2012

Pax5 Signatures: The Identification of Pax5 Isoforms in Developing and Activated B Cell Populations of Rainbow Trout

Elizabeth Anne MacMurray
College of William & Mary - Arts & Sciences

Pax5 Signatures: The Identification of Pax5 Isoforms in Developing and Activated B Cell Populations of Rainbow Trout

Elizabeth Anne MacMurray

Montclair, Virginia

Bachelor of Science, College of William and Mary, 2010

A Thesis Presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

Department of Biology

The College of William and Mary
August, 2012

APPROVAL PAGE

This Thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

Elizabeth Anne MacMurray

Approved by the Committee, July, 2012

Committee Chair
Professor Patty Zwollo, Biology
The College of William and Mary

Professor Lizabeth Allison, Biology
The College of William and Mary

Associate Professor Diane Shakes, Biology
The College of William and Mary

COMPLIANCE PAGE

Research approved by

Institutional Animal Care and Use Committee

Protocol number(s): 2009-03-03-5861-pxzwol

Date(s) of approval: 6/15/2011

ABSTRACT PAGE

Pax5 is an alternatively spliced transcription factor that regulates B cell development, activation, and differentiation. Although misexpression of Pax5 is associated with malignancies such as lymphomas, research has yet to elucidate the function of specific Pax5 isoforms and to further correlate these isoforms with disease or B cell developmental stages. Our study hypothesized that in the anterior kidney, blood, and spleen of rainbow trout, specific Pax5 isoforms characterize individual B cell populations as a means of modulating Pax5 activity. Using flow cytometric analyses with antibodies recognizing the Pax5 paired domain, exon 6 domain, and C-terminus, we demonstrate that B cell populations differentially express Pax5 isoforms. Additionally, using the developmental markers Xbp1-S, EBF, and HCmu, we reveal that Pax5 isoforms lacking a paired domain are present at the earliest stages of B cell development. Furthermore, we discover for the first time in trout that these early developing B cell populations exist in the secondary immune tissues, blood and spleen. With these findings, we propose a model in which the stages of B cell development and activation are delineated by combinations of Pax5 domain expression which we have termed the "Pax5 signature". The identification of Pax5 signatures with flow cytometry provides a means to assess aberrant molecular expression in B cell populations and has important implications for clinical diagnostics.

Table of Contents

Acknowledgements	ii
List of Tables	iii
List of Figures	iv
Chapter 1. Introduction	1
1.0 B cell development	2
1.1 Hematopoiesis and CLPs	3
1.2 Pro-B-mature B cell development	5
2.0 B cell activation	7
3.0 The role of Pax5 in B cell commitment	12
3.1 Pax5 and Pax family	12
3.2 Pax5 in B cell development and activation	14
3.3 Alternative splicing	15
4.0 Rationale for the current study	21
5.0 Flow cytometric analysis	23
5.1 Flow cytometer fluids	23
5.2 FSC and SSC	24
5.3 Fluorescence	24
5.4 Data interpretation	26
6.0 Hypothesis and research aims	28
Chapter 2. Manuscript	32
2.1 Introduction	32
2.2 Methods	35
2.3 Results	40
Chapter 3. R and S Fish	71
3.1 Introduction	71
3.2 Results	71
Chapter 4. Discussion	80
4.1 The Pax5 patterns of early developing B cells	80
4.2 The Pax5 patterns of late developing B cells	83
4.3 The Pax5 patterns of activated B cells	84
4.4 The Pax5 patterns of peripheral blood	86
4.5 Pax5 isoform model	87
4.6 Pax5 signatures	89
4.7 Flavobacterium resistant versus susceptible fish	91
4.8 Limitations to the current study	92
4.9 Future studies	94
References	97

Acknowledgements

I would first and foremost like to express my deep gratitude to my advisor, Professor Patty Zwollo, for her patience, guidance, and enthusiasm for this project. I am grateful for her constant encouragement and inspiration, without which this thesis would not have been possible. I would also like to extend my gratitude to my committee, Professors Lizabeth Allison and Diane Shakes, for their incredible support and useful critiques on this thesis. Thank you for challenging me and helping me perform to the highest capacity. Thank you all for showing me the value of a wonderful teacher.

I would like to thank the Kaattari lab (Steve and Ilsa Kaattari, and Mary Ann Vogelbein) for their invaluable guidance, support, and assistance with the fish dissections.

I would also like to thank the entire Zwollo lab for their awesome assistance and constant excitement for science. Thank you Laura Stephens, Emily Fruchterman, and Poorna Dharmasri for their hard work collecting data and for making the flow cytometry group amazing.

I would like to thank Chris Pallister who supported this thesis from the beginning and helped push it into reality. I wish to thank my fellow grad students and friends for their endless support while completing the project.

Lastly, I would like to thank my family, my parents, my sister Erin, and significant other Dan, for taking the time to read my thesis, support my crazy science thoughts, and listen to my science woes with a smile. I couldn't have done this without you.

List of Tables

Table	Page
1. Expression of Pax5 isoform and development markers during B cell stages	42
2. Average % frequency (S.E.) of Pax5 subpopulations across immune tissues	48
3. Average % frequency (S.E.) of LPS activated SPL cells	65
4. Average frequency of proliferating and non-proliferating Pax5.E10 cells (S.E.)	73
5. Average frequency of proliferating and non-proliferating Pax5.E6 cells (S.E.)	73

List of Figures

Figure		Page
1. 1	Stages of B cell development characterized by molecular patterns	11
1.2	Murine Pax5 isoforms shown as cDNA sequence	17
1.3	Trout Pax5 isoforms shown as cDNA sequence	20
1.4	Flow cytometer process	25
1.5	Flow data graphs	27
1.6	Model of Pax5 isoform modulations of B cell development	31
1.	Diagram of trout Pax5 antibody specificity	41
2.	Western blots analysis on trout spleen (SPL), blood (PBL), and anterior kidney (AK) using three Pax5-specific antibodies	43
3.	Two-color flow cytometry of AK, PBL, and SPL	45
4.	Two-color flow cytometry using Pax5.PD and Pax5.E10 antibodies	47
5.	Two-color flow cytometry using the Pax5.PD and Pax5.E6 antibodies	50
6.	Three-color flow cytometry for Pax5.E6, HCmu, and Xbp1 in AK, PBL, and SPL	53
7.	Two-color flow cytometry for cells cultured in *E. coli* LPS	57
8.	Change in relative intensity of Pax5.E6 and Pax5.E10 expression in LPS-activated SPL cells	59
9.	Change in relative intensity of Pax5.PD and Pax5.E10 expression in LPS-activated SPL cells	60
10.	Change in relative intensity of Pax5.PD and Pax5.E6 expression in LPS-activated SPL cells	62
11.	Two-color flow cytometry contours of LPS-activated SPL cells stained with anti-Pax5 and anti-HCmu antibodies	63
12.	Two-color flow cytometry of mouse bone marrow (BM) and spleen (SPL) cells, and trout antierior kidney (AK) and spleen (SPL) cells stained with anti-Pax5.PD and anti-Pax5.E10 antibodies	67
13.	Two color flow cytometry contour graphs for mouse and trout immune tissues	68
14.	The average frequency of proliferating cells in R line and S line blood (A) and spleen (B)	76
15.	Differences in early developing (A) and activated (B) B cell populations in R and S lines of trout	77
16.	Pax5 domain patterns in developing and activated B cell populations	90

Chapter 1. Introduction

The immune system is comprised of a multitude of cells capable of targeting foreign pathogens and altered self-cells such as cancer cells [1]. Innate immunity functions in both vertebrates and invertebrates to activate inflammatory responses leading to pathogen destruction [1]. In contrast, the acquired immune system is highly specific and found only in vertebrates. When activated, it operates in conjunction with the innate immune system by employing T lymphocytes, B lymphocytes and natural killer cells. The B lymphocyte, the focus of this study, is the antibody secreting cell of the humoral (antibody) response. Through a highly regulated process dependent on transcription factor cascades and the microenvironment, B cell progenitors develop into mature B cells that express membrane immunoglobulin (IgM). Upon antigen encounter, mature B cells activate and differentiate into Ig-secreting plasma cells, which secrete antibody that binds antigen with high specificity [1].

The phenotypic pathway of B cell development and activation is well delineated and fairly conserved across vertebrates. However, unique evolutionary differences in the molecular mechanisms and immune tissue locations of B cell development exist between higher and lower vertebrates. While mammalian B lymphopoiesis occurs in the bone marrow, lymphopoiesis in lower vertebrates occurs in alternative primary immune tissues. Of particular interest are the teleosts which lack bone marrow but instead maintain lymphopoiesis in the anterior kidney and launch an acquired immune response comparable to that of their mammalian counterparts [2-4]. The importance of both

similarities and differences between these two groups will be emphasized and explored throughout this chapter.

In addition to cross-species comparisons, the molecular mechanisms determining B cell differentiation, particularly the B cell specific factors, require further examination. The master regulator of B cell development is the paired domain transcription factor Pax5 [5-7]. Pax5 is translated from an alternatively spliced mRNA transcript and is necessary for the maintenance of the B cell phenotype [8, 9]. While the role of Pax5 in B cell development is conserved, researchers have yet to elucidate the function and expression of multiple Pax5 isoforms across immune tissues and species. Identifying the roles of individual Pax5 isoforms in B cell development may have important clinical implications, as the misexpression of Pax5 isoforms correlates with erroneous B cell proliferation and malignancy [10-12]. This chapter will present the current paradigms in B cell development and Pax5 alternative splicing, and will highlight important unanswered questions linking these mechanisms.

1. B Cell Development

B cell development is a highly regulated, sequential process dependent on the microenvironment. Transcription factors, which are DNA-binding proteins, function to both activate genes guiding B lymphopoiesis and to suppress genes driving alternative cell fates. Cytokines and their receptors also enrich the microenvironment of primary and secondary immune tissues and promote the acquisition of B cell competency (the ability of a cell to follow a particular cell lineage). In fact, each stage of B cell development

(Figure 1.1) is defined by distinct expression patterns of molecular markers, including transcription factors (reviewed in [13]), cytokine receptors, and Ig heavy (H) and light (L) chain gene rearrangements. As B cell progenitors progress through the developmental stages marked by these molecular patterns, they gradually lose their alternative lineage potential until finally acquiring a mature B cell phenotype.

1.1 Hematopoiesis and Common Lymphocyte Progenitors (CLPs)

Hematopoiesis is the process by which pluripotent hematopoietic stem cells either self-renew or acquire a progenitor fate capable of producing all immune cell types. In most vertebrates, hematopoietic tissues arise from the ventral mesoderm during fetal development [14]. During fetal development, the spleen functions as the primary hematopoietic site. At birth, mammalian hematopoiesis primarily occurs in the bone marrow and continues there throughout adulthood. While bone marrow serves as the primary immune tissue in mammals, other tissues, such as dorsal aorta and anterior kidney, serve as a site for hematopoiesis in lower vertebrates such as amphibians, chickens and teleosts (reviewed in [15]).

The rainbow trout anterior kidney (defined in [2, 16]) functions as the primary immune organ and site of hematopoiesis. These findings were primarily confirmed by antigen inhibition assays in which anterior kidney cells had reduced antigen recognition capabilities when compared to spleen cells. The results agreed with findings observed in murine bone marrow and spleen [4]. In a further parallel, both the anterior kidney of teleosts and the bone marrow of mammals house the adventitial, endothelial, and reticular

cells [17]. The anterior kidney also contains progenitor cells characterized by conserved early molecular markers, such as early B cell factor (EBF), recombination activating gene (RAG)-1,-2, and Ikaros [18-20]. Although murine and human hematopoietic cells are typically identified and defined by the antigenic surface marker CD34 (previously My-10), an equivalent cell surface marker for trout hematopoietic cells has yet to be identified [21, 22].

In all vertebrates, the pluripotent progenitor cells produced by hematopoiesis have the potential to differentiate into all blood cell lineages, but they are developmentally restricted to specific lineages by the expression of hormones and cytokines in the stroma of hematopoietic tissues. Erythropoietin, for example, restricts cells to erythrocyte fates while cytokines interleukin (IL)- 15 and IL-6 restrict cells to natural killer (NK) cell and granulocyte lineages respectively (reviewed in [23]). The lymphoid lineage, particularly B-lymphocytes, requires exposure to IL-7 [24, 25]. Cells driven toward NK, T- and B-lymphocyte fates are collectively known as the common lymphocyte progenitors (CLP).

Common lymphocyte progenitors biased towards the B cell fate are characterized by expression of the transcription factors Ikaros and EBF [13, 18, 26]. Ikaros expression occurs prior to the activation of B cell-specific genes and is required for lymphoid fate in both teleosts and mammals [18, 27]. Mice homozygous deficient for *Ikaros* lack NK, T-, and B-lymphocytes, a finding that demonstrates Ikaros is necessary as a lymphoid progenitor regulator [18, 28]. Similar to knock-down experiments for Ikaros, knockdown of EBF results in reduction of lymphoid fates and an increase in myeloid-derived fates. Additionally, ectopic expression of EBF directs pluripotent stem cells towards B cell

fates [29]. While Ikaros functions as a CLP regulator, EBF has a more specific role in B cell commitment as it regulates, and is regulated by, Pax5 [29] later in development.

Common lymphocyte progenitors are additionally defined by their level of Ig gene rearrangements. RAG1 locus activation occurs in early multipotent progenitor cells prior to B cell gene expression [30]. Concurrently, these CLP cells begin expression of terminal deoxyncleotidyl transferase (TdT) which is necessary for antigen receptor diversity [30, 31]. Prior to differentiation to non-lymphoid fates, CLPs begin to produce more D_H-J_H (diversity and joining gene segments) rearrangements [23], but are not necessarily committed to the B cell fate [30].

Recently, populations of early developing B cells including common lymphocyte progenitors have been compared using trout and murine primary immune tissues. Using flow cytometric analysis and antigenic markers of RAG1 and EBF, Zwollo et al. [32] demonstrated that similar early B cell populations exist in both murine bone marrow and trout anterior kidney. In addition to expressing these early progenitor cell molecular markers, these cell populations were of similar large size (i.e. high in forward light scatter- FSC), further emphasizing the evolutionary parallels in mammals and teleosts.

1.2 Pro-B –Mature B Cell Development

As with CLPs, the more differentiated stages of B cell development (Figure 1.1) may be identified by molecular changes in cell surface markers, transcription factors, and Ig rearrangements. Pro-B cells differ from CLPs since they are restricted to the B cell fate and can no longer produce T cells. In addition, murine pro-B cells are defined by cell

surface markers B220 and CD43 [33-35]. Both murine and teleost pro-B cells are marked by continued expression of EBF and RAG1 with the new expression of Pax5 [32]. Pax5 is the master regulator of B cell commitment and development; Pax5-/- cells arrest at the pro-B stage [7, 36]. Murine and teleost pro-B cells are also characterized by rearrangement of the DJ gene segments at both IgH loci [37, 38].

After the initial heavy chain rearrangement in pro-B cells, cells enter the pre-B stages of development. The pre-B stages are divided into two phases: pre-BI and pre-BII (includes large pre-BII and small pre-BII). During pre-BI, EBF and mammalian CD43 expression decline and the primary immune cells begin expressing heavy chain (HC) mu [32, 39-41]. Assembly of the pre-B cell antigen receptor (preBCR) begins as the surrogate light chain (LC) forms from the Igα/Igβ subunits [23, 36, 38, 42]. Cells then undergo positive selection for the preBCR and progress to the pre-BII stage.

In the pre-BII stage, pre-BII cells no longer express either EBF or RAG1 [32]. The variable (V) gene segment recombines to the DJ gene segment and the locus undergoes VDJ chain rearrangements [37]. During this stage, cells become smaller, shifting from the larger pre-BII cell morphology to smaller pre-BII cell morphology, as evidenced through the FSC of flow cytometric analyses [32]. Remarkably, the relative frequency of the small, late developing B cell populations (including small pre-BII, immature and mature B cells) is well conserved between mouse bone marrow cells and trout anterior kidney cells; approximately 1% of bone marrow and anterior kidney cells are in the late developing stages.

The end of pre-BII development and beginning of the immature B phase is characterized by LC gene rearrangement [37]. The immature B cell is the first of the B cell lineage to express surface IgM (reviewed in [43]). In mice, immature B cells will migrate from the bone marrow to the surrounding lymph nodes, upregulating mature B cell genes as they travel [44]. In teleosts such as trout, research suggests migration occurs from anterior kidney to either the posterior kidney or the spleen [2]. Once inside the secondary immune tissues, B cells will mature, continue to express functional membrane bound IgM, and remain in a resting state until antigen activation.

2. B Cell Activation- Plasmablasts, Plasma Cells, and Memory Cells

Upon antigen activation, B cells will terminally differentiate into one of three fates: short-lived plasma cell, long-lived plasma cell, or memory B cell. Similar to early B cell development, this process follows a regulated sequence of events, beginning with plasmablast proliferation. During terminal differentiation, B cells will either remain in their final secondary immune tissue location or return to the primary immune tissue where they can survive within supportive niches as long-lived plasma cells. As observed in earlier stages of B cell development, the process of terminal differentiation and plasma cell survival is highly dependent on both transcription factor expression and the microenvironment.

In the secondary immune tissues, mature resting B cells encounter antigen, activate, and then begin proliferating as antibody secreting plasmablasts. Plasmablasts are primarily found in the spleen or in the blood stream migrating to other tissues. In both

mammals and teleosts, proliferating plasmablasts are also present in bone marrow (anterior kidney for teleosts), spleen, and blood [16]. The peripheral blood of teleosts contains plasmablasts but lacks plasma cells, as exemplified by the lack of cell antibody secretion in the presence of the cell cycle inhibitor, hydroxyurea (reviewed in [45]) [3]. However, plasmablasts isolated from peripheral blood are incapable of differentiating to mature plasma cells *in vitro* [16], emphasizing the importance of microenvironmental factors such as the presence of cytokines, on B cell terminal differentiation [3]. It is not known whether peripheral blood B cell terminal differentiation occurs *in vivo*.

One key developmental marker of proliferating and differentiating B cells is the X-box binding protein, Xbp1. Xbp1 is a key regulator of the unfolded protein response and its expression allows antibody-secreting cells to accumulate proteins in the endoplasmic reticulum without triggering the unfolded protein response [46-48]. During mammalian and teleost plasmablast proliferation, Pax5 is downregulated, thus reversing the Pax-5 mediated inhibition of Xbp1 [49]. Xbp1 upregulation facilitates the production of IgM [48], the isotype of naive B cells. Flow cytometric analyses have utilized combinations of antigenic markers of Xbp1-S (the stable form of protein induced by antigenic stimulation), HCmu (the heavy chain of IgM), and Pax5 to distinguish subpopulations of differentiating plasma cells in both trout and mouse by their distinct expression patterns [16]. When compared to mouse cells, trout cells do not produce as high a frequency of activated B cells when cultured in LPS (see [50] for use), however both trout and mouse plasmablasts exhibit intermediate levels of Xbp and HCmu as Pax5 levels decrease. As plasmablasts differentiate to plasma cells, they express very high

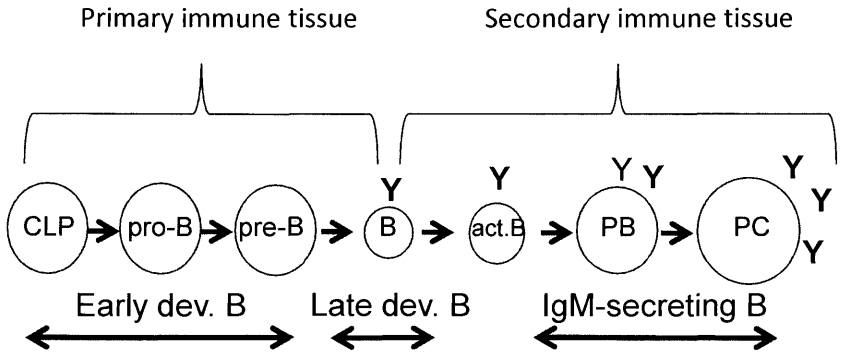
levels of Xbp1 and HCmu and no longer express Pax5. Thus, the patterns of Xbp1 expression and other conserved molecular markers provide conclusive evidence that plasmablasts are a conserved population distinct from plasma cells.

A plasmablast's differentiation into a short-lived plasma cell is accompanied by important changes in IgM expression. Plasma cells are antibody-secreting cells that downregulate membrane IgM and they therefore no longer bind antigen. Instead, mammalian plasma cells upregulate and secrete Ig of various isotypes including IgM (reviewed in [51]). These short-lived plasma cells can be identified by their lack of somatic hypermutations; B cells with less specific binding to the antigen will undergo Ig somatic hypermutations to increase specificity. Teleosts also upregulate secreted forms of Ig, but with less isotype switching. Initially, IgM was proposed to be the only secreted teleost Ig, but recent studies indicate the additional presence of secreted IgD and IgT [52-54]. The lack of mutational variability within the secreted Ig population is important as it suggests primary antigen exposure and can be used to distinguish short-lived plasma cells from long-lived plasma cells and memory cells.

Long-lived plasma cells (LLPCs) are plasma cells that have been previously exposed to antigen and thus secrete high affinity antibody [51]. The term "humoral memory" is applied to LLPCs as they can maintain active antibody secretion for months to years without re-exposure to antigen. In mice, LLPCs arise from a distinct plasma cell precursor population in the bone marrow [55]. These precursors differentiate to LLPCs and are maintained in a bone marrow niche of specialized stromal cells [51, 55]. Just as the mouse bone marrow harbors LLPCs, evidence suggests a similar role for the trout AK

[reviewed in[56], [3, 57]. Upon antigen stimulation, these cells within the anterior kidney will continue to secrete antibodies weeks beyond those in the blood and spleen [3]. Furthermore, they do not require further antigen stimulation to remain active, Ig-secretors. Although not yet reported, it is likely these cells are supported within anterior kidney niches similar to those within mouse bone marrow [56].

