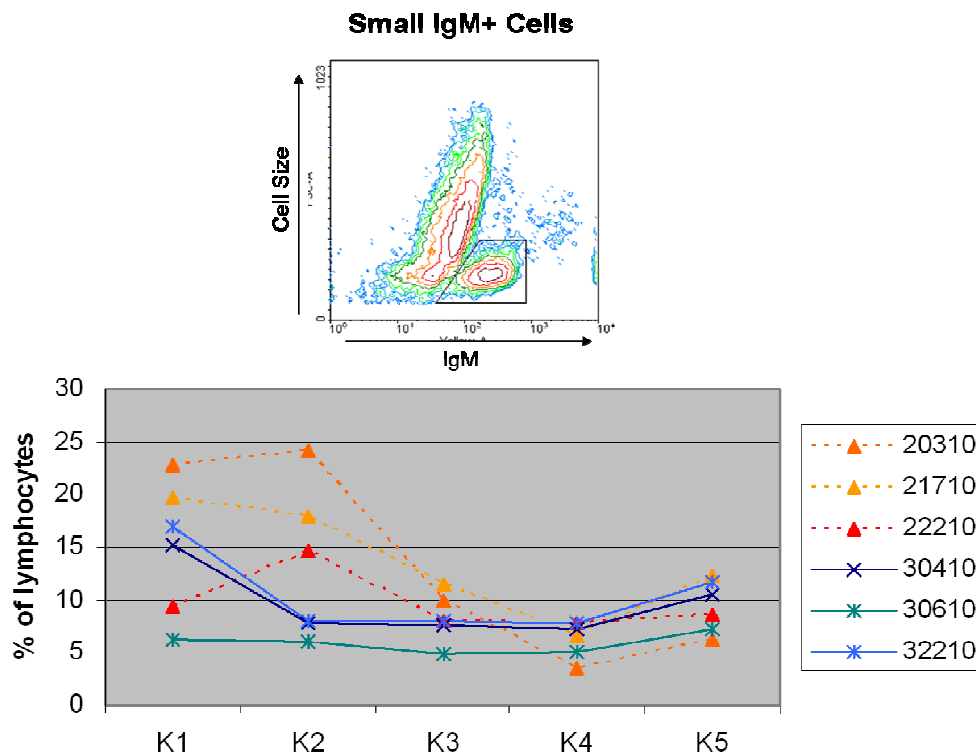
**Figure 12.** This graph shows the percent of lymphocytes which are *Xbp-1+* and *RAG-1+*. These cells are *proB* or *preB*. All three experiments display a decrease in percentage from K1 to K2, variation in K3 and K4, and a decrease in K5.

### 3.6 IgM positive populations

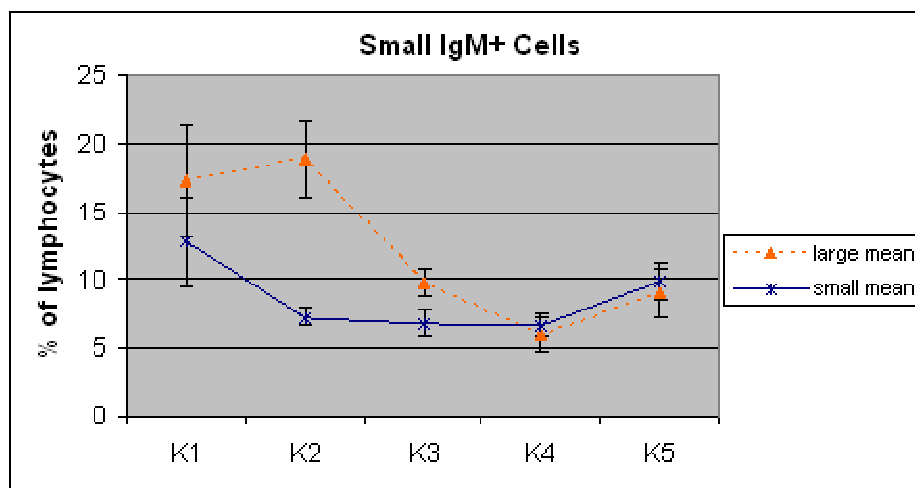
In addition to the coexpression of proteins, cell size can help define cell populations. As described in Figure 4C, there are three populations of IgM<sup>+</sup> cells, based on the amount of IgM each cell displays, as well as the cell size. The small cells which are IgM<sup>+</sup> are defined according to Table 2 as immature or mature B cells, as well as some early activated cells. This population was analyzed along the kidney gradient as well (Figure 13A). There is a large variety in the relative population size in K1 among the experiments, but this variation decreases down the gradient. The larger fish all show a decrease from K2 to K3 and to K4, and every experiment had an increase from K4 to K5. The means and standard errors for the small and large experiments are shown in Figure 13B. There is a significant difference between the small and large fish in K2 and K3. At K2, the large fish mean is near 18%, and the small fish mean is only about 7%. The other population analyzed along the kidney gradient was the high IgM<sup>+</sup> population consisting of intermediate sized cells (Figure 14). These cells are in the plasmablast stage or long-lived plasma cells. They are not likely plasma cells, as these are usually large. Although there are no noticeable changes along the kidney gradient, two of the larger fish had a large population of cells displaying this phenotype in all sections, while none of the small fish did.