In addition to differentiating into short-lived and long-lived plasma cells, activated B cells may also acquire a memory cell phenotype. Mammalian memory cells are distinct from plasma cells in that they continue to express membrane Igs. However, these memory cells have often undergone both isotype switching and somatic hypermutation of their variable gene segments (reviewed in [23]). Mammalian memory cells are further defined as cells that rapidly increase antibody production upon antigen re-exposure and produce higher affinity antibodies than those generated during the primary immune response. The teleost immune system also exemplifies memory responses as production of a secondary immune response in trout immune cells increases antibody secreting cells without altering the number of antigen-sensitive clones. However, unlike mammalian memory cells, teleost memory cells do not appear to exhibit affinity maturation (the production of antibodies with higher antigen affinity) [23, 58].



	CLP	pro-B	early pre-B	late pre-B/ (im)mat.B	act. B	PB	PC
Ikaros	+	+	+	+/–	+/–	+/ –	–
E2A	+	+	+/–	–	–	+	–
EBF1	+	+	+/–	+/–	–	–	–
Pax5	–	+	+	+	+	+	–
Blimp1	–	–	–	–	–	+	++
Xbpl	++	++	+	+/–	++	++	++
RAG1	+	+	+/–	–	–	–	–
cHCmu	–	–	–	+	+	++	++
BrdU	+/–	+/–	+	–	–	+	–
FSC	high	high	high	low	low	high	high

Figure 1.1. Stages of B cell development characterized by molecular patterns (adapted from [3]). +/- delineates positive/negative expression of antibody in cell. CLP: common lymphocyte progenitor; B: mature B cell; PB: plasmablast; PC: plasma cell; FSC: forward angle light scatter. High FSC is characteristic of large cells.

3. The Role of Pax5 in B Cell Commitment

The transcription factor Pax5 is considered to be a key regulator of B cell lineage commitment and is additionally expressed in both the developing central nervous system (CNS) and the adult testis [59]. As a member of the paired box (Pax) family of transcription factors, Pax5 shares a conserved DNA-binding domain of 128 amino acids known as the paired domain. Pax5 is necessary in developing B cell populations as it activates genes involved in B cell receptor formation and represses genes directing alternative lineage fates [60-63]. Its subsequent downregulation is critical for plasma cell proliferation and terminal differentiation. Given its crucial role in the early proliferative stages of B cell development, it is not surprising that misexpression of Pax5 leads to oncogenesis. Additionally, Pax5 is one of the many genes processed via alternative splicing, although the role of multiple Pax5 isoforms in B cell development, activation, and cancer formation remains poorly understood.

3.1 Pax5 and Pax Family

The Pax family consists of nine transcription factors which express a conserved, bipartite paired domain and some or all of two additional motifs (see Figure 1.2 for Pax5) [64]. X-ray crystallography demonstrates that the two sub-regions of the paired domain include an N-terminal PAI domain encoded by exon 2 and a C-terminal RED domain encoded by exon 3 [65]. These domains bind two half-sites on adjacent DNA major grooves [66]. The Pax family is further divided into four classes by the presence of a conserved homology region called the homeodomain and an octapeptide repeat region

(reviewed in [67]). Class one, consisting of Pax1 and 9, contains the octapeptide region, but no homeodomain. Class two, consisting of Pax2, 5, and 8, has the octapeptide region and a partial homeodomain which lacks the ability to bind DNA. Class three, consisting of Pax3 and 7, has both the octapeptide region and a full homeodomain. Finally, class four contains Pax 4 and 6, displaying the full homeodomain and no octapeptide repeat.

Pax5 falls into a transcription factor family with unique homology region properties and transactivating potential. The partial homeodomain homology region allows for interaction with the TATA-binding protein at the gene promoter, enabling Pax5 to directly manipulate gene transcription [68]. This region also contains a binding motif for the retinoblastoma gene product which is important in tumor suppression [68, 69]. The Pax5 octapeptide motif interacts with the Groucho family of corepressors to repress gene transcription [70]. The C-terminal domain of Pax5 contains a 55 amino acid transactivating domain which is serine/threonine/proline-rich. The transactivating domain is negatively regulated by inhibitory sequences in the extreme C-terminus [71].

In addition to their homology regions, Pax family members share commonalities in their conservation across species, alternative splicing patterns [9, 72-74] and oncogenic properties [67]. Pax genes are shared amongst mammals and lower vertebrates such as zebrafish, *Xenopus*, trout, and amphioxus [71, 72, 75-77]. Alternative splicing (reviewed later in this chapter) has been identified for each of the classes with the majority of isoforms containing an exon 2 deletion (for review, see [72]), resulting in a non-functional paired domain.

3.2 Pax5 in B Cell Development and Activation

Functional studies of Pax5 using knockout and targeted mutation approaches demonstrate its role in B cell development and activation. Immune cells of mutant mice homozygous for Pax5 deletions are unable to proceed past the pro-B developmental stage [7]. These cells are capable of undergoing D-J rearrangements at the HC locus, but express significantly reduced V-D-J rearrangements (reviewed in [78]) [5]. Arrested pro-B cells are not restricted to the B cell lineage: culturing these cells in the presence of lineage-specific cytokines allows redirection of cell fate. Furthermore, Pax5 re-expression via viral induction is sufficient to reverse the pro-B arrest and completion of B cell differentiation [6].

Pax5 alters B cell development through a mechanism of gene activation and repression [63]. Delogu et al. [61] identified 110 genes repressed by Pax5, and demonstrated that continuous Pax5 mediated repression of alternative lymphoid and myeloid genes was required to maintain the B cell lineage. It is therefore unsurprising that release of Pax5 repression is necessary for terminal plasma cell differentiation. In addition to genes related to cell adhesion and cellular migration, Pax5 also represses genes involved in isotype expression. Specifically, murine Pax5 binds and represses the enhancer of HC 3α, as demonstrated via transient transfection studies of pro-B, pre-B and mature B cell lines [79, 80]. Pax5 also suppresses transcription of the Ig J chain gene, a process which must be reversed before cytokines such as interleukin-2 [81] and the transcriptional repressor Blimp-1 [82] can induce terminal plasma cell differentiation.

More recently, Schebesta et al. [60] used microarray technology to identify 170 Pax5-activated genes in murine pro-B cells, half of which are subsequently downregulated in plasma cells (reviewed in [83]). One of the potential mechanisms of Pax5 gene activation is histone modification, as evidenced by ChIP-on-chip studies [60]. These studies define active histones by the patterns of histone lysine methylation and acetylation (i.e. H3K4me2, H3K4me3, and H3K9ac). Microarray studies demonstrate the necessity of the Pax5 DNA binding domain for these active histone marks: upon Cre-mediated conditional knockout of the Pax5 DNA binding domain, pro-B cells exhibit fewer active histone marks than pro-B cells with intact versions of wildtype Pax5 [83]. Recent studies have further demonstrated that Pax5 induces changes in histone activation by recruiting chromatin remodeling and histone modifying complexes [62]. Pax5 is also necessary to activate genes involved in cell cycle regulation and metabolism [60], suggesting the expansive role of the transcription factor in developmental processes.

3.3 Pax5 Alternative Splicing

Alternative splicing is a mechanism by which the exons of an RNA transcript are differentially combined to produce different functional protein products. By recombining RNA exons in multiple ways, alternative splicing enables an economical means by which a single gene can encode multiple protein isoforms. The Pax family is one of several transcription factors subjected to this process [9, 72-74]. The first Pax isoforms observed were those of Pax8; four alternatively spliced Pax8 mRNA transcripts were identified in human kidney cells lines via PCR amplification and RNAse protection assays [73].

Further analysis with transient transfection assays demonstrated that each isoform has distinct C-terminal transactivating properties. Unique patterns of Pax8 mRNA transcripts were also identified in mouse cell lines representing different stages of embryogenesis, suggesting the isoforms are carefully regulated during development.

Shortly after researchers identified Pax8 alternative splicing, similar observations were made for the additional Pax family members, including Pax5. The murine Pax5, identified in pro-B cell lines, mature B cell lines and spleen cells, has four distinct isoforms - Pax5a (full length: FL), b, d, and e (see Figure 1.2) [9]. Pax5b and e have deletions in exon 2, leading to a truncated, non-functional DNA-binding domain. Studies of plasma cell lines suggest that low levels of Pax5b are present in the cell line SP20, one of three plasmacytoma lines tested [9]. In contrast, Pax5a is completely down-regulated in this same cell line. Subsequent functional studies demonstrated that although Pax5e alone is non-functional, in combination with Pax5a it may increase reporter gene activity [9, 84].

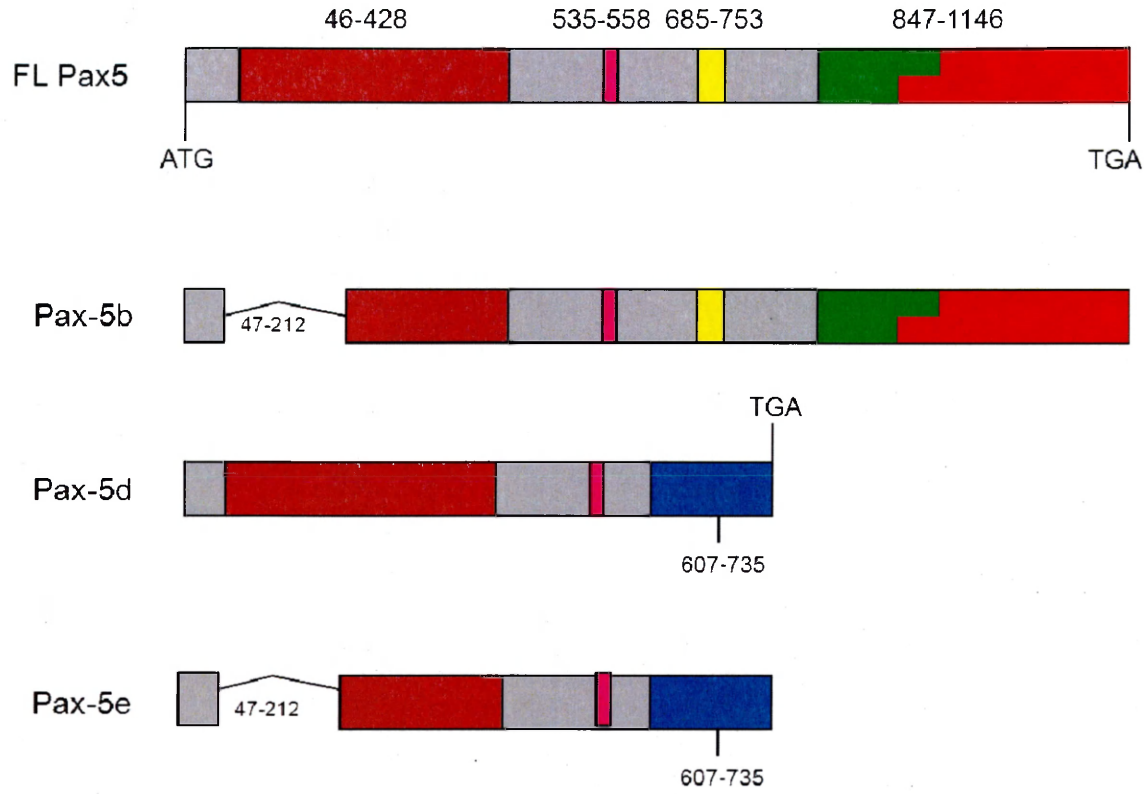


Figure 1.2 [9]. Murine Pax5 isoforms shown as cDNA sequence. Numbers represent nucleotide number. Red box: paired domain (exons 2 and 3); purple: octamer region (exon 5); yellow: homeodomain homology region (exon 6); green: transactivation domain (exons 8 and 9); bright red: repressor domain (exons 9 and 10); blue: novel C-terminus.

Similar to Pax8, murine Pax5 isoforms d and e have alternatively spliced C-termini, creating novel C-termini and deleted homeodomains. The novel C-terminal region contains significantly fewer Ser/Thr/Pro residues than Pax5a, altering its transactivating potential [9]. Functional studies of Pax5d reveal that the isoform binds DNA with the same affinity as Pax5a and thus acts as an inhibitor of Pax5a in a dose-dependent manner [84]. The inhibitory role of Pax5d is further emphasized by its decreased presence in LPS activated cells [84]. As Pax5a is needed for cell proliferation, it is logical that the inhibitor of Pax5a would be downregulated during B cell activation. Interestingly, while the Pax5a DNA-binding affinity is decreased in B lymphocytes of aged mice, the Pax5d DNA-binding affinity remains constant, further suggesting a unique role for Pax5d in B cell processes [85].

Remarkably, similar Pax5 isoforms are observed across species, including but not limited to humans, mice, fish (see Figure 1.3; Zwollo, unpublished data), and amphioxus [9, 12, 72]. Humans [12], trout (Zwollo, unpublished data), and amphioxus [72] have isoforms that either include or exclude exons 7-9, drastically altering their transactivating potential. Likewise, conserved deletions of exon 2 (the DNA-binding domain) in amphioxus, trout (Zwollo, unpublished data), mouse, and human isoforms [9, 10, 72] are particularly noteworthy. However, beyond a few functional studies of isoforms lacking exon 2, a definitive role for expression of this isoform has yet to be identified.

Numerous studies have examined the role of aberrant Pax5 isoform expression in cancer development. Pax5 is considered a proto-oncogene since its misregulation often correlates with lymphomas and leukemias [86]. Haploinsufficiencies of full length Pax5

correlate with acute lymphoblastic leukemia (ALL; [87]) whereas alternatively spliced Pax5 variants correlate with cancer formation. Robichaud et al. [12] found that human lymphoma samples express a reduced number of Pax5 isoforms, specifically the full length Pax5 and the Pax5δ8 (lacking exon 8), in comparison to healthy samples which express multiple isoforms. Additionally, the expression of a novel Pax5 exon discovered by Borson et al. [11] is downregulated in patients with multiple myeloma. Despite the promise in these results, other studies have been unable to identify a single pattern of isoform expression that could be used to distinguish normal from malignant B cells [10]. Similarly, researchers have cautioned the testing of alternative splicing in cancer cells as unrelated stress factors such as overnight sample shipping may induce increased Pax5 isoform expression [88]. Thus, although multiple splicing patterns have been reported in malignant cells, further analysis is required to establish any conclusive or diagnostic correlations.

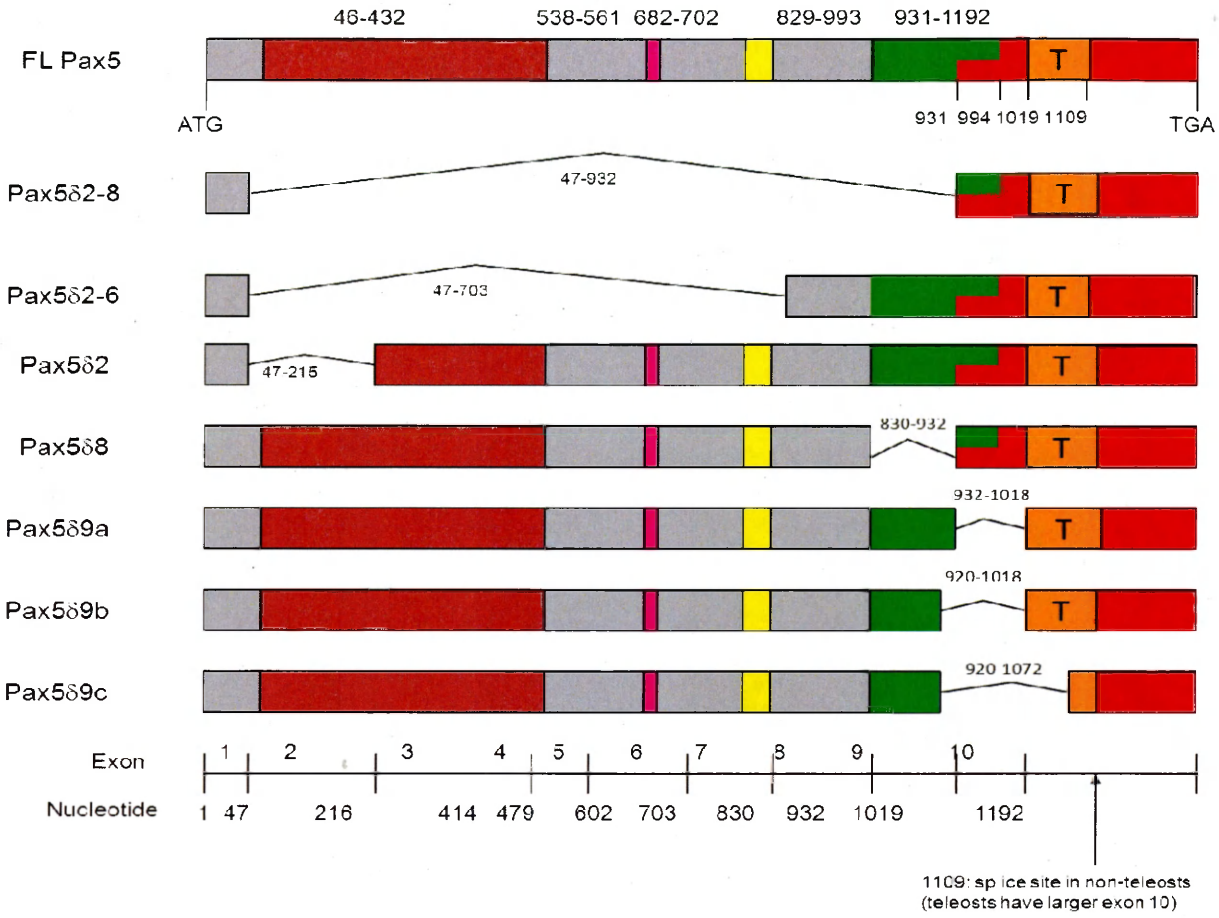


Figure 1.3. Trout Pax5 isoforms shown as cDNA sequence (adapted from Zwollo, unpublished data). Red box: paired domain; purple; octamer region; yellow: homeodomain homology region; green: transactivating domain; bright red: repressor domain; T: additional C-terminal amino acids.

4. Rationale for the Current Study

Although the role of Pax5 in B cell development and activation has been well described, the temporal-spatial expression patterns, functions, and consequences of multiple Pax5 isoforms in these processes remain unknown. Numerous studies emphasize the importance of Pax5-regulated gene activation and repression in B-cell development [60, 61]. Through conditional knockouts targeting Pax5 exon 2, repressor studies reveal the necessity of the DNA-binding domain in the activation of B cell commitment genes [60]. Additionally, full length Pax5 (Pax5 FL) knockouts reveal the importance of Pax5 inhibition. Without the transcription factor, cells re-express previously Pax5-inhibited genes. However, what these studies fail to address is the function of specific Pax5 isoforms, including Pax5δ2, in gene targeting. Functional studies exploring Pax5 isoforms with paired domain deletions and novel C-termini have indicated a role for these isoforms in increasing Pax5a gene activation [84]. Therefore, it is likely these isoforms contribute to B cell developmental processes and mechanisms of regulation.

Additionally, identifying the function of these isoforms across species will help elucidate the evolutionary forces driving immune system development. A cross-species analysis may also enable researchers to identify the most relevant isoforms in B cell development and disease. As previously stated, the rainbow trout is an excellent model organism for cross-species comparisons. Despite lacking bone marrow and lymph nodes, its immune system functions similarly to that in humans. Furthermore, almost no published studies have compared B cell development in teleosts versus mammals, including studies of Pax5 isoform expression and function.

The first step in evaluating the functional potential of Pax5 isoforms is to identify their existence in individual B cell populations. Previous studies have primarily utilized a traditional or nested PCR approach requiring RNA from pooled cell samples to identify novel Pax5 isoforms [9, 72, 89]. While this approach is sufficient to identify the existence of Pax5 isoforms, it does little to assess their expression within individual developing, proliferating, and differentiating B cell populations. The frequency of cells representing specific B cell sub-populations is potentially quite small [2, 16, 76]. If individual isoforms function in these small, transient populations, a PCR approach will be unable to demonstrate the relative abundance (and potential importance) of these isoforms compared to isoforms found in larger B cell populations. Thus, a novel approach utilizing flow cytometry is necessary to elucidate the expression and potential role of Pax5 isoforms in B cell development.

Studies have previously employed flow cytometry to establish "B cell signatures" or cytometric patterns representing the frequency and intensity of conserved molecular markers that demarcate B cell populations in different immune tissues [16]. As a fingerprint identifies an individual, flow cytometric analysis has successfully distinguished between trout and mouse immune tissues by demonstrating each immune tissue's distinct frequency of developing B cell populations, including CLPs, pro-B, pre-B, mature B, proliferating, and plasma cells [2, 32, 76]. Furthermore, lymphocyte cells stained with antibodies against the Pax5 paired domain and analyzed by flow cytometry demonstrate the patterns of paired domain expression in B cell populations previously delineated in the literature. Therefore, flow cytometry provides a reliable and

reproducible means of characterizing both B cell populations and whole immune tissues. Therefore, the aim of the current study was to use flow cytometry to evaluate patterns of Pax5 isoform expression in developing mouse and trout B cell populations and to characterize the "Pax5 signatures" of mouse and trout immune tissues.

5. Flow Cytometric Analysis

Flow cytometry is a technique used to identify cells by the key molecular markers of individual cells comprising a larger population. Cells from tissues are stained with fluorescent-tagged antibodies and are then run single file through a laser beam, collecting information on the cell's fluorescence, its forward light scattering, and its side light scattering. Each cell passing through the laser is considered an "event" and the fluorescence and light scattering information for each event is captured in a computer graphic that can be easily read by the researcher.

5.1 Flow Cytometer Fluids

The simple set up for flow cytometry is diagrammed in Figure 1.4. Cells are drawn up from a 96-well plate, mixed with sheath fluid (a proprietary buffer saline solution with detergent), and pushed through a flow chamber via laminar flow. In the flow chamber, cells are aligned single file by hydrodynamic focusing [90]. After passing through a laser beam, the cells are discarded into a waste container.

5.2 Forward Angle Light Scatter (FSC) and Side Angle Light Scatter (SSC)

As cells are excited by a laser, light is scattered based on the size and complexity (indication of shape due internal cellular components) of the cell. FSC is light diffracted from the cell in the direction of the laser beam. Larger cells will have greater FSC than smaller cells. The FSC light is detected by a lens and translated into electronic pulses by a photodiode [90]. SSC is light reflected and refracted at 90° and is based on the cell's topography (membrane roughness), cytoplasmic granularity, and shape of organelles. SSC is only 10% of total light scattering and therefore must be detected by a more sensitive device such as a photomultiplier tube (PMT), which will amplify the weak signal.

5.3 Fluorescence

The BD FACSArray Bioanalyzer can detect fluorescence from four regions of the light spectrum: infrared (IR), yellow, red, and far red. The emitted sample fluorescence is directed towards the optical filters using a series of mirrors and beam splitters. The filtered light is then amplified with a PMT and converted into electrical signals which can be read by the computer. If fluorochromes overlap in their emission spectra, BD FACSArray provides a compensation tool to collect data from fluorochromes only within a specific wavelength range.

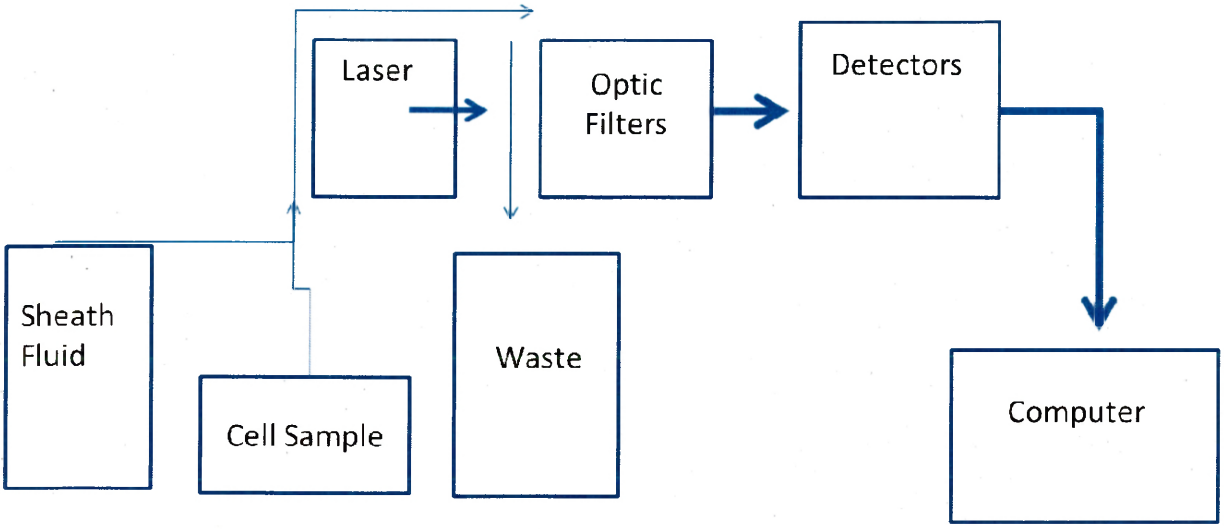


Figure 1.4. Flow cytometer process. Light blue arrows delineate cell pathway; bright blue arrows represent laser pathway. Single cell suspensions are mixed with sheath fluid and cells are passed individually through a laser beam. As cells pass through the beam, light is scattered and the direction of the scattering is detected by an optic lens. Additionally, the laser excites antibody fluorochromes. The light emitted from individual fluorochromes is passed through optic filters to detectors. The information is then passed to a computer which presents the intensity of light scattering and fluorochrome fluorescence in a graphic image.

5.4 Data Interpretation

Information from each cell is presented graphically on the computer as it is passed through the laser. A typical experiment will collect around 30,000 events, a high number designed to detect even small cell populations, such as plasma cells. During a typical experiment, multiple graphs will be used at once to examine the size of the cells versus the complexity, the size of the cells versus their fluorescence, expression of one fluorochrome-labeled antibody versus a second fluorochome-labeled antibody, and a histogram for the number of cells expressing a fluorochrome at any given intensity of fluorescence.

When studying lymphocytes, the lymphocyte population is first gated based on a graph of FSC versus SSC. Previous experiments have already identified how lymphocyte populations should appear on the FSC v. SSC graphs [2]. Figure 1.5 provides an example. This graph can be examined both in dot plot form (Figure 1.5a), where each cell is represented as one dot on the graph, or in contour form (figure 1.5b), where populations of cells are represented by varying degree of intensity. Once the lymphocyte cells are gated, the population of cells may be examined for their fluorescence intensity of a particular antibody (see Figure 1.5c). In the example provided, the cells have been stained for two antibodies: one in the yellow emission wavelength and one in the red wavelength. The frequency of cells in a given population can be identified based on the four criteria listed above. Thus, flow cytometry provides a valuable means to characterize tissues composed of diverse cell populations.

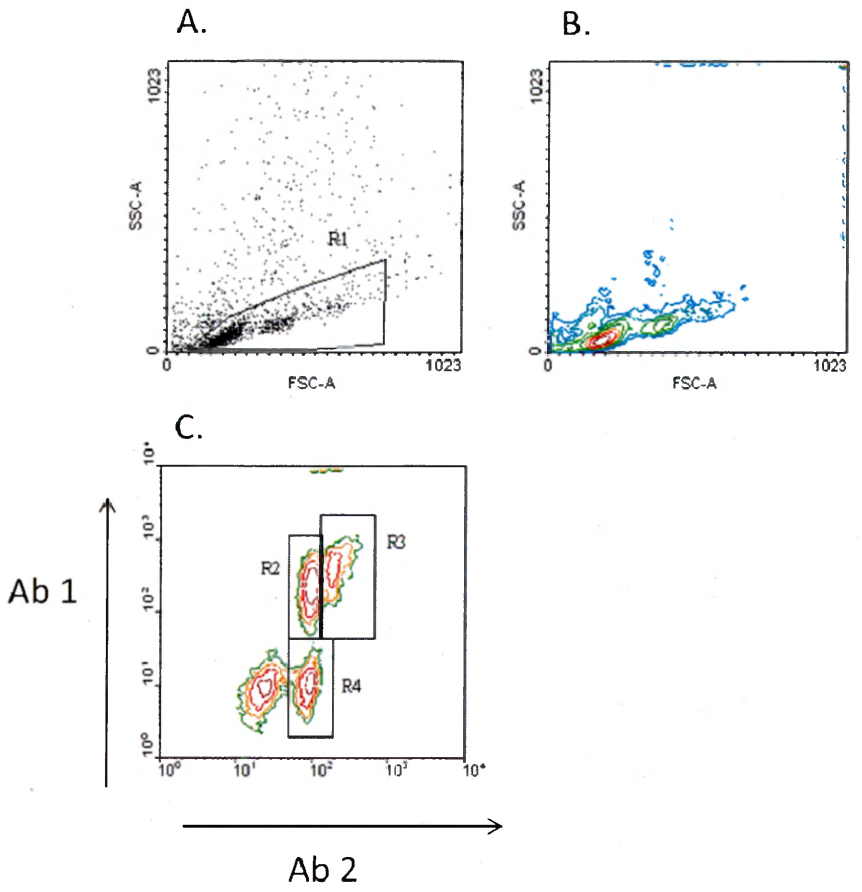


Figure 1.5. Flow data graphs. *Ab* = antibody. A) Cells individually recorded as "events" and separated according to size (FSC) and complexity (SSC). The region of lymphocytes is gated as population R1. B) The same population of lymphocytes is represented in contour form. C) Four populations of cells stained for two antibodies are presented. Ab 1 emits light in the yellow wavelength and Ab 2 emits light in the red wavelength. The populations of interest are boxed. Population R2 has high Ab 1 staining and medium Ab2 staining. R4 has low or no Ab 1 staining and medium Ab 2 staining. R3 is high in Ab 1 staining and high in Ab2 staining. The unboxed region represents a double negative for Ab 1 and Ab 2 staining.

6. Hypothesis and Research Aims

Pax5 regulates B cell development through a process of coordinated gene activation and repression. Pax5 isoforms are able to enhance and inhibit full length Pax5's ability to activate gene expression, thus potentially altering the course of B cell development. Therefore, this study predicted that Pax5 isoform patterns are differentially expressed in developing and activated B cell populations, forming unique Pax5 signatures. This study hypothesized that specific Pax5 isoforms modulate Pax5 activity, thus altering B cell development and activation (see model in Figure 1.6). For example, Pax5 FL is able to activate genes necessary for B cell development; other isoforms such as the Pax5δ2 (lacking exon 2) may inhibit the activation of these genes. Similarly, Pax5 FL is downregulated to allow for plasma cell terminal differentiation. Isoforms lacking exon 2 may induce plasma cell differentiation.

To address this hypothesis, this study presented four research aims:

1) Identify trout Pax5 isoforms in developing B cells using two-color and tri-color flow cytometry.

   We stained single cell suspensions with combinations of antibodies against specific Pax5 domains in addition to markers of B cell development. We distinguished populations of developing B cells across the anterior kidney, blood, and spleen using established B cell flow cytometric patterns. We analyzed the frequency of B cell populations expressing specific Pax5 domains and the intensity of Pax5 domain staining within a given B cell population.

2) Examine trout Pax5 isoform expression after LPS activation using two-color flow cytometry.

We cultured trout blood and spleen cells in the presence of B cell mitogen *E. coli* lipopolysaccharide. We analyzed Pax5 domain expression and cell proliferation exemplified by EdU incorporation across tissues.

3) Compare and contrast murine and trout Pax5 isoform expression during development and activation.

We analyzed mouse bone marrow and spleen cells using two-color and tri-color flow cytometry. We obtained the frequencies of developing B cell populations expressing specific Pax5 domains and compared these to trout B cell populations.

4) Identify the preliminary differences in Pax5 isoform expression in trout resistant and susceptible to flavobacterium.

We identified B cell populations using the above listed methods. We compared the frequencies of B cell populations across immune tissues and the frequencies of populations expressing specific Pax5 domains between fish. Additionally, we

identified changes in B cell populations after LPS activation and we analyzed differences in proliferating populations based on EdU incorporation.

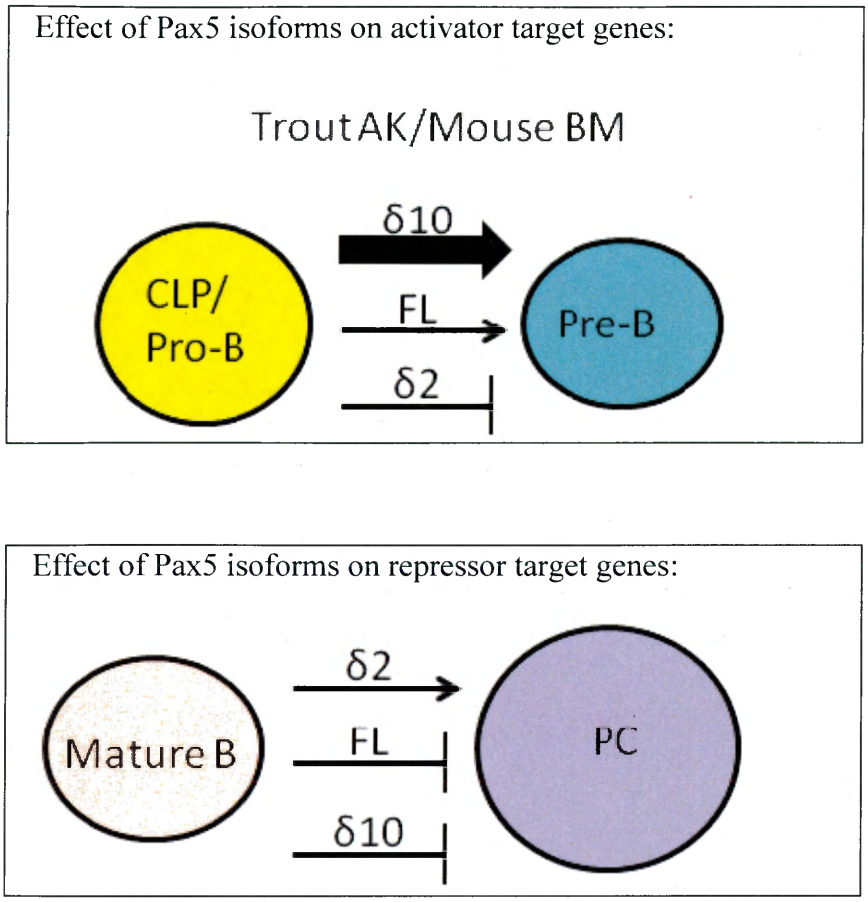


Figure 1.6. Model of Pax5 isoform modulations of B cell development. Top: During development, full length (FL) Pax5 is necessary to activate genes promoting B cell development. Isoforms that inhibit Pax5.FL may inhibit pre-B stages of development. Bottom: B cells must downregulate Pax5 repression of genes necessary for terminal plasma cell differentiation. Isoforms lacking exon 2 are unable to bind DNA and therefore cannot inhibit plasma cell differentiation. Isoforms lacking the repressor domain will permit Pax5 transcription, inhibiting plasma cell differentiation.

Chapter 2. Manuscript

2.1 Introduction.

The mechanisms of B cell development and activation are highly complex and the tissues supporting B cell development are uniquely diverse across species. Even with such species diversity, the stages of B cell development and molecular players are fairly conserved. In adult mammals, the bone marrow functions as the primary immune organ and site of hematopoiesis. In this tissue, hematopoietic stem cells produce common lymphocyte progenitors (CLPs) with the competency to form B cells. B cells progress through stages of immunoglobulin chain rearrangements, delineated as progenitor-B (pro-B), pre-B1, large-pre-BII, small pre-BII, and immature B cell stages [13, 15]. Once B cells acquire functional B cell receptors, they migrate to the spleen (SPL) or other secondary immune organs where they function as mature B cells, and may encounter antigen, proliferate and differentiate into antibody-secreting plasma cells.

While previous research has extensively examined mammalian B cell development, only recently have researchers made significant insights into the teleost B cell system [2-4, 16, 32, 50, 76]. In contrast to mammals, teleosts lack bone marrow and alternatively utilize the anterior kidney (AK) as their primary immune organ [2, 3]. Similar to mammals, mature teleost B cells reside in the spleen, encounter antigen, and differentiate into plasma cells [3]. Additionally, some plasma cells will migrate back to the primary organ where they will reside as long-lived plasma cells [3].

Although the surfeit of mammalian serological reagents is unavailable for deciphering teleost stages of B cell development, recent research has employed suitable

alternatives [2, 16, 32, 76]. Conserved transcription factor expression demarcates stages of B cell development in both mammals and teleosts [13]. Early developing B cells are characterized by high expression of Ikaros, EBF, Xbp1, and RAG1/2 (see Table 1). Mature B cells express Pax5 combined with low levels of IgM, while antibody-secreting cells express high levels of IgM but no Pax5 [32].

Pax5 is a particularly intriguing transcription factor because of its conserved role in regulating B cell commitment in vertebrate species [7, 63]. It is transcribed from an alternatively-spliced RNA transcript and similar Pax5 isoforms are observed in humans, mice, trout and amphioxus [2, 71, 72, 77]. Pax5 expression is first observed at the pro-B stage and Pax5 homozygous deficient B cells will arrest at this stage [5, 7]. During B cell development and activation, Pax5 targets genes necessary for B cell commitment while downregulating genes necessary for alternative lineages [60-63]. Expression of Pax5 continues until terminal B cell differentiation. During B cell differentiation, the transcription factor Blimp1 represses Pax5, allowing expression of the B cell activation marker Xbp1 [49].

The developmental role of the full length Pax5 isoform has been described by many groups, but little is known about the potential function of alternatively spliced Pax5 isoforms. Using RT-PCR approaches, such isoforms have been observed and examined in primary cells and malignant cell lines [10-12, 86, 87]. Thus far, no strong correlations have been observed between isoform type and cell strain or malignancy, although researchers have demonstrated the differences in transactivating potential of multiple isoforms in addition to their modulating effects on full length Pax5 [12, 84].

Current studies are limited in their ability to correlate Pax5 isoforms with distinct developing B cell populations due to the use of pooled cells, either at the RNA level (RT-PCR) or protein level (western blot analysis). However, recent research in our lab has made use of flow cytometric analyses and antibodies against conserved, B cell specific transcription factors to begin identifying individual rainbow trout B cell populations at the various developmental and activated stages [2, 16, 32, 76]. Our research has demonstrated that early developing B, late developing B, and antibody-secreting B lymphocyte populations can be characterized through the combinatorial expression of specific developmental markers. These expression patterns form "B-cell signatures", or flow cytometric patterns that characterize a particular immune tissue.

On the basis of our prior studies, we hypothesize that certain Pax5 isoforms are present during limited, specific stages of B cell development as a means of modulating Pax5 activity. We predicted that we could further classify rainbow trout B cell populations by their patterns of Pax5 domain expression. Here, we report on the flow cytometric analysis of both resting and activated trout immune cells using Pax5 domain-specific antibodies. Our results reveal several unique B lymphocyte populations based on expression of different Pax5 proteins. We demonstrate that early developing, late developing, and activated B cells differentially express three Pax5 domains, encoded by exon 6 (Pax5.E6), exon 10 (Pax5.E10), and the paired domain (Pax5.PD). Examining the combinatorial expression of Pax5 proteins with EBF, Xbp1-S, and HCmu, we provide evidence for the first time that early developing populations are present in the blood and spleen of rainbow trout.

2.2 Methods.

Animals and facilities.

Rainbow trout (*Onchorhynchus mykiss*) were generous gifts from Dr. Steve Kaattari (Virginia Institute of Marine Science). Fish were maintained in 100 gallon tanks with a recirculating system employing biologically-filtered well water. Water was maintained at 12° C. C57BL/6J mice between 3-5 months of age were obtained from Jackson Laboratories.

Isolation of immune cells.

Fresh trout anterior kidney cells were obtained using methods previously described [2]. Briefly, the trout kidney was divided into subsections of seven vertebrae beginning at the posterior end (K5). The K1 region was collected in 5 mL sterile HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM $Na_2HP0_4{\cdot}2H_20$, and 20 mM Hepes at pH 7.05). Similarly, mouse bone marrow and mouse and trout spleen were collected in HBSS as described previously [84]. Single cell suspensions were created by passing cells through a 10 mL syringe followed by filtration through a 40 nm nylon filter (Falcon; BD Biosciences). Cells were then pelleted at 250 *g* for 10 minutes and resuspended in cold HBSS. Cells were then either prepared for culturing (see cell culture and mitogens) or washed in 1X PBS (1.9 mM $NaH_2P04{\cdot}H_20$, 8.1 mM $Na_2HP0_4{\cdot}7H_20$, 137 mM NaCl, and 2.6 mM KCl, pH 7.4) containing 0.02% sodium azide in preparation for fixation (see Fixation). Blood cells were washed in cold HBSS and layered onto Histopaque 1077

cushions (Sigma Aldrich) and spun at 500 *g* at 4 °C for 45 minutes. The peripheral blood (PBL) cell layer was removed and cells were either washed in cold HBSS for culturing or in PBS containing 0.02% azide for fixation.

Cell culture and mitogens.

Freshly isolated trout PBL and spleen cells were cultured at $1.0x10^7$ cells/mL in trout culture medium (TCM) consisting of RPMI 1640 with 10 mM $_L$-glutamine, 10% FCS, 50 μg/ml gentamicin, 50 μM 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (final concentration 10 μg/ml; Sigma-Aldrich). Cells were maintained at 18 °C in the presence of blood gas and fed every other day with one tenth of the culture volume of a 10X tissue culture cocktail containing 500 μg/ml gentamycin, 10X essential amino acids (aas), 10X non-essential aas, 70 mM $_L$-glutamine, 70 mg/ml dextrose, 10× nucleosides, and 33% fetal bovine serum (FBS). B lymphocytes were activated using mitogen LPS (from *Escherichia coli* 055:B5, pasteurized for 30 min at 70 °C in distilled water) at 100 μg/ml. For cell proliferation analysis, cells were incubated in the presence of 3 μM 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) for 17 hours immediately before collection.

Antibodies.