The model in Figure 1 proposes that the development of B cells in the kidney follows the gradient. In other words, the earliest cells are the most anterior, and mature cells are more posterior. With this hypothesis, it is expected that there would be an inverse relationship between precursor and mature cells along the kidney gradient. This trend was analyzed by using the Pax5<sup>+</sup>/IgM<sup>+</sup> cells to represent the immature and mature B cell population, and the EBF<sup>+</sup>/IgM<sup>-</sup> cells to represent the precursor population (Figure 15). Because of the different trends observed

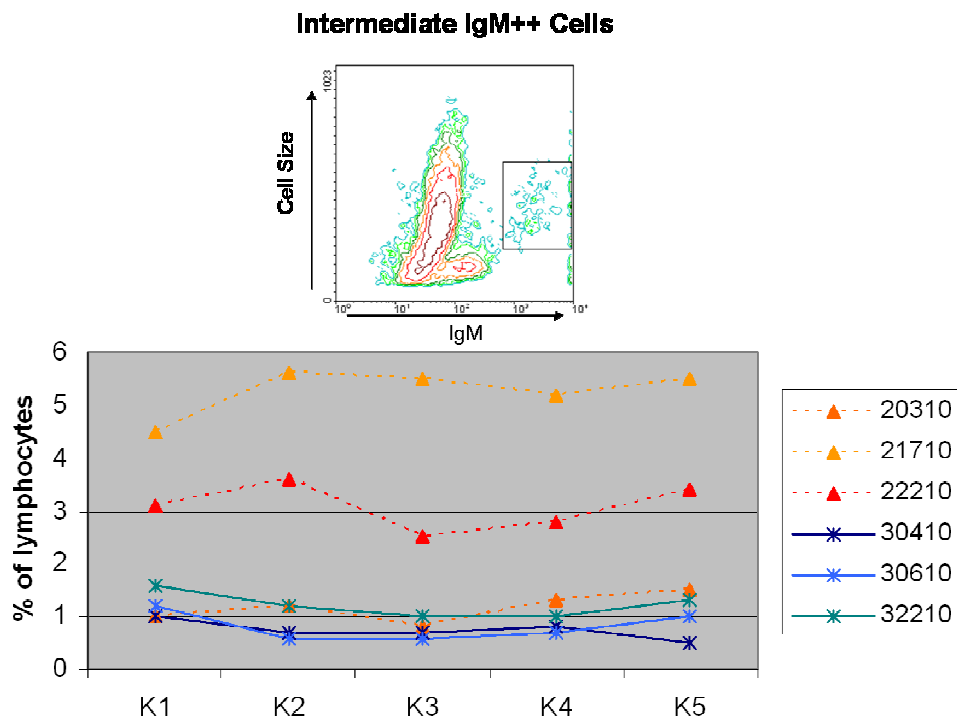
between the small and large fish mature B cell populations, these two groups were kept separate. In K1 of large fish, the mature and precursor populations make up equal proportions of the lymphocytes. However, at K2, the precursor population decreases, while the mature population increases. The other difference is the secondary peak in precursor cells at K4, where mature populations have decreased from K3. The mature cells in small fish do not seem to show this inverse relationship, as both the precursors and mature cells decrease from K1 to K3. Only from K4 to K5 do the populations act inversely. The precursor population decreases in this section, and the mature population increases.