The polyclonal anti-paired domain (Pax5.PD) antibody (previously called ED-1) recognizes trout and mouse Pax5 and has been described previously [91]. The rabbit anti-trout Pax5.E6 polyclonal antibody was raised against the injected Pax5 peptide

HGPGGRDFLRKQMRGDL. The rabbit anti-trout Pax5.E10 polyclonal antibody was raised against the peptide sequence AASRGAGPAATATASAYDRH. Both antibodies were generated with GenScript. The monoclonal mouse anti-trout immunoglobulin heavy chain mu (HCmu I-14; [92]) was a generous gift from Greg Warr. The tXbp1–S antibody (referred to here as anti-Xbp1) binds the stable form of Xbp1 protein resulting from antigenic stimulation and has been described previously [16]. The polyclonal goat-anti-human Pax5.E10 IgG (Pax5-C20) detects the 20 C-terminus amino acids of human Pax5. The mouse Pax5.E10 and the rabbit-anti-human EBF IgG (H300; detecting amino acid residues 1–300 of EBF1, and reactive with mouse/human EBF2, 3, and 4) were purchased from Santa Cruz Biotech. The rat-anti-mouse IgM-PE was purchased from BD Biosciences. Isotype control antibodies included rabbit IgG, goat IgG (Molecular probes), or mouse IgG (eBiosciences) conjugated to Alexa Fluor 555 or Alexa Fluor 647. For flow cytometric analyses, unlabeled antibodies were conjugated (Alexa Fluor 555 and/or Alexa Fluor 647) using protein labeling kits according to manufacturer's instructions (Molecular Probes). Antibody aliquots were stored in 1% BSA at −20 °C

Fixation and permeabilization of cells for flow cytometry.

Cell pellets were resuspended in freshly prepared 1% ice-cold paraformaldehyde (10% stock, EM-grade; Electron Microscopy Sciences) in PBS and fixed on ice for 15 minutes. Cells were then washed in 1X PBS and resuspended drop wise (while vortexing) in 1 mL ice-cold 80% methanol. Cells were incubated overnight at -20 ˚C and stored at -20 ˚C until use (up to 3 weeks).

Flow cytometry.

Cells were removed from -20 ˚C and an equal volume PBS plus 0.02% azide was immediately added. Cells were then pelleted and resuspended in 1 mL PBS and 0.02% azide. Cells were then resuspended in 1 mL permeabilizing solution (BD perm wash in PBS, BD Biosciences) containing 2% FBS and then incubated in perm wash + 5% FBS at a concentration of $1x10^7$ cells/mL at 4 ˚C with gentle shaking. 0.5×10^6 cells were then transferred to each well on a 96-well polystyrene round bottom plate (Fisher). Fluorescent Abs were added to the cell suspensions to a final antibody concentration of 0.5–2 μg/0.5×10^6 cells/50 μl final volume, and cells incubated at 4 °C for 90 min in the dark with gentle shaking. Cells were next washed in 260 μl of perm wash + 2% FBS followed by a 10 min incubation in the dark, shaking, at room temperature. The wash and incubation was repeated once. For three-color flow cytometry, an indirect staining approach was used for the third antibody by staining cells with a biotinylated HCmu in conjunction with steptavidin APC-750 (Sigma). For cell proliferation analysis, the nucledoside analog EdU and a Click-It kit (Invitrogen) was used according to manufacturer's instructions. Cells were pelleted and resuspended in 200 μl perm wash containing 2% FBS and immediate analyzed. Approximately 30,000 events were acquired per sample using a BD FACSArray (BD Biosciences). Duplicate samples were analyzed for each experiment. Experiments were repeated a minimum of three times.

Western blot analysis.

Trout cell suspensions were centrifuged in aliquots of 0.6×10^5 cells or 1.7×10^5 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 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 containing 0.5% Tween (PBST) for 1 h, followed by a 2 h incubation in blocking solution in the presence of primary antibody: ED-1 (Pax5.PD; 1µg/mL final dilution), Pax5.E10 (2 µg/mL final dilution) or Pax5.E6 (2 µg/mL). Four 5 minute washes in PBST were then followed by a 1 h incubation with the secondary antibody goat anti-rabbit IgG-HRP conjugate (0.1 µg/ml; Zymed Laboratories) in blocking solution, and membranes were washed four more times for 5 minutes each in PBST and filters developed using a chemiluminescence kit (ECL-Plus; GE Healthcare Life Sciences).

Flow cytometric data analysis.

Contour graphs were generated using WinMDI 2-8 (J. Trotter 1993–1998) software at a 2/0.1 threshold/smooth setting. Contour graphs are shown as log algorithms with intervals of 50%. Quadrants for LPS-activated samples were established by placing Q3 on the double negative population of the D0 sample. All other gates were created by following contour lines. Statistics were obtained from WinMDI 2-8 software. Arithmetic means of population frequencies and standard errors were calculated for each experiment

using Excel software. The change in relative intensity of antibody staining over days of LPS activation was calculated for each experiment as follows: the average fluorescence of a given antibody within the population of interest was calculated using WinMDI software and the highest fluorescence of the antibody within the experiment was given a 100% intensity value. All other average fluorescent values were calculated as a proportion of the highest fluorescent value. In this manner, we were able to examine the relative changes in average fluorescence across days of activation.

2.3 Results and Discussion.

Previous studies have extensively examined Pax5 alternative splicing in immune tissues across species. However, these studies fail to identify the expression of Pax5 isoforms in individual developing and activated B cell populations due to the use of pooled cells for RT-PCR. Here, we describe an alternative approach using flow cytometry on freshly isolated rainbow trout tissues. Fixed anterior kidney (AK), peripheral blood (PBL) and spleen (SPL) cells were permeabilized and stained with fluorescent antibodies targeting specific B cell markers and transcription factors (Figure 1). Initially, three distinct antibodies were used to explore the presence of Pax5 subpopulations in trout immune tissues. The Pax5.PD antibody recognized the paired domain of Pax5 and has been used in previous studies [2, 16, 32, 76]. A second anti-Pax5 antibody recognized the region encoded by exon 6 of Pax5, named Pax.E6. A third antibody, Pax5.E10, is specific to the C-terminal region of Pax5, encoded by exon 10.

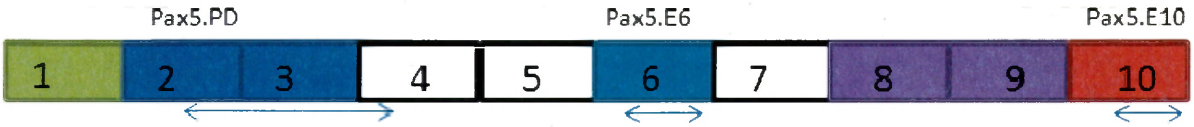


Figure 1. Diagram of trout Pax5 antibody specificity. Pax5.PD antibody recognizes the paired domain of Pax5; Pax5.E6 recongizes a region encoded by exon 6; Pax5.E10 recognizes the C-terminus (repressor domain). Each number represents the exon encoding the respective Pax5 domain.

To demarcate the identified B cell subpopulations, these three Pax5 antibodies were then used in combination with antibodies for the transcription factors X-box binding protein 1 (the processed form of Xbp1; [16]), EBF [32], and the marker of immunoglobulin heavy chain mu (HC mu; see Table 1; [2, 76]). Each of these antibodies had been used successfully in previous studies to define trout B cell populations. Xbp1 is expressed at high levels in very early B cell stages (CLP, pro-B), is down-regulated during B cell maturation, and is strongly induced again during terminal B cell differentiation (see Table 1). HCmu is first expressed in immature B cells and expression increases throughout terminal B cell differentiation. EBF is highly expressed in CLP and pro-B cells, followed by downregulation as the B cell matures [16].

Table 1. Expression of Pax5 isoform and development markers during B cell stages.

	Xbpl-S	Pax5.E10	Pax5.PD	Pax5.E6	EBF	RAG1	EdU	HCmu	FSC
CLP	++	-	–	++	+	+	+/–	–	H
Pro-B	+	+	+/–	+	+	+	+/–	–	M/H
Small pre-B	+	+/-	+	+/-	–	–	–	+/–	L
Mature B	+	+	+	+	–	–	–	+ or –	M
Early Act. B	+	++	+	+	–	–	–	+	M
Act. B	++	++	++	++	–	–	–	++	M
Plasmablast	++	+	+	+	–	–	+	++	H
T-Plasma cell	++	+	+/–	+	–	–	–	+++	H
M-Plasma cell	++	?	–	?	–	–	–	+++	H

Note: H: high; M: medium; L: low. Act.: activated. Expression demarcated by (+) sign, increased expression by (++) and highest expression by (+++).

2.3.1. Western blot analysis.

To verify that the three Pax5 antibodies recognized Pax5 protein, western blots of freshly isolated rainbow trout spleen, peripheral blood, and anterior kidney were prepared (Figure 2). Figure 2A shows that the Pax5.PD antibody recognizes the full length Pax5 protein, a protein with an approximate molecular weight of 48 kD which must represent full length Pax5. Using the same antibody, the level of Pax5 protein is significantly lower in anterior kidney compared to spleen and blood. Conversely, both the Pax5.E10 antibody (Figure 2B) and the Pax5.E6 antibody (Figure 2C) stained the 48 kD (full length Pax5) protein in all three tissues. Additionally, Pax5.E10 recognized a lower molecular weight isoform in all three tissues. These results demonstrate that the

three Pax5 antibodies recognize the target Pax5 protein and furthermore show that each antibody recognizes Pax5 in a unique manner across tissues.

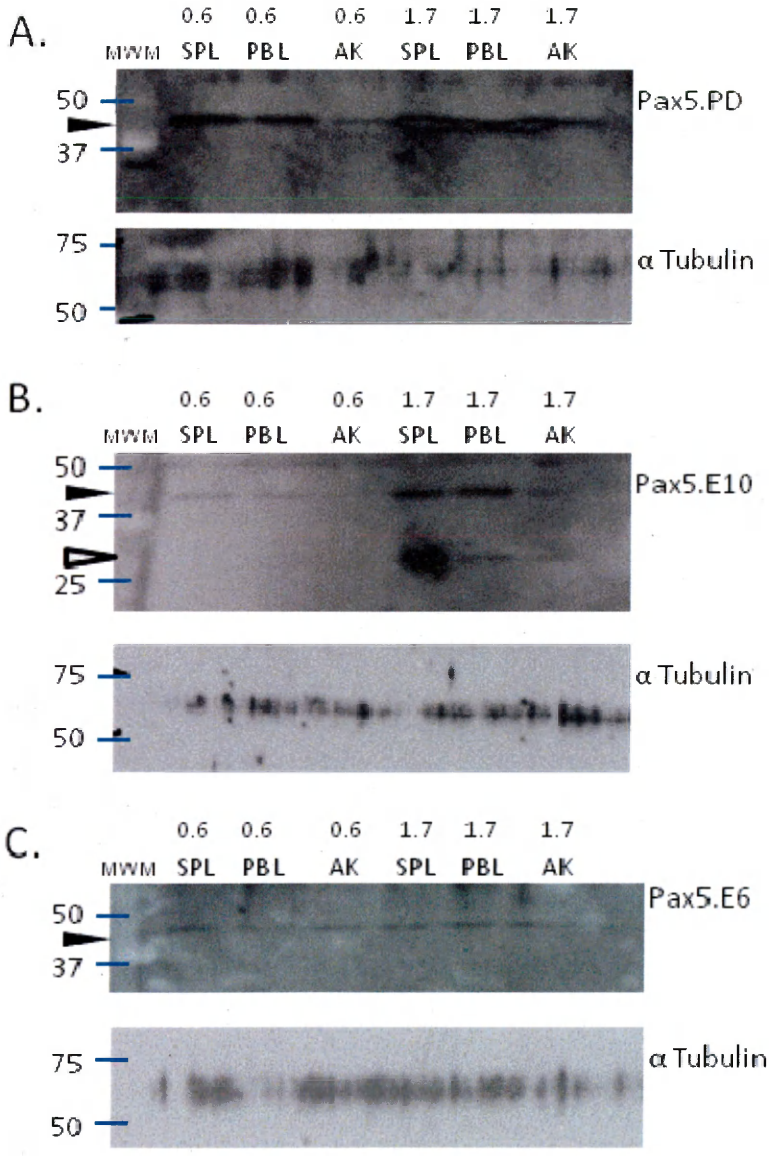


Figure 2. Western blot analysis of trout spleen (SPL), blood (PBL), and anterior kidney (AK) using three Pax5-specific antibodies. Closed Triangle: full length Pax5; open triangle: Pax5 isoform of lower molecular weight. Molecular weight markers (MWM) shown in kDA on left. First three lanes contain $0.6x10^5$ cells/lane; last three lanes contain $1.7x10^5$ cells/lane. A. Pax5.PD stain. B. Pax5.E10 stain. C. Pax5.E6 stain.

2.3.2. Patterns of Pax5-expressing cells using three distinct trout Pax5 antibodies.

Multiple lymphocyte populations were identified in the anterior kidney, blood and spleen as shown in Figure 3A. In the anterior kidney, a low and high FSC population is present, as reported previously [32]. These populations are predicted to contain late and early developing B cells respectively. Blood was highly variable, consisting of two to three lymphocyte populations with unique low and high FSCs. Spleen contained one main population of lymphocytes and a smaller population of low FSC cells.

Permeabilized anterior kidney, peripheral blood, and spleen cells were first co-stained with the anti-Pax5.E6 and anti-Pax5.E10 antibodies and analyzed by two-color flow cytometry (Figure 3B). For each tissue, three Pax5 subpopulations were identified: a Pax5.E6 high/Pax5.E10 low population (presumably all isoforms lacking the C-terminus of Pax5), a Pax5.E6 high/Pax5.E10 high population (cells expressing intermediate to high levels of both E6 and E10 regions of Pax5), and a Pax5.E6 low/Pax5.E10 high population (cells lacking exon 6 staining). Table 2 lists a summary of each subpopulation's mean frequencies and S.E.s.

When comparing the Pax5.E10 and E6 expression patterns between tissues, the most abundant population in all three immune tissues consisted of smaller lymphocytes that co-stained with both antibodies (the Pax5.E6 high/Pax5.E10 high population). For PBL and spleen, the frequencies of this co-staining population are consistent with previous findings [76], and likely represent resting mature B cells. For anterior kidney, the relatively high frequency of this population was unexpected.

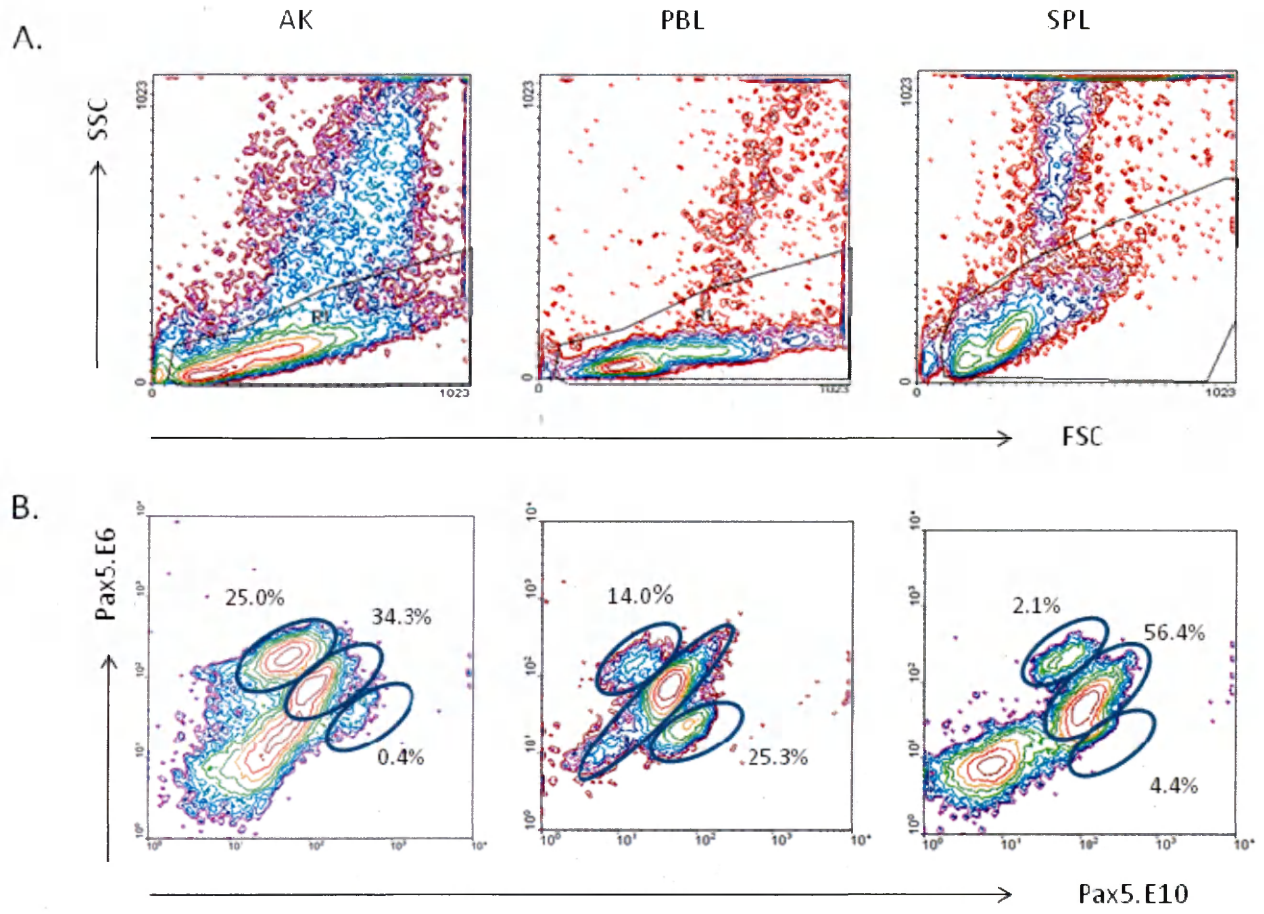


Figure 3. Two-color flow cytometry of anterior kidney (AK), peripheral blood (PBL), and spleen (SPL). A) FSC and SSC of gated lymphocyte populations. B) Patterns for Pax5 using the Pax5.E6 and Pax5.E10 antibodies. Frequencies of each population are given as mean % gated lymphocyte population. See Table 2 for all means and SEs.

Using the same two antibodies, a second population of Pax5-expressing cells had high Pax5.E6 staining, but lacked a C-terminus (Pax5.E6 high/Pax5.E10 low). This population was most abundant in anterior kidney (25.0% ± 3.5) and least abundant in spleen (2.1% ± 0.7). This subpopulation consisted of large cells (high FSC), supportive of their representing an early developing B cell population [32]. The possible presence of

developing B cells expressing both exon 6 and exon 10 in secondary lymphoid tissues (PBL and spleen) was investigated and is discussed below.

Conversely, cells that expressed Pax5 proteins that *lacked* the region encoding exon 6 but which contained a C-terminus (Pax5.E6 low/Pax5.E10 high population) were present at low relative frequencies in anterior kidney (0.4%) and spleen (4.4%), and at high frequencies in the PBL (25%). These findings suggest that isoforms expressing the C-terminus may be more prominent in activated, terminally differentiating B cells. The high frequency of presumably activated/terminally differentiated (Pax5.E6 low/Pax5.E10 high) cells in PBL was unexpected.

Next, trout tissues were stained with the same anti-Pax5.E10 antibody but now in combination with anti-Pax5.PD (the paired domain-specific antibody). Data are shown in Figure 4. In the spleen, the majority of cells co-stained (43.9%) with the two antibodies, and similarly, PBL showed high (32.8 %) co-staining. Together with the Pax5.E6/E10 data (Figure 3), this suggests that the main Pax5 isoform expressed in secondary immune tissues is the full-length form (PD+/E6+/E10+).

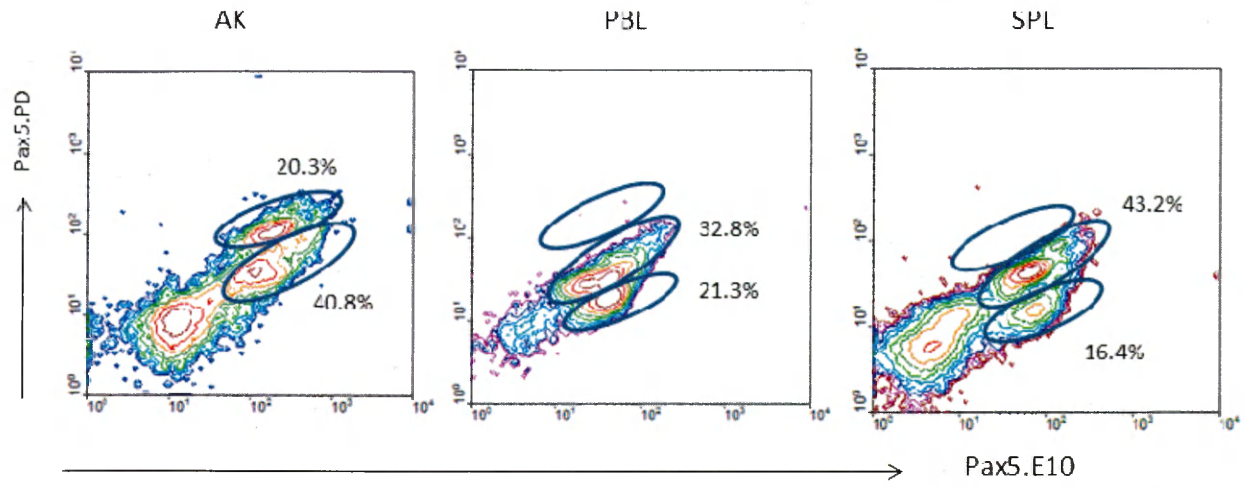


Figure 4. Two-color flow cytometry using Pax5.PD and Pax5.E10 antibodies. Mean frequencies represent % gated lymphocytes. See Table 2 for all means and SEs.

Furthermore, using the same two antibodies, an anterior kidney-unique Pax5-positive population was detected which contained the paired domain, but not the C-terminus of Pax5 (Pax5.PD high/Pax5.E10 low; mean of 20.3%). While both spleen and PBL lack this population, both tissues have a population of Pax5-expressing cells that are somewhat the opposite: they lack the paired domain, but do contain the C-terminus (PD low/ E10 high). In spleen an average of 16.4% of lymphocytes had this phenotype. Furthermore, these spleen cells exemplified a medium FSC, suggesting they are mature B cells high in C-terminal Pax5 expression and lower in paired domain Pax5 expression. Together with the data using Pax5.E6 and Pax5.E10 antibodies, this supports a role for the C-terminus of Pax5 in mature or activated B cells.

Table 2. Average % frequency (S.E.) of Pax5 subpopulations across immune tissues.

Combination	AK	PBL	SPL	Figure*
Pax5.E6 high/Pax5.E10 low	25.0 (3.5)	14.0 (5.4)	2.1 (0.7)	3B
Pax5.E6 high/Pax5.E10 high	34.3 (3.8)	53.1 (7.8)	56.4 (5.3)	3B
Pax5.E6 low/Pax5.E10 high	0.4 (0.2)	25.3 (4.3)	4.4 (1.4)	3B
Pax5.PD high/Pax5.E10 high	20.3 (1.7)	0.0 (0.0)	0.0 (0.0)**	4
Pax5.PD high/Pax5.E10 high	40.8 (6.8)	32.8 (13.1)	43.2 (3.8)	4
Pax5.PD low/Pax5.E10 high	0.0 (0.0)**	21.3 (9.9)	16.4 (2.8)	4
Pax5.PD high/Pax5.E6 int.	30.3 (4.4)	31.3 (4.1)	44.7 (2.5)	5A
Pax5.PD high/Pax5.E6 high	0.5 (0.2)	6.1 (2.8)	2.4 (0.3)	5A
Pax5.PD low/Pax5.E6 high	27.8 (3.5)	10.7 (3.0)	1.3 (0.4)	5A

Next, two-color flow cytometry was performed using the remaining combination of anti-Pax5 antibodies, namely anti-Pax5.PD and anti-Pax5.E6 (Figure 5). All three tissues show significant populations of high paired domain, intermediate exon 6 expressing (Pax5.PD high /Pax5.E6 int.) cells: spleen has 44.6%, blood 31.3%, and anterior kidney 30.3%. This suggests that these cells express full length Pax5 and represent mature B cells in spleen and blood, and either pre-B or immature B cells in the anterior kidney. It should be noted that upon tissue isolation, the anterior kidney tissue contains a fairly high amount of blood, which may affect the anterior kidney B cell frequencies and explain the relatively high percentage of paired-domain expressing (immature and mature B) cells in this tissue.

Paired-domain-lacking populations were present in all three tissues: in anterior kidney, 27.8% of the lymphocytes stained strongly for Pax5.E6, but weakly for Pax5.PD (Pax5.PD low/Pax5.E6 high). Both PBL (10.7%) and spleen (1.3%) also contain a paired-domain-less Pax5 population (Figure 5). For this antibody combination, the expression pattern for Pax5.E6 expressing cells follows the patterns shown above (see Figure 3), with the highest frequency of Pax5.E6 expressing, but paired domain lacking and C-terminus lacking population (PD–/E6+/E10–) in the anterior kidney, and the lowest frequency in the spleen, suggesting that this population contains mostly early developing B cells. This is supported by FSC data; as shown in Figure 5B, the Pax5.PD low/Pax5.E6 high population in anterior kidney primarily consists of large, high FSC cells, typically representing progenitor cells.

Lastly, using Pax5.PD and Pax5.E6 antibodies, a small population of high co-staining cells is present in all tissues, with the lowest frequency in anterior kidney (0.5%) and highest in the PBL (6.1%; Figure 5A). These cells likely contain full-length Pax5 and have increased Pax5 expression; hence, they may represent activated or terminally differentiating B cells. This agrees with other reports that observe increased Pax5 expression upon B cell activation [82], as well as with our own observations. Furthermore, the cell size of these high co-staining cells (Pax5.PD high/Pax5.E6 high) in spleen and PBL is considerably larger (medium to high FSC) than those of the mature B cells (Pax5.PD high/Pax5.E6 int. cells), supportive of their being in a more activated state. The higher frequency of this subpopulation in the PBL compared to spleen suggests that PBL contains more activated B cells than spleen.

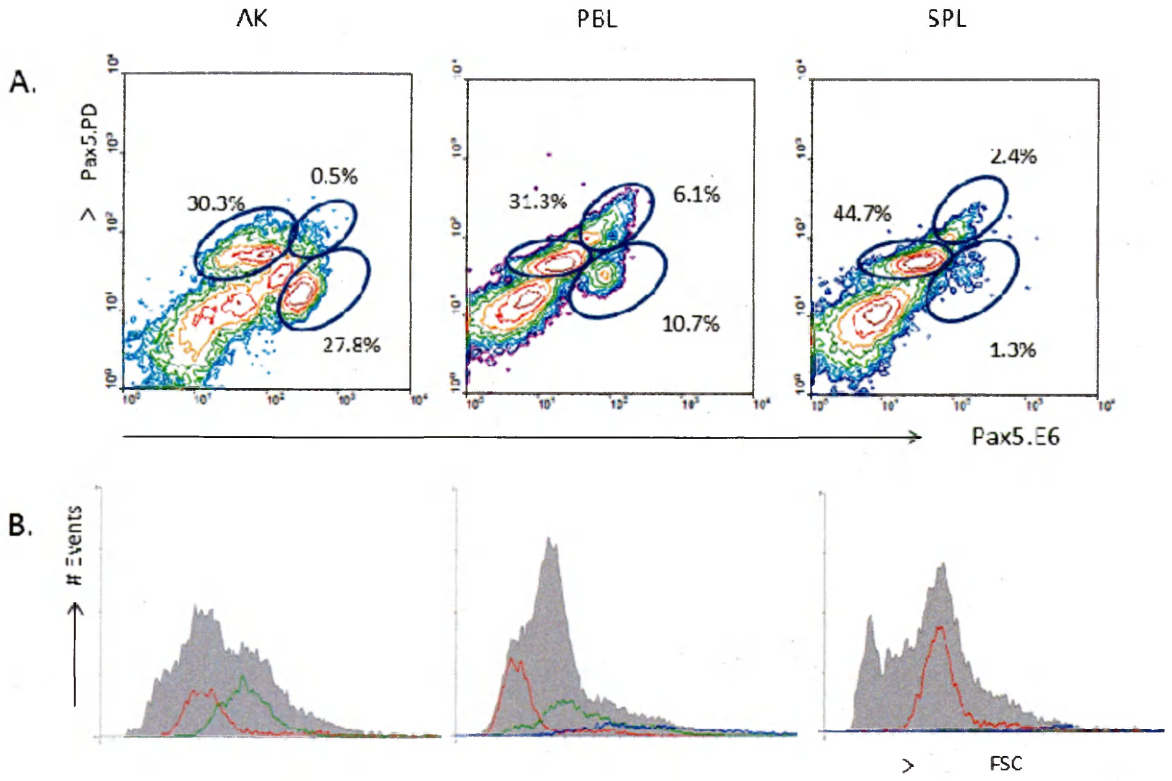


Figure 5. Two-color flow cytometry using the Pax5.PD and Pax5.E6 antibodies. A) Flow graphs depicting mean % frequency of each Pax5 subpopulation. See Table 2 for all means and SEs. B) FSC of each gated Pax5 population. Shaded line: all lymphocytes; red line: Pax5.PD high/Pax5.E6 int. population; green line: Pax5.PD low/Pax5.E6 high population; blue line: Pax5.PD high/Pax5.E6 high population. Each corresponding histograph is present below each flow graph.

In summary, using the three trout-Pax5 antibodies, we observed several patterns. We hypothesize that:

1. Some early developing B cells in the anterior kidney already express Pax5 isoforms that lack the paired domain and the C-terminus. The Pax5 pattern for such cells is PD–/E6+/E10– with a B cell signature of PD–/E6+/E10–/HCmu–/Xbp1+/EBF+.
2. The spleen and blood contain a minor population of early B cell progenitors with the Pax5 pattern PD–/E6+/E10+ with a B cell signature PD–/E6+/E10+ /HCmu–/Xbp1+/EBF+ . These cells are LPS sensitive and differentiate upon LPS activation.
3. The majority of mature resting B cells express full-length Pax5 protein.
4. A subpopulation of mature B cells has higher Pax5 C-terminus expression and activated B cells upregulate full-length forms of Pax5.
5. Blood may contain more activated B cells than spleen.

2.3.3. Identification of early developing B cells with the Pax5 pattern PD–/E6+/E10– in the trout anterior kidney.

To test the prediction that some very early developing B cells in anterior kidney express Pax5 isoforms that lack both the paired domain and C-terminus, we stained cells with a combination of Pax5 antibodies and the tXbp1-S, HCmu, and EBF antibodies. First, to provide evidence that cells expressing high Pax5.E6 represent early lymphoid progenitors, we stained anterior kidney cells with both anti-Xbp1 and anti-Pax5.E6 antibodies. Results showed that Xbp1 exhibited perfect co-staining with Pax5.E6 in all

three tissues (Figure 6): cells that express high Xbp1 also express high Pax5.E6, and cells that express low Xbp1 also express low Pax.E6. Next, to distinguish between early developing B cells and IgM-secreting plasmablasts/pre-plasma cells (both of which co-stain with Pax.E6 and which express high levels of Xbp1), we performed three-color flow cytometry with anti-Pax5.E6, anti-Xbp1, and anti-HCmu antibodies (Figure 6). Three Pax5.E6/Xbp1 co-staining populations were gated: high, intermediate, and no-staining, named R2, R3, and R4 respectively (see Figure 6A). Histograms were then generated for HCmu intensity of each gated population (Figure 6B). Importantly, the results show that a subpopulation of cells with the highest co-staining for E6 and Xbp1 (R2) lacked HCmu (Figure 6B, red line), in support of the prediction that most of these are early developing B cells. Cells from the intermediate population (R3) expressed intermediate to high levels of HCmu, suggesting these are the late developing B cells, while as expected, the R4 region also lacked HCmu expression. When the same experiment was repeated using anti-Pax5.PD instead of Pax5.E6, it was found that PD high cells do not express high levels of Xbp1 (data not shown). Together with the Pax5.PD data, this suggests that a subpopulation of anterior kidney cells express high levels of a Pax5 isoform that contains exon 6, but lacks a paired domain, while expressing high levels of Xbp1; such cells must represent early developing B cells, because they do not express immunoglobulin [16]. The FSC histogram supports this assumption, as the high co-staining subpopulation (R2) consists entirely of larger (high FSC) cells, a characteristic of early progenitors and transitional plasma cells. In contrast, the Pax5.PD cells are smaller, more mature cells (data not shown).

Formally, it is still possible that the PD–/E6+/Xbp1 +/HCmu– cells are antibody-secreting cells that secrete an isotype different from IgM. To further establish early B cell identity, we used the anti-EBF antibody in combination with anti-Pax5.E6, anti-PD, or anti-Xbp1. A population of large cells (high FSC) that co-stained E6 and EBF, and Xbp1 and EBF, was detected in anterior kidney, supporting that these are early developing B cells in the anterior kidney with the Pax5 pattern PD–/E6+/E10–.

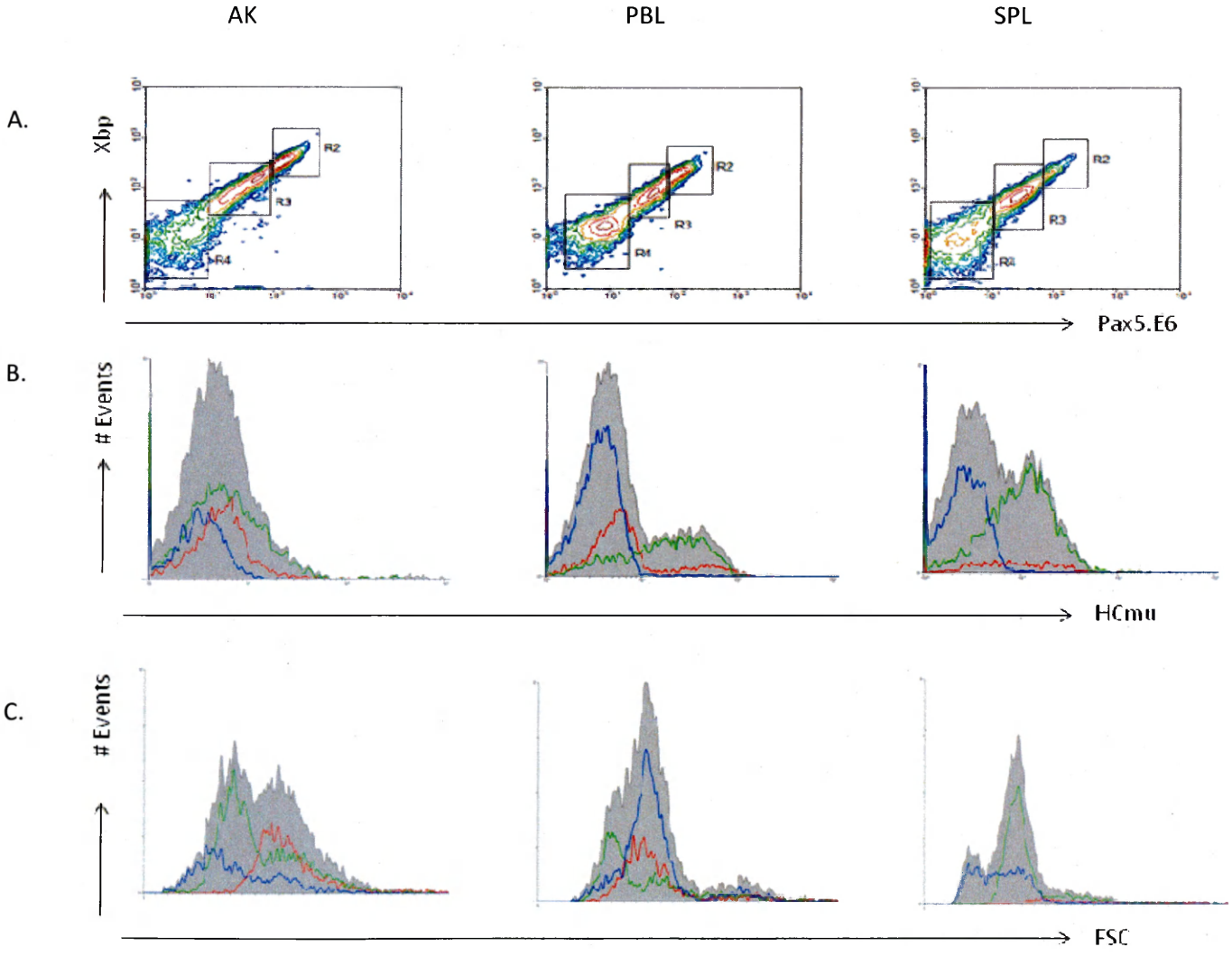


Figure 6. Three-color flow cytometry for Pax5.E6, HCmu, and Xbp1 in AK, PBL, and SPL. A) Flow graphs depicting the coexpression of Pax5.E6 and tXbp1. Regions R2, R3, and R4 utilized for histogram analysis. B) Histogram of number of cells within Pax5.E6/Xbp1 regions expressing intensities of HCmu. C) Histogram of FSC for cells within Pax5.E6/Xbp1 regions. Grey shaded line: all gated lymphocytes; red line: R2; green line: R3; blue line: R4.

2.3.4. Identification of early developing B cells with the PD–/E6+/E10+ Pax5 pattern in the trout spleen.

Next, the presence of early developing B cells (Pax5.E6 high/Xbp1 high) was tested in secondary immune sites. As expected, a population of mature B cells (high HCmu-expressing cells) was observed in the intermediate (R3) population in both spleen and PBL (Figure 6B; green line). However, the highest co-staining population (R2, red line) contained both HCmu-staining and non-HCmu-staining populations. The HCmu-staining population likely includes IgM antibody-secreting cells, either transitional or mature plasma cells [32].

To address the possibility that some of the Pax5.E6 high/Xbp1 high/ HCmu negative (R2) population in spleen contained early B cell progenitors, cells were stained with both Pax5.E6 and the early B cell marker EBF. Indeed, a small population of E6 high/EBF high cells was detected (data not shown). This supports our hypothesis that trout spleen cells house a small population of early developing B cells.

We additionally tested if cells high in Pax5.E10 expression also co-stained EBF. In the anterior kidney, none of the EBF positive cells express HCmu [32]. In both the anterior kidney and spleen, EBF co-stained with cells expressing the Pax5-C terminus, suggesting that some early developing B cells have a Pax5 pattern PD–/E6+/E10+. These cells likely represent a population of early progenitor B cells as they were larger in

size, but did not costain with cells expressing the highest E6 and Xbp1. In the spleen, this subpopulation had a frequency of 2-4%. Hence, our prediction that spleen contains a population of early developing B cells with the Pax5 pattern PD–/E6+/E10+ holds true. This population is different from the PD-/E6+/E10- population (Figures 3B and 5).

2.3.5. Differential expression of Pax5 isoforms during *in vitro* LPS-activation of trout spleen cells.

Three predictions needed to be tested in spleen cells: (1) that resting mature spleen cells express full length Pax5, (2) that spleen cells contain a small population of early developing B cells with the Pax5 pattern PD–/E6+/E10+ which differentiate upon activation, and (3) that activated B cells have increased expression levels of full-length Pax5. To test these predictions, we cultured spleen cells in the presence of mitogen LPS and analyzed cells on days 2, 4, and 7 after activation (see Figure 7).

Figure 7A displays the change in frequency of the major Pax5 lymphocyte population, the population that co-stained with Pax5 antibodies recognizing exons 6 and 10 (Pax5.E6 and Pax5.E10), and which presumably express the full-length form of Pax5. The abundance of this co-staining subpopulation *decreased* significantly as cells became activated by LPS, with frequencies dropping from 52.7% on day 0 to 21.0% on day 7 (see Table 3 for mean frequencies and SEs). Conversely, a population of cells staining for exon 10 but not exon 6 (Pax5.E6 low/Pax5.E10 high), *increased* in frequency, from 8.4% on day 0 to 20% by day 7. A similar *increase* was observed when the Pax5.E10 antibody was used in combination with Pax5.PD: cells staining with exon 10 but not PD. This

Pax5.PD low/Pax5.E10 high subpopulation increased from 14.6% on day 0 to 30.7% on day 7 (Figure 7B). In agreement with these patterns, the Pax5.PD/Pax5.E10 co-staining population (containing PD and exon 10) *decreased* in frequency during activation, demonstrating that cells stained with Pax5.PD decrease in frequency. Figure 7C represents the change in cell populations expressing paired domain and exon 6 across days of activation. The frequency of the Pax5.PD high/Pax5.E6 low subpopulation also *decreases* during activation (consistent with the trends observed in Figure 7B) as does the Pax5.PD high/Pax5.E6 high subpopulation. The graphs also demonstrate a noteworthy decrease of the Pax5.PD low/Pax5.E6 high early developing population (Figure 7A and 7C red circles) previously described in this study.

Together, these findings suggest that the frequency of cells expressing the paired domain decreases during activation. However, the frequency of cells expressing the Pax5 C-terminus, but not the paired domain (Pax5.PD low/Pax5.E10 high), increases during activation. Lastly, the Pax5.E6 high early developing cells decrease during activation, suggesting they differentiate upon LPS activation, although cell death could also account for this decrease.

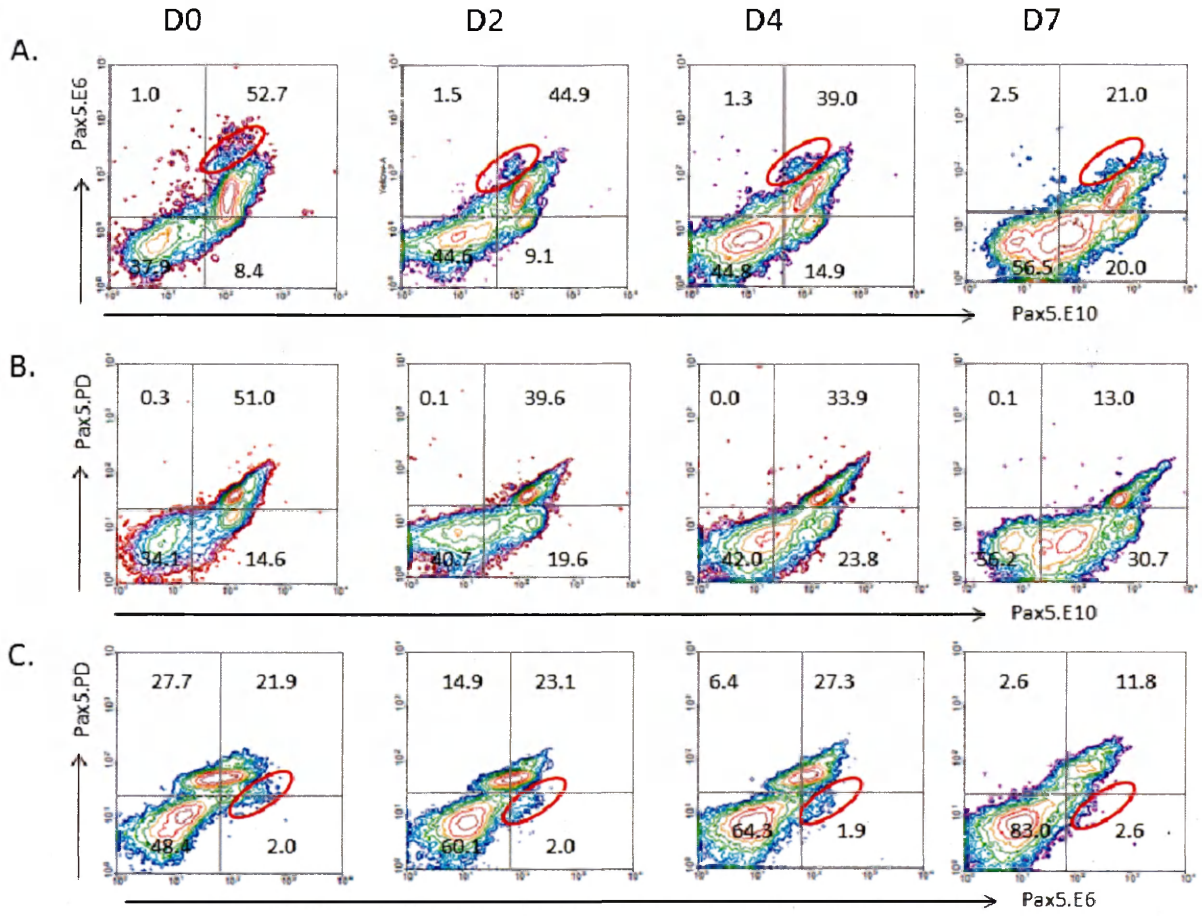


Figure 7. Two-color flow cytometry for cells cultured in *E. coli* LPS. Days (D) represent day fixed after LPS exposure. Red circles indicate Pax5.E6 high early developing cells. Frequencies represent average frequency of cells in quadrant. A) Cells stained with anti-Pax5.E6 and anti-Pax5.E10 antibodies. B) Cells stained with anti-Pax5.PD and anti-Pax5.E10 antibodies. C) Cells stained with anti-Pax5.PD and anti-Pax5.E6 antibodies.