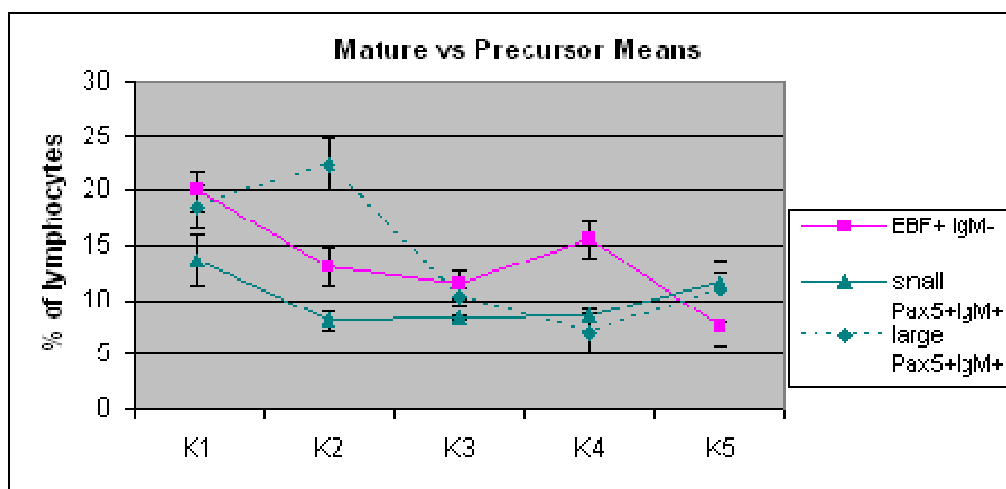
**Figure 13A.** This figure shows the percent of lymphocytes in each kidney section which are small and positive for IgM. Dotted lines are large fish, solid lines are small fish. As defined in Table 2, These cells could be immature B cells or mature B cells. Some may also be early activated cells. There is a large variety in the relative population size in K1, but this variation decreases down the gradient. The larger fish all show a decrease from K2 to K3 and to K4, and every experiment had an increase from K4 to K5.



**Figure 13B.** The means and standard errors of the small IgM+ population in small and large fish. There is a significant difference between the small and large fish in the K2 and K3 sections.



**Figure 14.** This figure shows the percentage of lymphocytes which have high levels of IgM, and are of intermediate size. Dotted lines are large fish, solid lines are small fish. These cells are likely activated cells in the plasmablast stage or long-lived plasma cells. Their size indicates that they are not likely plasma cells. Although a trend along the kidney gradient is not noticeable, two of the larger fish had a large population of cells displaying this phenotype.



**Figure 15.** The means of a mature and precursor population. The precursor population is the mean of all experiments, the large and small fish mature B populations are separate. The large fish show an inverse relationship between the precursor and mature populations, but the small fish do not display this relationship.



## IV. Discussion

The main goal of this project was to identify the developing B cell populations in the trout kidney. The anterior kidney has been previously described as the functional equivalent of the mammalian bone marrow and is expected to generate the majority of B cells [17, 18]. There have been some reports on B cell populations and hematopoiesis in the anterior kidney but very little is known about the distinction of B cell populations throughout the kidney's length. This lab's previous work created a model (Figure 1) based on separation of cells by cell density and the ability of these populations to be activated [21]. However, distinctions between the earliest B cell development stages within the kidney were not made. This project developed through the need to further explore the B cell developmental gradient along the trout kidney in conjunction with an activation gradient. In accordance with the model, it is hypothesized that a developmental gradient exists from the anterior to posterior kidney, and this creates an inverse relationship between the precursor and mature B cell populations along the gradient. It is also hypothesized that Xbp-1 expression in the anterior kidney is occurring in precursor B cell populations or long-lived plasma cells.