To explore these patterns further, we measured if the changes in a population's cell frequency during activation were accompanied by changes in the relative intensity of Pax5 exon 10 expression in individual cells. We focused on two small, but dynamic, populations. First, the predicted early developing Pax5.E6 high and Pax5.E10 low population was analyzed (Figure 8). For this population of cells, the intensity of Pax5.E6 did not change significantly during LPS-activation, while the intensity of Pax5.E10 expression *increased* throughout the activation period. Based on earlier findings, the increase in Pax5.E10 supports the idea that these cells are differentiating during activation. A second minor population Pax5.E6 low/Pax5.E10 high (predicted to be mature or activated B cells), also showed little change in intensity of exon 6- expression, while the intensity of E10 again increased throughout the activation period.

Together, these data demonstrate that in addition to increased *cell frequency* of E10 staining cells during activation, cells also exhibit a significant increase of Pax5 C-terminus intensity. This could be an indication that cells are not losing usage of exon 6 expression as much as they are accumulating more Pax5 protein that contains a C-terminus.

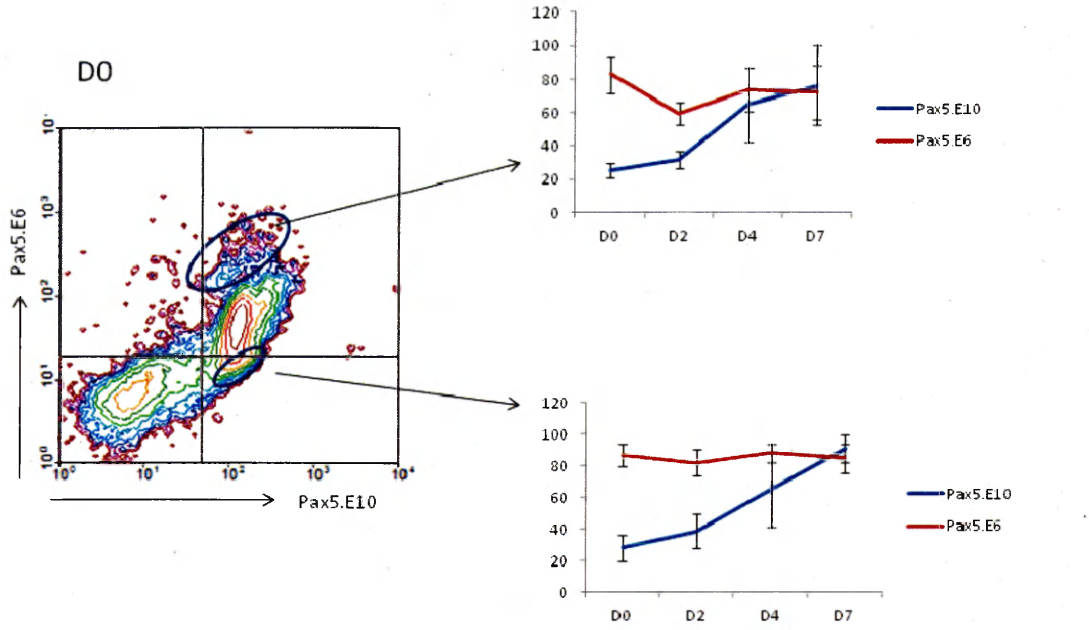


Figure 8. Change in relative intensity of Pax5.E6 and Pax5.E10 expression in LPS-activated SPL cells. Top graph: change in relative expression of the Pax5.E6 high/Pax5.E10 low population. Bottom graph: change in relative expression of the Pax5.E6 low/Pax5.E10 high population.

Similarly, when experiments are performed using Pax5.E10 and Pax5.PD antibodies, the high co-staining population of cells upregulate Pax5.E10 expression as they reach terminal B cell differentiation, while levels of paired domain in the cell remain relatively constant during that period (Figure 9). Furthermore, Pax5.PD low/Pax5.E10 high cells not only *increase* their Pax5.E10 intensity during activation, but simultaneously *decrease* their Pax5.PD intensity. This further emphasizes an increased role for the C-terminus and decreased role for the paired domain as early activated B cells differentiate toward antibody-secreting cells.

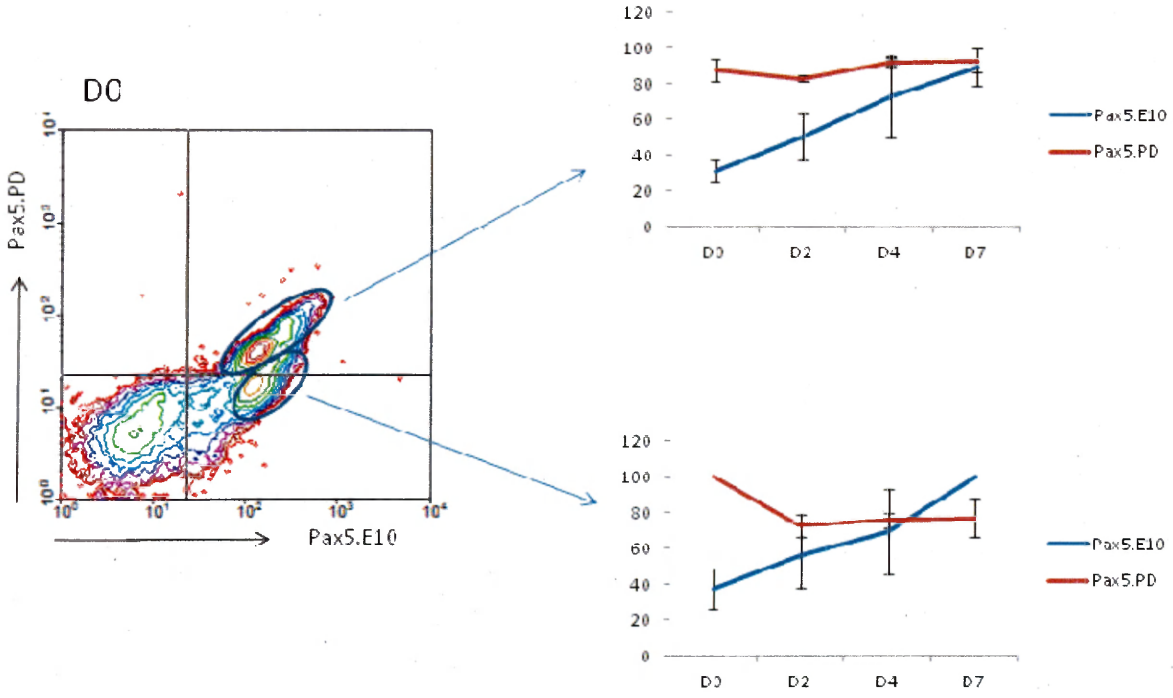


Figure 9. Change in relative intensity of Pax5.PD and Pax5.E10 expression in LPS-activated SPL cells. Top graph: change in relative expression of the Pax5.EPD high/Pax5.E10 low population. Bottom graph: change in relative expression of the Pax5.PD low/Pax5.E10 high population.

Lastly, we analyzed changes in Pax5.PD/Pax5.E6 intensity during LPS activation (Figure 10). Upon LPS-activation, the main Pax5 population, which represents mature B cells (Pax5.PD high/Pax5.E6 int.), had increased intensity of both Pax5.PD and Pax5.E6 domains. This increase in intensity corresponded with a transient increase in frequency of cells expressing high levels of both paired domain and exon 6 through day 4 of LPS activation. These patterns suggest that early activated B cells increase the amount of full-length Pax5 protein in their cells prior to downregulation of Pax5 during the final differentiation towards plasma cells.

The minor population of exon 6 possessing, but paired domain-lacking cells (Pax5.PD low/Pax5.E6 high) appears to lose even more Pax5.PD expression as the cells become more activated. Pax5.E6 expression per cell also decreases during activation. This population disappears over time, and consequently, this population could not be analyzed on day 7 after LPS activation. It is possible that this population represents T-plasma cells, which also have low expression of Pax5.PD [32]. Alternatively, a subpopulation of these E6 high cells is early developing cells because they co-stain with EBF and possibly develop into mature B cells during activation.

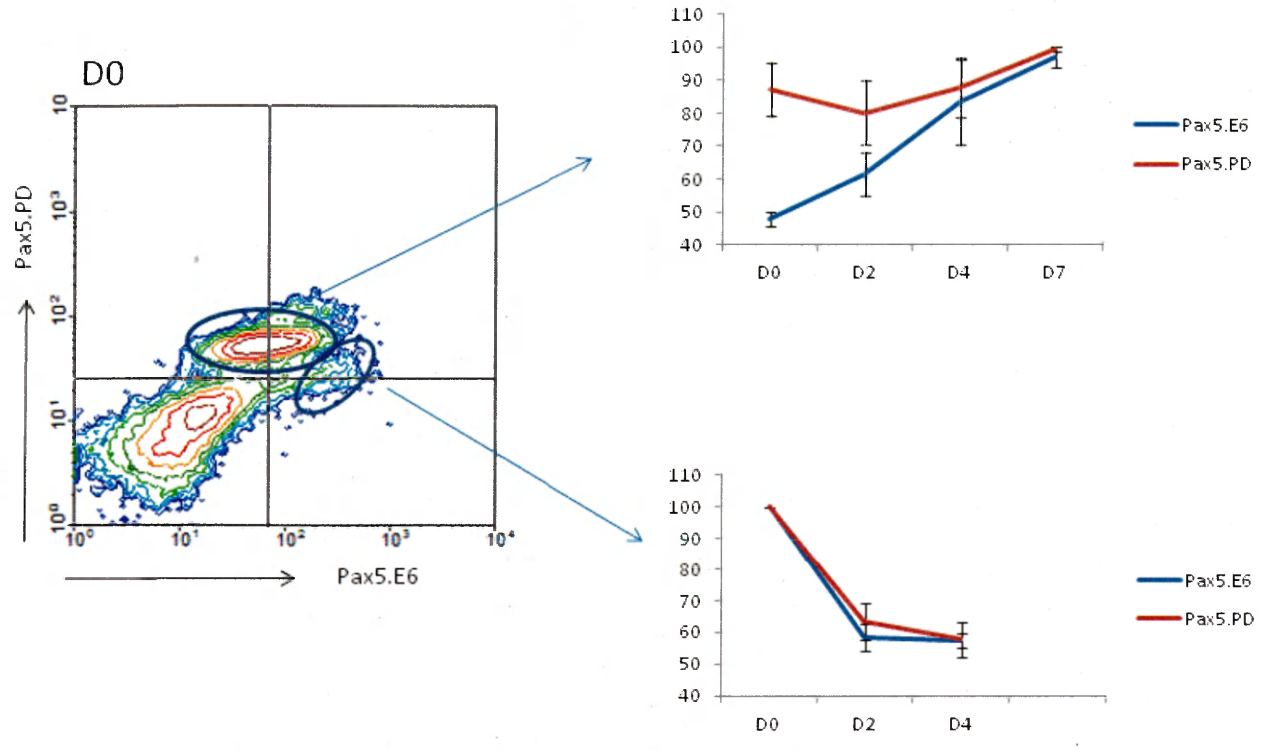


Figure 10. Change in relative intensity of Pax5.PD and Pax5.E6 expression in LPS-activated SPL cells. Top graph: change in relative expression of the Pax5.EPD high/Pax5.E6 int. population. Bottom graph: change in relative expression of the Pax5.PD low/Pax5.E6 high population.

2.3.6. Expression of Pax5 subpopulations with HCmu during LPS-activation.

So far, our findings suggested that Pax5 isoforms lose their paired domain during B cell activation, while some of these cells maintain their Pax5 C-terminus. To further test the validity of these findings, we stained spleen cells with combinations of Pax5 antibodies and the IgM-detecting (HCmu) antibody during LPS-activation, as presented

in Figure 11. Figure 11A and 11B demonstrate unique flow cytometry patterns for Pax5.E6/HCmu expression and Pax5.E10/HCmu expression, respectively.

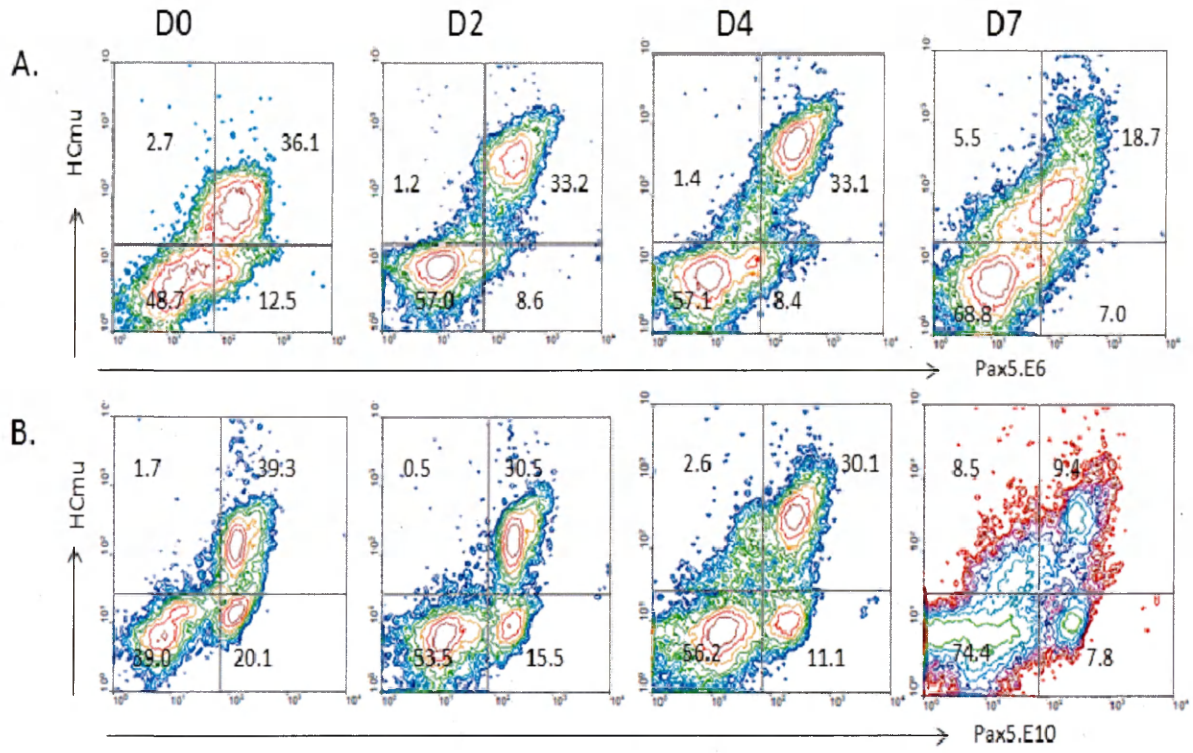


Figure 11. Two-color flow cytometry contours of LPS-activated SPL cells stained with anti-Pax5 and anti-HCmu antibodies. D: Day A) Cells stained with anti-Pax5.E6 and anti-HCmu antibodies across days in culture. B) Cells stained with Pax5.E10 and HCmu across days in culture.

The majority of Pax5.E6 positive cells co-stained for the HCmu antibody, while Pax5.E10 positive cells split into a IgM-expressing (HCmu high) and IgM-lacking (HCmu low) population (Figure 11B). As the graphs demonstrate, both the Pax5.E6 high/HCmu high population and the Pax5.E10 high/HCmu high population increase levels of HCmu per cell during the activation period, suggesting that cells expressing

both Pax5 domains (E6 and E10) are activated B cells. It is unlikely that any of the high IgM-expressing (HCmu++) cells have differentiated into plasma cells by day 7 [16, 32], and this corresponds with continued expression of Pax5 up to that time point. However, EdU proliferation analyses reveal that some of these cells are plasmablasts that express all three Pax5 antibodies (data not shown).

Two interesting additional observations can be made: first, when staining with Pax5.E10, there is a very significant shift from most cells being HCmu+ and Pax5.E10 double-positive cells, to almost equal numbers of IgM+ and IgM- cells that all contain the C-terminus of their Pax5 protein. Although some these IgM- cells additionally express the paired domain (data not shown), the frequency of the Pax5.E10+/IgM- population is consistently higher than the Pax5.PD/IgM- population. This further supports an important role for exon 10 during terminal B cell differentiation.

A second observation is that a minor, high Pax5.E6/low HCmu staining population is downregulated during B cell activation and is undetectable by D7. This is likely the same population observed earlier, Pax5.PD low/Pax5.E6 high (Figure 7). Hence, during terminal B cell differentiation, a population of paired domain-lacking, exon 6 expressing, but IgM-lacking B cells, (PD–/E6+/Hcmu–) disappears. It is possible that this population represents one of the two early developing B cell populations (PD–/E6+/E10+ or PD–/E6+/E10–) described above.

Table 3. Average % frequency (S.E.) of LPS activated SPL cells.

Pax5.E6/Pax5.E10	**Q1 (E6+/E10-)**	**Q2 (E6+/E10+)**	**Q3 (E6-/E10-)**	**Q4 (E6-/E10+)**
D0	1.0 (0.3)	52.7 (11.5)	37.9 (12.1)	8.4 (1.6)
D2	1.5 (0.9)	44.9 (2.0)	44.6 (2.5)	9.1 (1.5)
D4	1.3 (0.9)	39.0 (5.3)	44.8 (12.0)	14.9 (7.3)
D7	2.5 (1.1)	21.0 (0.9)	56.5 (7.3)	20.0 (9.3)
Pax5.PD/Pax5.E10	**Q1**	**Q2**	**Q3**	**Q4**
D0	0.3 (0.1)	51.0 (5.4)	34.1 (8.1)	14.6 (4.0)
D2	0.1 (0.0)	39.6 (1.3)	40.7 (2.5)	19.6 (3.3)
D4	0.0 (0.0)	33.9 (4.6)	42.0 (1.2)	23.8 (5.6)
D7	0.1 (0.0)	13.0 (0.8)	56.2 (12.3)	30.7 (13.0)
Pax5.PD/Pax5.E6	**Q1**	**Q2**	**Q3**	**Q4**
D0	27.7 (5.1)	21.9 (4.3)	48.4 (1.5)	2.0 (0.3)
D2	14.9 (5.6)	23.1 (3.7)	60.1 (5.0)	2.0 (0.5)
D4	6.4 (1.8)	27.3 (5.3)	64.3 (6.0)	1.9 (0.6)
D7	2.6 (0.4)	11.8 (3.3)	83.0 (3.7)	2.6 (1.0)
HCmu/Pax5.E6	**Q1**	**Q2**	**Q3**	**Q4**
D0	2.7 (1.3)	36.1 (3.8)	48.7 (3.6)	12.5 (2.6)
D2	1.2 (0.6)	33.2 (4.7)	57.0 (6.3)	8.6 (2.8)
D4	1.4 (0.8)	33.1 (4.3)	57.1 (5.6)	8.4 (2.7)
D7	5.5 (3.1)	18.7 (4.1)	68.8 (6.7)	7.0 (2.1)
HCmu/Pax5.E10	**Q1**	**Q2**	**Q3**	**Q4**
D0	1.7 (0.4)	39.3 (4.4)	38.9 (4.9)	20.1 (1.0)
D2	0.5 (0.1)	30.5 (3.7)	53.5 (6.4)	15.5 (2.9)
D4	2.6 (0.7)	30.1 (4.7)	56.2 (5.1)	11.1 (1.2)
D7	8.5 (1.9)	9.4 (2.8)	74.4 (6.8)	7.8 (2.2)

Note. D: day fixed after LPS activation.

2.3.7. Comparative expression of Pax5 subpopulations in the mouse and rainbow trout.

Much of the previous work on Pax5 alternative splicing has been performed using murine models. To further test our findings in the teleost model and to elucidate important evolutionary differences between teleosts and mammals, we performed two-color flow cytometry on freshly isolated mouse bone marrow (the functional equivalent to the anterior kidney) and mouse spleen cells. Cells were stained with a mouse equivalent of anti-Pax5.E10 antibody (C20) and the conserved anti-Pax5.PD antibody (ED-1). We do not currently have a mouse equivalent of the Pax5.E6 marker and therefore cannot use this for comparison. However, to further ascertain the identity of early developing, Pax5-expressing-B cell subpopulations, cells were stained with anti-EBF and anti-HCmu in combination with Pax5 antibodies.

Based on our findings in trout, we predicted that the mouse bone marrow would possess an early developing subpopulation with the Pax5 pattern PD–/E10+/HCmu–/EBF+. To test this prediction, we first stained mouse bone marrow cells for Pax5.PD and Pax5.E10. Figure 12 demonstrates that the flow cytometry patterns for bone marrow cells stained with this combination are different from the patterns identified earlier for trout anterior kidney cells. In contrast to anterior kidney, the bone marrow maintains a high frequency (27.0%) of cells lacking the paired domain (Pax5.PD negative) but expressing the Pax5 C-terminus (Pax5.E10 high cells). As presented earlier, the anterior kidney maintains a high frequency (20.3%) of cells expressing the paired domain but with low C-terminus expression. Previous research demonstrates that murine bone marrow consists of more early developing B cells and fewer late developing B cells than the trout anterior

kidney [32]. The higher frequency of the Pax5 C-terminus expressing cells may be attributed to the fact that mouse bone marrow contains more early developing B cells.

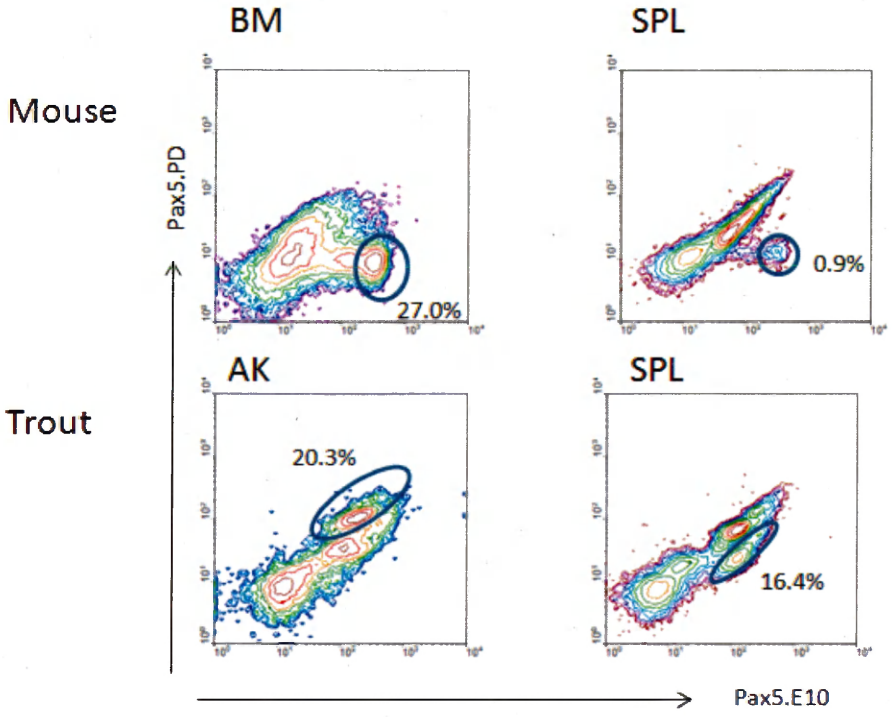


Figure 12. Two-color flow cytometry of mouse bone marrow (BM) and spleen (SPL) cells, and trout antierior kidney (AK) and spleen (SPL) cells stained with anti-Pax5.PD and anti-Pax5.E10 antibodies.

Surprisingly, when the PD/E10 combination in mouse spleen was tested, a small population of larger (high FSC) cells expressing the Pax5 C-terminus, but not the paired domain was identified. This population is similar to the small PD–/E10+ population identified in trout spleen. While the frequency of the mouse spleen Pax5.E10 high population is 0.9%, the trout spleen Pax5.PD low/Pax5.E10 high subpopulation is 16.4%.

The difference in frequencies may be explained by the earlier finding that trout spleen consists of a Pax5.E10 high mature/activated B cell population in addition to a Pax5.E10 high early developing B cell population.

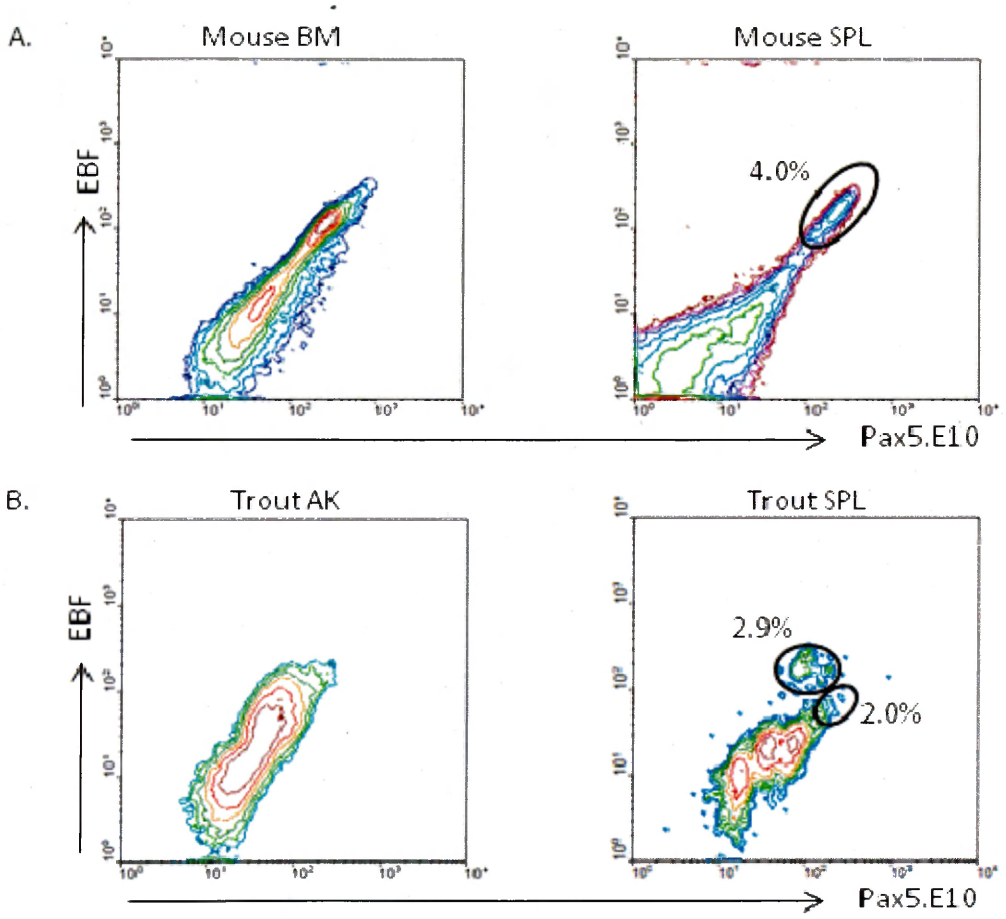


Figure 13. Two-color flow cytometry contour graphs for mouse and trout immune tissues. A) Mouse bone marrow (BM) and spleen (SPL) using anti-EBF and anti-Pax5.E10 antibodies. B) Trout anterior kidney (AK) and SPL using anti-EBF and anti-Pax5.E10 antibodies.

To test whether mouse Pax5.E10 high populations represented early developing B cells, we stained mouse cells with a combination of the anti-EBF and anti-Pax5.E10 antibodies. Remarkably, in both mouse bone marrow and spleen, the Pax5.E10 high/EBF high populations co-stained EBF and exemplified flow cytometry patterns comparable to the trout anterior kidney and spleen (Figure 13). In mouse spleen, the frequency of the Pax5.E10 high population was 2-4%. In comparison, in trout spleen we had observed two EBF high populations that co-stained with Pax5.E10, one at 2.9% and one at 2.0%. Together with the previous data, these patterns suggest that both species have a population of early developing B cells that express their Pax5 C-terminus, but do not express the paired domain (Figure 12). Furthermore, in mouse as in trout, the Pax5.E10 high/EBF high population does not co-express HCmu (data not shown). From these findings, we can conclude that although mouse and trout may differentially utilize isoforms containing the C-terminus during B cell maturation and activation, both species possess an isoform containing exon 10, but lacking paired domain, during early B cell development.

2.3.8. Concluding Remarks.

In conclusion, we have demonstrated that antibodies targeting three distinct domains of Pax5 can be used with flow cytometry to identify individual B cell populations in rainbow trout using flow cytometric analysis. Early developing B cells, and potentially common lymphocyte progenitors, lack the paired domain and are characterized by high exon 6 expression. Early pro-B cells may similarly have high exon

6 expression, but are further characterized by high exon 10 expression. These cell populations are observed in both the anterior kidney and the spleen.

The majority of mature B cells expressed in the spleen are characterized by full length Pax5 expression. However, there is an additional population of mature/activated B cells that are low in Pax5 paired domain and high in Pax5 C-terminus. By staining LPS activated cells, we have demonstrated that terminal differentiation is characterized by the loss of the paired domain and potential maintenance or increase of the C-terminus. Additionally, we have shown that activated B cells, there is a transiently increase in the expression of full length Pax5 expression. Finally, using a murine model, we have provided support for our novel finding that Pax5 isoforms lacking the paired domain are observed in early developing B cell populations. Furthermore, we have demonstrated that the use of these isoforms in developing B cells is potentially conserved between mammalian and teleost species.

Chapter 3- R and S Fish

3.1 Introduction.

Flavobacterium psychrophilum is the pathogen responsible for bacterial cold water disease (BCWD), a sickness which contributes to high mortality rates in aquaculture [93]. Recent work at the National Center for Cool and Cold Water Aquaculture has attempted to identify traits of rainbow trout resistant to BCWD and to selectively breed resistant trout through three generations [94]. The selection process has proved promising, but it has yet to be uncovered which molecular mechanisms underlie trout flavobacterium resistance. One potential mechanism contributing to resistance is a change in humoral immune response. Therefore, to investigate the humoral immunity of resistant trout, our lab is currently utilizing flow cytometry to compare and contrast the B cell populations composing the resistant and susceptible trout immune tissues. In this chapter, we present key preliminary results regarding the proliferating, developing, and activated B cell populations.

3.2 Results.

To further understand the molecular basis of bacterial cold water disease resistance, our lab examined B cell development and activation in trout bred to be genetically resistant (R line) or susceptible (S line) to flavobacterium. We first created single cell suspensions from either freshly isolated R line or S line trout immune tissues. We then fixed, permeabilized, and stained cells with antibodies targeting developmental transcription factors and HCmu. We additionally performed primary cell culturing of

cells in the presence of mitogen LPS and proliferation marker EdU prior to cell fixation. Two-color flow cytometry was performed to analyze differences in cell proliferation and B cell development and activation.

3.2.1 Differences in LPS activated B cell proliferation.

To test the differences in B cell proliferation, R line and S line trout blood and spleen were LPS activated over seven days. Prior to collection, cells were cultured in the presence of nucleoside analog EdU (Invitrogen). The cells were then fixed, permeabilized, and stained with either the anti-Pax5.E10 or Pax5.E6 antibodies (for methods, see Chapter 2).

As expected, the majority of proliferating blood and spleen cells expressed both exon 6 and exon 10 of Pax5, suggesting these cells were in the plasmablast stage of activation (see Q2 of Tables 4 and 5). The preliminary results do not suggest a trend in the frequency of proliferating B cells expressing Pax5.E10 versus the frequency of proliferating cells expressing Pax5.E6. However, observations from one experiment may suggest that more proliferating cells are Pax5.E10 negative across activation than Pax5.E6 negative. This means that as B cells terminally differentiate, they lose isoforms containing exon 10 of Pax5. Although Pax5.E10 is maintained in a higher frequency of cells during activation than Pax5.E6, it could be the case that only Pax5.E6 is expressed later in the transient plasma cell stage.

Table 4. Average frequency of proliferating and non-proliferating Pax5.E10 cells (S.E.).

	R-PBL			**S-PBL**			**R-SPL**			**S-SPL**		
	D2	D4	D7	D2	D4	D7	D2	D4	D7	D2	D4	D7
Q1 E10+/EdU-	19.3 (7.9)	38.5 (9.0)	46.2 (11.7)	23.9 (10.9)	38.9 (9.3)	41.1 (14.7)	34.4 (9.0)	28.7 (5.4)	18.1 (3.1)	50.3 (10.4)	47.4 (9.2)	27.2 (5.1)
Q2 E10+/EdU+	**0.3** (0.2)	**1.7** (0.4)	**0.7** (0.4)	**0.2** (0.0)	**3.7** (1.2)	**2.6** (1.3)	**0.2** (0.1)	**1.2** (0.5)	**0.4** (0.2)	**0.3** (0.0)	**1.1** (0.5)	**0.7** (0.3)
Q3 E10-/EdU-	80.1 (7.7	59.3 (8.7)	53.0 (11.3)	75.8 (11.0)	56.7 (10.0)	54.8 (13.4)	65.4 (8.9)	69.8 (4.8)	81.4 (3.3)	49.4 (10.4)	51.1 (9.5)	71.9 (5.20
Q4 E10-/EdU+	0.3 (0.2)	0.5 (0.3)	0.0 (0.0)	0.1 (0.0)	0.7 (0.5)	1.4 (1.3)	0.1 (0.0)	0.3 (0.2)	0.0 (0.0)	0.0 (0.0)	0.3 (0.2)	0.2 (0.2)

Note. S.E. represented in parentheses. D: Day of fixation after LPS activation

Table 5. Average frequency of proliferating and non-proliferating Pax5.E6 cells (S.E.).

	R-PBL			**S- PBL**			**R-SPL**			**S-SPL**		
	D2	D4	D7	D2	D4	D7	D2	D4	D7	D2	D4	D7
Q1 E6+/EdU-	33.31 (11.4)	42.0 (12.1)	47.7 (4.6)	32.8 (6.9)	38.3 (10.4)	49.5 (8.7)	40.4 (10.3)	32.9 (6.6)	25.9 (7.3)	43.0 (10.1)	39.7 (7.0)	31.9 (3.8)
Q2 E6+/EdU+	**0.1** (0.0)	**0.4** (0.2)	**0.1** (0.0)	**0.1** (0.0)	**2.1** (2.4)	**1.2** (0.7)	**0.2** (0.0)	**0.7** (0.3)	**0.3** (0.2)	**0.2** (0.0)	**0.8** (0.3)	**0.5** (0.3)
Q3 E6-/EdU-	66.6 (11.4)	57.5 (12.2)	52.1 (4.6)	67.1 (6.9)	48.7 (12.6)	49.3 (9.4)	59.3 (11.8)	66.4 (6.3)	73.8 (7.4)	56.7 (10.1)	59.5 (6.8)	67.5 (3.6)
Q4 E6-/EdU+	0.0 (0.0)	0.1 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.1)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	0.1 (0.1)

Note. S.E. represented in parentheses. D: Day of fixation after LPS activation

Total proliferation of cells independent of Pax5 expression was also identified for each experiment. The results show that blood had the highest proliferation and expressed proliferating cells differently between resistant and susceptible lines of trout. The frequency of proliferating cells during activation is higher in the S line blood versus the R line blood (Figure 14A). In contrast to blood, there was very little cell proliferation in the spleen and there appeared to be no differences in spleen cell proliferation between the R line and S line (Figure 14B). These findings were unexpected due to initial observations during our earlier experiments with younger trout. In these first experiments, we observed greater increases in cell proliferation in blood and spleen of the R line compared to the S line. However, in later experiments with older fish, we observed the opposite for blood and the lack of proliferation in spleen cells. One possible explanation for this change could be that the older resistant fish contained more erythrocytes than susceptible fish. The increased presence of erythrocytes in these R line spleens could possibly alter the ability of cultured lymphocytes to uptake the EdU. A consequence of this would be a false negative proliferation result. However, it is still possible that earlier experiments were outliers. A larger sample size will be necessary before any conclusions can be drawn for these proliferation studies.

3.2.2 Differences in B cell development.

Our initial study of Pax5 isoform expression in B lymphocytes revealed a population of early developing B cells lacking the Pax5 paired domain but expressing Pax5 exon 6. This population was found (at different frequencies) in the anterior kidney,

blood, and spleen. The presence of this developing population in both primary and secondary immune tissues lead us to believe it would be an interesting candidate population to compare between R and S lines of trout. We therefore analyzed the frequency of cells in the Pax5.PD low/Pax5.E6 high population across tissues. By using this combination, we ensured that we were examining an early developing Pax5 population lacking in paired domain. The results show an interesting trend: the developing population is present at a higher frequency in R line anterior kidney and blood compared to S line (Figure 15A). Conversely, in spleen, the developing population has a higher frequency in the *S line* than the R line.

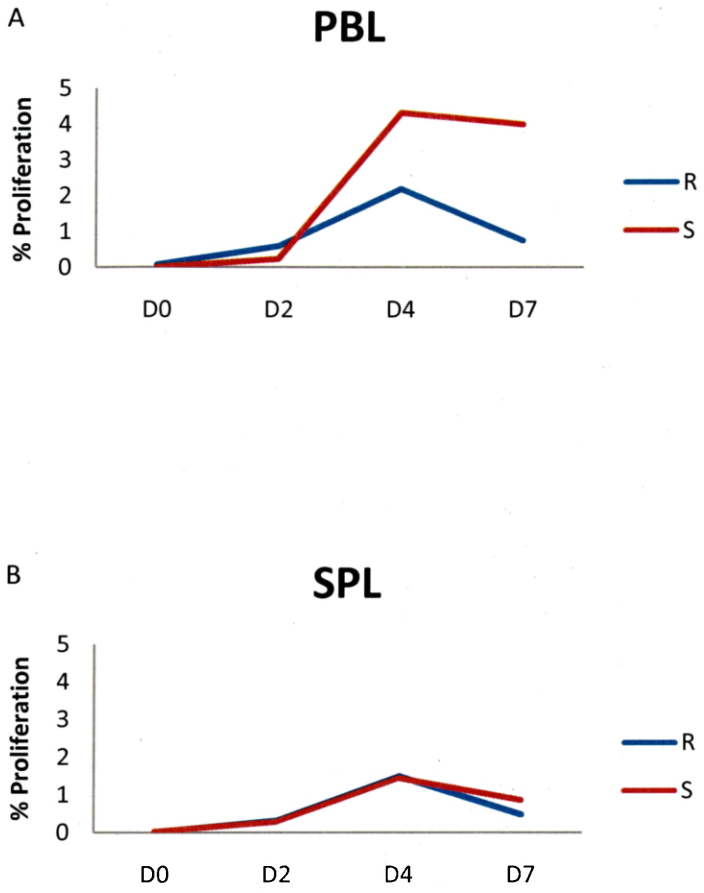


Figure 14. The average frequency of proliferating cells in R line and S line blood (A) and spleen (B).

There are several potential explanations for the differences in developing B cell populations. Previous studies demonstrated increases in murine spleen hematopoiesis at the expense of bone marrow hematopoiesis after viral or bacterial infection [95-97]. It is possible that infection in rainbow trout induces a response resulting in increased splenic B cell development. In contrast to spleen, the increase in developing B cells in the anterior kidney and blood of resistant fish could provide a means to protect against

infection. If this is true, then this follows our expectation that susceptible fish have a lower frequency of developing B cell populations in the anterior kidney and blood. However, this is again speculation; larger sample sizes and statistical analysis will reveal if these findings reflect a true biological phenomenon.

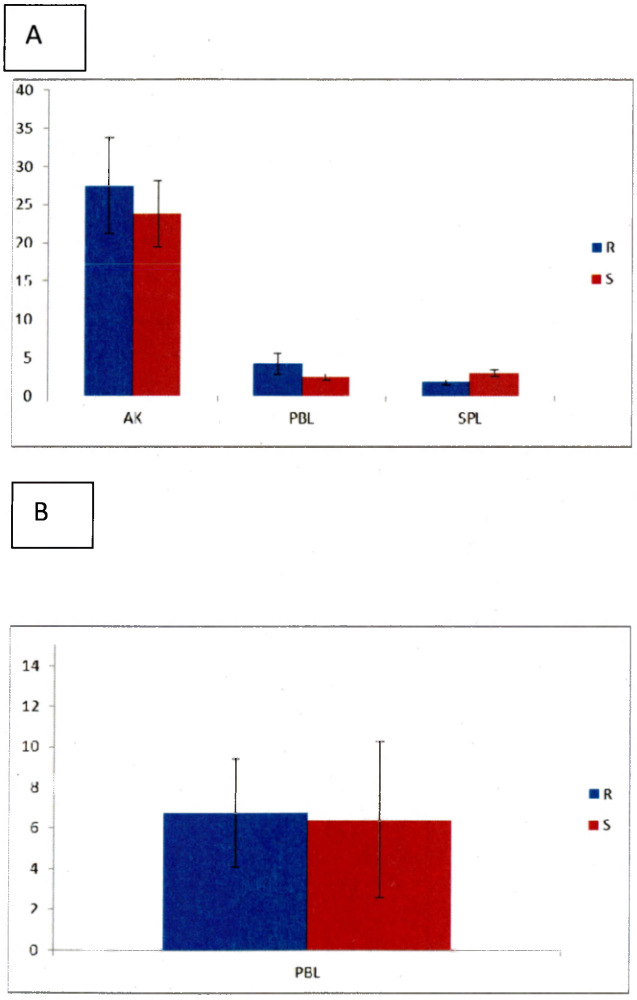


Figure 15. Differences in early developing (A) and activated (B) B cell populations in R and S lines of trout.