### 4. 1 Differential expression of Xbp-1

Initial flow cytometry experiments in the anterior kidney provided surprising results using the Xbp-1 marker. Preliminary testing on small (<10 inch) fish showed significant populations of Xbp-1+ cells in the spleen and anterior kidney, but more recent samples using larger adult fish did not detect this population in the kidney, although it was present in spleen. Therefore, it was determined that reagents were not affecting these results, and the fish themselves must account for this discrepancy. For that reason, a major focus of this project is the

difference between the large and small fish B cell populations, in particular, those cells which express Xbp-1.

If the discrepancy in Xbp-1 expression was due to a significant difference in the presence of a specific B cell population, expression of other proteins in those stages should also differ significantly. The expression of Xbp-1 in early developing cells is not very well studied, although Brunsing et al detected the function of the kinase which cleaves Xbp-1 mRNA in developing B cells. The action of this kinase, which is specific for the Xbp-1 mRNA in the unfolded protein response pathway, is a strong indication that Xbp-1 is being expressed and functioning in these cells [16]. This study indicates that there are some differences in the percent of developing B lymphocytes which are positive for EBF and IgM. However, none of the other antibodies detected such a striking result as Xbp-1. The Xbp-1/EBF and Xbp-1/RAG-1 costain experiments showed that all RAG-1+ and EBF+ cells also express Xbp-1. The Xbp-1+ cells which do not express RAG-1 or EBF could be activated or plasma cells, or non B cells. The connection between Xbp-1 expression in progenitor cells and a lack of an Xbp-1+ population in large fish remains poorly understood.

#### 4. 2 Defining the IgM subpopulations

Interesting observations were made by analyzing the pattern of expression of different IgM+ populations. Because the small IgM+ cells make up the majority of IgM+ cells, their expression across the gradient is reflected in the Figures 6C and 6D of total IgM+ cells. The intermediate size cells which express high levels of IgM are of interest because Zwollo previously described these cells using flow cytometry and PCR [18]. These cells could be plasmablasts or long-lived plasma cells, which are known to home to the bone marrow in

mammals [30]. If this population is, indeed, a long-lived plasma cell population, this would be yet another functional characteristic that anterior kidney shares with the primary immune organ of mammals, the bone marrow. These intermediate size cells with high IgM expression are detectable at significant amounts in all areas of the kidney for two of the large fish samples, but none of the small ones (Figure 14). Large fish are more likely to have had previous infections, and therefore have created pools of long-lived plasma cells. Young fish may not have faced much antigen challenge in their lifetime, and have not yet built up a pool of these cells. It is important to note that the IgM<sup>+</sup> populations do not necessarily reflect the entire mature or activated B cell population. At least three other isotypes have been described in teleosts: IgD, IgT, and IgZ, [33, 34] but no antibodies are yet available for these isotypes.

#### 4.3. The immature and mature B cell population

The markers of mature B cells, IgM and Pax5, display similar trends in the positive populations. There is a marked difference in the expression pattern in small fish and large fish. This is especially obvious for IgM, as the large fish have significantly larger populations of IgM<sup>+</sup> cells in K2 than the small fish have (10.1% compared to 26.8% on average for large and small fish respectively). There is also a difference in the pattern along the gradient between the two groups. Large fish have an increase in the relative amount of cells which express IgM from K1 to K2, but sharply decrease through K4. Small fish actually show a decrease from K1 to K2, but both groups show an increase from K4 to K5. This may mean that as fish age, the function of the anterior kidney sections changes, while the posterior kidney continually harbors similar B cell populations throughout life.

With the exception of one experiment, the trend along the kidney gradient of the Pax5+ population mimics the trend of the IgM+ population. Two differences are noticeable. The large and small fish do not differ as much for Pax5 as they did for IgM, particularly in K2. In addition, the percentages are significantly higher for Pax5 (5-10% increase) compared to the IgM percentages. This is likely due to the presence of cells which express other Ig isotypes instead of IgM, but still express Pax5.

Along the kidney gradient, the Pax5+/IgM+ population trend reflects the pattern of the single stains for both proteins. The Pax5+/EBF- population, representing immature or mature B cells) does not show the peak in K2 except in one experiment. The trends in this population more closely reflect the hypothesized movement of maturing cells proposed in the model, because there is a large relative increase to K5 from the mid kidney sections. Data published in 2008 also indicate a presence of mature B cells in the anterior kidney, with an IgM+ population which expressed Pax5, and some also expressed low levels of secreted Ig [18]. This project did not explore secreted Ig levels independent of total Ig, as the secreted levels are expected to be relatively low in the kidney. However, the size of the cells and the low levels of total Ig indicate that the population defined in this study is, indeed, mostly composed of mature B cells [4].

#### 4.4 Precursor/mature B cell dynamics

The model proposes a spatial specialization within the kidney. If the anterior kidney is more specialized for hematopoiesis and the earliest lymphoid development stages and the posterior kidney acts as a secondary lymphoid organ, with a gradient in between the two, it is expected that an inverse relationship would emerge between the precursor and mature B cell populations along the gradient. Figure 15 displays this relationship using two of the defining

populations, EBF+/IgM- and Pax5+/IgM+. The precursor population includes all experiments because the pattern is not dependent on the size of the fish. The mature B cell population was divided between the two groups because of the large differences in certain sections of the kidney. Interestingly, the inverse relationship is clear for the entire kidney in large fish. In K1 and K2, the large fish have more mature B and the small fish have more early progenitors. In K3 through K5, the inverse relationship between developing B and mature B cells is independent of fish size. It is possible that younger fish have not yet developed the spatial specialization as seen in the older fish. It is important to also recognize the precursor population in the K4 area, as observed in Figures 10B, 11B, and the expression of RAG-1 or EBF alone. The presence of this population may indicate another region of hematopoiesis in K4, which could be due to several factors including a possible difference in renal function in this area, or some other factor which lowers the level of B cell development in the K2 and K3 sections.

#### 4.5 Directions for future studies

In some ways the data in this project uphold the proposed model, such as the clear presence of developing B cells in the anterior kidney, and their decrease towards the posterior kidney (Figures 10B, 11B). In addition, some samples contained what are likely long-lived plasma cells which returned back to the kidney following an infection (Figure 14). However, continued experiments on this project may lead to some changes in the model. The data in this study indicate that a second site of hematopoiesis and B cell development exists in K4. With additional studies, this could be added to the model. The model should also include a distinction between juvenile and adult fish.

The reason for a lack of Xbp-1 expression in the kidney of larger fish is still unclear. The precursor populations do not seem to differ greatly between the two groups, based on EBF and RAG-1 expression. Therefore, a decrease in hematopoiesis and precursor populations cannot account for the difference in Xbp-1 populations. However, continuing to determine the full extent of the differences between the juvenile and adult fish could be very useful in treatment protocols of aquaculture animals, as well as in comparative immunology studies. Ontogeny research in teleosts generally terminates the study shortly after hatching. This project sheds light on continued development of the adult immune system through the first several months of life, and should encourage ontogeny research to extend their studies through the juvenile stage.

A lack of reagents, especially teleost-specific antibodies, has been a major obstacle in this study. For example, the EBF/Pax5 costain showed very few cells which expressed both proteins. According to mammalian studies, Pax5 is expected to be turned on early enough to costain with EBF in the late proB and preB cell stages [31]. Pax5 has multiple alternatively spliced forms, and their expression at early developmental stages may be detected by PCR techniques, but not by this antibody in flow cytometry. Our lab is currently testing antibodies which detect these spliced forms by flow cytometry. Unpublished data by Dr. Zwollo on comparative studies in mice reveal the presence of these spliced forms in the CLP and proB stages, as detected with the new antibodies. Therefore, the lack of EBF and Pax5 costaining with our current antibody may be due to its inability to detect developing B cells which are still expressing EBF but are also making spliced Pax5 transcripts. In order to continue distinguishing the specific stages of B cell development in teleosts by flow cytometry, the design and development of the antibodies for the alternative spliced Pax5, other B cell surface markers and the light chain of the B cell receptor

will be critical. This will help achieve the final goal of creating a B cell profile in teleosts which can be used for the identification of health in lymphocyte populations of individuals.

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