3.2.3. Differences in blood B cell activation.

Using three-color flow cytometry, we previously demonstrated that a population of blood cells expressing high paired domain (Pax5.PD) and Pax5 exon 6 (Pax5.E6) were additionally high in HCmu expression. The HCmu expression indicated this population was highly activated. Interestingly, this population had the highest frequency in the blood, suggesting a role for more activated B cells in this tissue. Therefore, we decided to investigate changes in this activated population between R and S line blood using two-color flow cytometry for Pax5.PD and Pax5.E6 expression. The results demonstrate a slight increase in the frequency of the activated population in resistant fish (Figure 15B). An increase in activated cells could be a mechanism allowing the fish to have B cells more readily available to fight pathogen. However, this result does not likely represent a statistically significant finding as the standard error was large in both resistant and susceptible fish samples. Therefore, in our preliminary experiments there appears to be no difference in the Pax5.PD/Pax5.E6 population between trout strains. However, as the blood is a highly variable tissue and our sample size is small, it may be premature to conclude no differences exist.

3.2.4. Concluding remarks.

Our preliminary results suggest two major differences in B cell populations between R and S lines of trout. First, the susceptible fish may express a higher frequency of proliferating blood plasmablasts after day four of LPS activation, as demonstrated by cells' combinatorial expressions of Pax5.E10 (or Pax5.E6) and EdU. The reason for

higher proliferation in susceptible trout is unclear, but one potential explanation could be that the fish are inducing a greater humoral response to pathogen more frequently than the resistant fish. Our preliminary data also suggest a role for early developing B cells in anterior kidney and blood (but not spleen) in resistant line trout. With a higher frequency of early developing B cells, resistant fish may be able to more readily fight pathogen by inducing a more rapid developmental program.

Although no differences were identified when examining the activated Pax5.PD/Pax.E6 high B cell population, our observations indicate there still may be differences in activated B cells between fish strains. The data from other Pax5 combinations in addition to LPS studies suggest resistant fish more often express higher frequencies of cells in many of the activated B cell populations (data not shown). The spleen will be a particularly interesting tissue to examine in this context and other studies of resistant fish have already demonstrated that spleen sizes, particularly larger spleens, correspond to flavobacterium resistance [98]. Overall, the potential to identify differences in developing B cell populations between resistant and susceptible trout is promising. Identifying the molecular context of flavobacterium resistance is essential for understanding the implications of genetic alterations and is invaluable knowledge for future aquaculture engineering.

Chapter 4-Discussion.

The expression and function of Pax5 isoforms remains an enigma in the immunological community. Thus far, the use of RT-PCR has enabled researchers to identify multiple transcripts that encode N- and C-terminal Pax5 isoforms, many of which are conserved across species. Such studies have revealed that trout, mice, humans, and amphioxus all express a subset of Pax5 isoforms that lack either the paired domain or C-terminus. Transfection studies have further demonstrated these isoforms are not only translated, but also have functional transactivating capabilities. Despite these advances, research has yet to demonstrate a definitive pattern of Pax5 isoforms across cell lines, immune tissues, and malignancies. Furthermore, investigators still do not understand the biological function of these distinct isoforms or the relevance of their expression patterns. Here, for the first time, we support a role for Pax5 isoforms by demonstrating differential isoform expression in individual developing and activated B cell populations in rainbow trout. Although our flow cytometric approach has limitations, it reveals that distinct early developing and activated populations can be characterized by their combined expression of transcription factors, surface markers, and Pax5 domains. Collectively, we call this expression pattern the "Pax5 signature" of a tissue.

4.1 The Pax5 patterns of early developing B cells.

The anterior kidney is a complex immune tissue composed of early developing, late developing, and long-lived plasma cells ([2, 3, 32, 76]. Previous studies have separated anterior kidney populations by their diverse expression of EBF, HCmu and

most relevantly, the Pax5 paired domain [32]. The majority of *early* developing B cells are characterized by high FSC, high EBF expression, and low Pax5 paired domain expression [32]. As the paired domain is essential for the transcription of genes necessary for B cell fate [8, 60], it is remarkable that our results identified a large population of the earliest developing B cells, including potential CLPs, that express isoforms of Pax5 that contain exon 6 but lack both the paired domain and the C-terminus. These cells have a Pax5 pattern PD-/E6+/E10- with a B cell signature PD-/E6+/E10-/HCmu-/Xbp1+/EBF+. This high FSC population consistently co-stained high levels of Xbp1 and EBF, and remained negative in heavy chain immunoglobulin expression, suggesting it constituted the earliest of B cell populations (see Table 1).

Most importantly, the earliest B cell population suggests a conserved developmental role for Pax5 isoforms lacking exon 2 and exon 10. In general, one proposed function for differential isoform expression is to regulate the activity of full length proteins by competing with full length isoforms for protein/DNA binding partners. This mechanism has been observed for isoforms of the alternatively spliced transcription factor Ikaros (reviewed in [99]). Supporting this hypothesis, studies of murine Pax5 show that the Pax5-d isoform may act as a dominant negative regulator; when co-transfected with full length Pax5 *in vitro*, it downregulates the activity of the full length protein [84]. Cross-species analyses reveal vast differences in Pax5 isoform expression; however, almost all species examined share isoforms lacking a functional paired domain. Recent research suggests that although Pax5 isoforms lacking a paired domain are incapable of binding DNA, they may still indirectly regulate transcription at anti-sense promoters via

the Pax5 partial homeodomain located on exon 6 [100]. Research has yet to elucidate if this mechanism of regulation is prominent in early developing B cells.

The lack of exon 10 expression in the earliest developing B cells demonstrates that in addition to a lack of DNA binding capability, early B cells also lack a repressor domain. Although the function of the repressor domain remains poorly understood, studies demonstrate that deletion of the last 22-33 amino acids of the Pax5 C-terminus results in increased Pax5 transactivating potential. Thus, the Pax5 transactivation domain may be strongly regulated by this adjacent inhibitory domain [71]. The lack of this domain in early developing cells suggests a potential role for this inhibitory domain in B cell activation, a hypothesis which is addressed later in this discussion.

An additional subpopulation of cells lacking the paired domain but possessing regions encoded by both exons 6 and 10 further supports a role for paired-domain-lacking isoforms in development. This population had a Pax5 pattern PD-/E6+/E10+ and a B cell signature PD-/E6+/E10+/HCmu-/Xbp1+/EBF+. However, these cells expressed lower levels of Xbp1 as compared to the earliest developing B cells, suggesting that they have progressed to a later stage of B cell development.

The role of cells lacking the Pax5 paired domain but expressing the Pax5 C-terminus in development was further evidenced by our murine model. Mouse bone marrow cells displayed similar patterns to the trout anterior kidney cells; the bone marrow had a population of cells that lacked the Pax5 paired domain but co-expressed EBF and the Pax5 C-terminus. As we do not currently have an equivalent antibody for the exon 6 domain of murine Pax5, we do not know whether bone marrow cells express

isoforms with the Pax5 C-terminus solely in progenitor B cells or if earlier developing cells also express isoforms with the C-terminus. In the anterior kidney, the majority of early developing cells expressed both exon 6 and exon 10 of Pax5, suggesting these two exons are expressed on similar isoforms.

The anterior kidney represents the primary site of hematopoiesis [3]. However, our results demonstrated that trout spleen also houses a population of early developing cells. Currently, there are no available surface markers, such as CD43 and CD34, to identify trout hematopoietic cells, but our findings suggest that hematopoiesis occurs in this secondary immune tissue. While this discovery of early developing cells in spleen is the first report of such a population in teleosts, it reflects similar findings in mammals which have demonstrated human and porcine hematopoiesis occurring in the adult spleen [101]. Importantly, we show that in both mouse and trout spleen, these early developing B cells lack the paired domain and express the Pax5 repressor domain (E10), further supporting a role for Pax5 isoforms lacking the paired domain in development.

4.2 The Pax5 patterns of late developing B cells

Late developing B cells of the anterior kidney include the small pre-B, immature, and mature B cells [32]. These cells have previously been characterized as having high Pax5 expression, HCmu expression, and low FSC. This trend was further supported by our data. In the anterior kidney, we consistently observed a population of cells expressing paired domain isoforms that were low in FSC (Figure 11). What is interesting about this population of developing B cells is that they had *higher* paired domain expression than

exon 6 and exon 10 expression. This late developing population is composed of approximately 20-30% lymphocytes, consistent with previous findings that late developing cells expressing Pax5 constitute approximately 18.2% of the anterior kidney [32]. The higher expression of the Pax5 paired domain in these late developing cells suggests there is an upregulation of isoforms expressing the paired domain (including full length Pax5) as early as the pre-B stage of development. An additional subpopulation of these late developing cells co-stained all three Pax5 isoforms and presumably expresses full length Pax5. Although similar patterns are observed in trout and mouse, it should be noted that we do not observe the same degree of co-staining in murine bone marrow. Consistent with earlier studies from our lab [32] one possible explanation for this is that the trout anterior kidney consists of more late-developing B cells (18.2%) than murine bone marrow (13.4%).

4.3 The Pax5 patterns of activated and differentiating B cells.

A great majority of lymphocytes in the trout spleen (and presumably blood) are either mature or activated B cells [2, 16]. Such cells co-expressed each of the three tested Pax5 domains, suggesting that mature B cells express the full length Pax5. A subpopulation of mature B cells upregulated its Pax5 C-terminus expression but not its paired domain expression. These cells were larger than resting B cells and were possibly partially activated B cells. This notion is supported by the LPS data, which demonstrates that, in activated B cells, C-terminus expression per cell is upregulated while the paired domain expression per cell remains constant. When mature B cells were LPS activated,

we observed a relative increase in expression for all three Pax5 domains, suggesting that the full length isoform is upregulated upon initial activation of mature B cells. Murine splenocytes also upregulate Pax5 upon initial activation, as demonstrated by increases in Pax5 RNA expression [82]. This is the first time a similar trend has been observed for Pax5 protein in trout using flow cytometry.

The role of the C-terminus in Pax5 function remains largely unknown, with the exception that it contains an inhibitory domain adjacent to an upstream transactivation domain [71]. In mice, deletion of the last 22-33 amino acids of the C-terminus results in increased Pax5 transactivating potential, suggesting that the Pax5 transactivation domain is strongly regulated by the adjacent inhibitory domain [71]. However, the loss of the C-terminus does not affect the ability of Groucho family members to repress Pax5 transactivation of target protein [70]. Thus, it is difficult to establish how maintenance of the C-terminus (as opposed to the maintenance of the paired domain) is important for Pax5 function during LPS activation.

There are several further pieces of evidence from our lab that B cell activation by LPS, results in an increase in the frequency of cells lacking a paired domain. First, using RT-PCR and primers targeting the full length Pax5 isoform and isoforms lacking exon2 (paired domain) in LPS activated spleen cells, we identified an increase in the ratio of paired domain-less isoforms to paired domain containing isoforms across days of activation (Bruce and Zwollo, unpublished data). Furthermore, we were able to correlate this with an increased ratio of secreted IgM to membrane IgM. Together, these results

suggest that as cells are activated and differentiate into antibody-secreting plasma cells, they are expressing more isoforms lacking the paired domain.

In addition to this *in vitro* approach examining isoform expression, our lab has also observed *in vivo* changes in isoform expression using spawning *O. nerka* samples (Bruce, Clister, Epp, Schouten, and Zwollo, unpublished results). Spawning fish are known to have increased immune activation. Using RT-PCR, our lab has observed an increase in the ratio of exon 2 lacking isoforms to exon 2 containing isoforms in these spawning fish relative to non-spawning trout controls. Furthermore, this ratio correlates with an increased ratio of secreted to membrane IgM. Together, these findings correlate with findings of the present study which demonstrate that during terminal B cell differentiation, activated B cells will shift towards the paired domain lacking forms of Pax5.

4.4 The Pax5 patterns of peripheral blood.

The peripheral blood is a highly variable and dynamically changing population, potentially harboring resting, mature B cells while transporting activated and long-lived plasma cells to their destinations [3, 76]. Our results demonstrate that in addition to mature and activated B cells, the blood also potentially contains early developing B cells. These cells lack Pax5 paired domain expression yet still express high levels of protein expressing exon 6 (Figure 11). We have not yet tested if this population co-stains the early B cell marker EBF, but it does exhibit high Xbp1 expression (Figure 12) and RAG (an early developmental marker similar to EBF), suggesting it truly represents early

developing cells (Barr and Zwollo, unpublished). Additionally, the blood also contained a population of cells that were low in paired domain and exon 6 expression, yet consisting of the Pax5 exon 10. It is possible that these cells represent the population of early activated B cells described earlier. However, due to the variability in blood, it is difficult to truly ascertain the identity of these populations, a point discussed further in the limitations of this study.

4.5 Pax5 isoform model.

Our initial hypothesis proposed that Pax5 isoform expression modulates the activity of Pax5 to affect B cell development and activation. We predicted that specific isoforms would be present in both developing and activated B cell populations and that the presence or absence of a particular isoform would either result in the progression or inhibition of the B cell developmental pathway (see Chap. 1, Figure 1.6). Although we do not yet know the function of individual isoforms, we have discovered that particular B cell stages are characterized by the expression of specific Pax5 protein domains (Figure 16). Our preliminary model suggested that isoforms containing the Pax5 paired domain would drive B cell development and that those isoforms lacking the paired domain would inhibit it. Our current model (Figure 16) supports this prediction; the paired domain is progressively turned on throughout early B cell development, starting at the pre-B cell stage, and remains part of Pax5 until the plasmablast stage. However, isoforms lacking in paired domain expression, including isoforms containing the repressor domain and exon 6 domain, may already be present at the earliest stages of B cell development including

the common lymphocyte progenitor stage. Importantly, in contrast to our original model (Chap. 1 Figure 1.6), Pax5 isoforms containing the C-terminal repressor domain may be *necessary* to promote B cell development rather than inhibit it. This model is supported by our observation that C-terminal containing isoforms are present during the pro-B cell stage of Pax5 (Figure 16).

Our preliminary model further suggested that the C-terminus was necessary for B cell terminal B cell differentiation and that the absence of the repressor domain would inhibit this process. The LPS activation experiments support this hypothesis. The frequency of mature and activated cells expressing the C-terminus increased throughout activation, as did the relative number of Pax5.E10 positive isoforms per cell. In contrast, the frequency of these cells expressing the paired domain decreased and additionally, the number of isoforms per cell expressing Pax5.PD remained constant. This supports the prediction that the isoforms lacking the paired domain are necessary for terminal B cell differentiation, while isoforms containing the repressor domain keep their function even later in the terminal differentiation process, possibly until the transitional plasma cell stage.

Our results also suggest that specific Pax5 isoforms may characterize other possible novel B cell populations, such as phagocytic B cells (Li et al., 2006) or memory B cells (Ye et al., 2011). In this study, we identified an interesting population of cells low in Pax5 paired domain expression, high in Pax5 C-terminus expression, and lacking HCmu expression. We propose that this population may represent phagocytic B cells lacking in IgM expression. As the current literature has not examined Pax5 expression in

phagocytic B cells, this possibility should be addressed in future studies (see later section).

4.6 Pax5 signatures.

By characterizing the B cell populations of a tissue through their flow cytometric patterns of Pax5 domain expression and delineating the frequencies of these B cell populations, we can create a Pax5 signature for each unique immune tissue. The Pax5 signature of a tissue is a means by which researchers can identify aberrant molecular expression via changes in the frequencies of key B cell populations. The Pax5 signature can be used to compare immune tissues across species or to compare different tissues within a species (i.e. anterior kidney and blood). It can also be used in diagnostics by comparing the Pax5 signature of a tissue exposed to pathogen versus a control (as demonstrated in the next section), or by examining a malignant tissue compared to a healthy tissue. As research further elucidates the patterns of Pax5 isoform expression in developing B cell populations, the more these patterns can be applied to examine immune tissues as a whole. The ability to apply a Pax5 signature to immune tissues will be beneficial to both future comparative and clinical immunology studies.

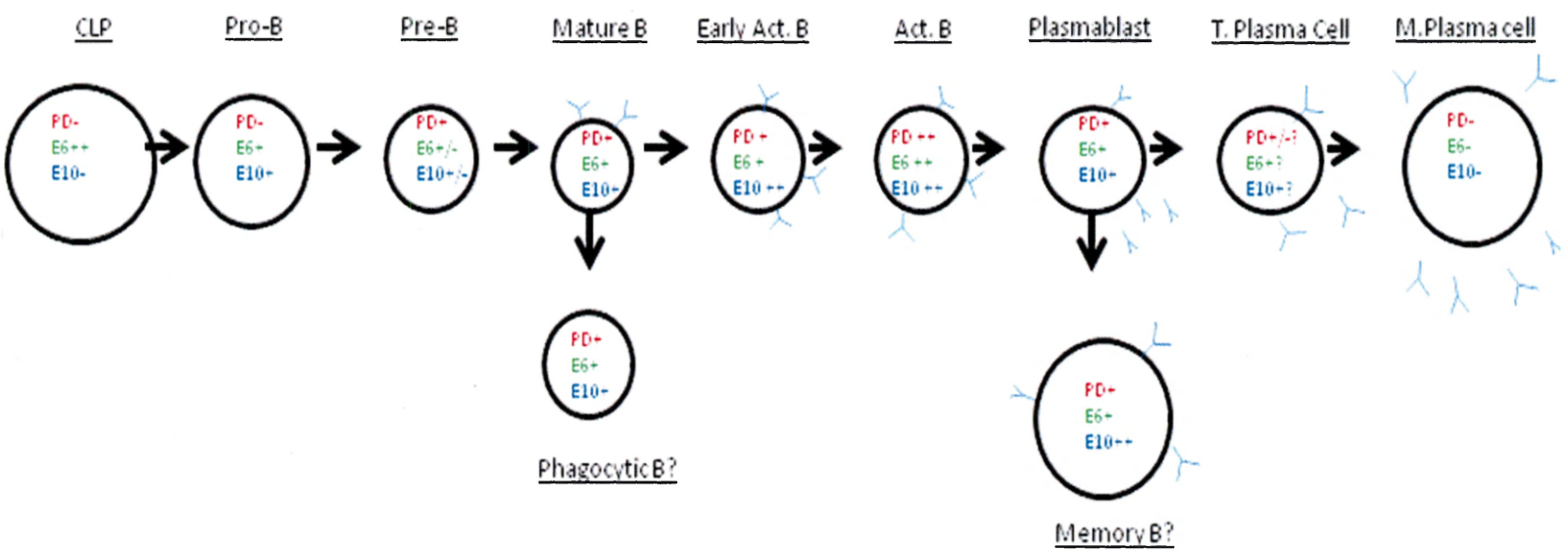


Figure 16. Pax5 domain patterns in developing and activated B cell populations. T. plasma cell: transitional plasma cell; M. plasma cell: mature plasma cell.

4.7 Flavobacterium resistant versus susceptible fish.

Bacterial cold water disease (CWBD) is prevalent in aquaculture and is responsible for high mortality rates in salmonid species [93]. Understanding the molecular mechanisms underlying fish resistance to this disease will better equip the aquaculture industry to prevent disease. Our preliminary results demonstrate that changes in B cell development and the humoral response may contribute to the mechanisms leading to flavobacterium resistance. Our data showed a higher frequency of early developing B cell populations in the anterior kidney and blood of fish resistant to CWBD compared to susceptible fish. Although more experiments are required to determine if these changes are statistically significant, the early developing B cell population (containing exon 6 of Pax5) remains an excellent candidate population to study as it is present in all immune tissues. Additionally, preliminary observations of this same population in the anterior kidney of splenectomized trout demonstrate it is a dynamic population: there is a lower frequency of exon 6 expressing B cells in splenectomized trout anterior kidney compared to control trout (Zwollo, unpublished data). This suggests that the early developing B cell population responds to atypical changes in the immune tissues as it needs mature cell to replace those mature B cells lost from the splenectomy. It supports preliminary evidence that *higher* frequencies of early developing B cell populations may be characteristic of fish resistant to infection.

4.8 Limitations to the current study.

The current flow cytometric approach enabled us to demarcate novel B cell populations in rainbow trout and to demonstrate that these populations differ in their patterns of Pax5 domain expression. However, there are populations in the current study that remain elusive without the availability of further immunological markers. One such population is the Pax5 positive populations that lack IgM HCmu expression. Approximately 15-20% of gated lymphocytes constituted this population in spleen (Figure 10) and the population was additionally observed in anterior kidney and blood (data not shown). This same population has previously been reported by our lab [32, 76]. Recent research has identified the presence of phagocytic B cells and B-1 cells in trout [102, 103]. However, each of these studies utilizes IgM as a B cell marker. So far, no research has examined Pax5 expression in phagocytic B cells. It is possible that our population of cells expressing Pax5 but lacking in IgM constitutes some of the IgM lacking phagocytic B cells. Alternatively, these cells could represent an early developing B cell population; however, this is unlikely as these cells did not co-stain for EBF. Additional experiments to test the phagocytic hypothesis are presented in the next section.

The flow cytometric patterns of Pax5 subpopulations were remarkably consistent across experiments. Although we were able to utilize these patterns to identify individual B cell populations, it remains formally plausible that observed patterns are not a reflection of alternative splicing events. Instead, because multiple regulatory proteins interact with Pax5 to modify transcription throughout B cell development, it is formally

possible that protein-protein interactions block available Pax5 sites for antibody binding in the context of flow cytometry experiments. .

Although protein-protein interactions may account for our findings, independent data from Western blots using the three anti-Pax5 antibodies (Figure 2) supports that alternative splicing is responsible for the observed flow cytometric patterns. Notably, these blots reveal that anterior kidney contains less of the paired domain containing isoforms at the molecular weight for full length Pax5, suggesting that full length Pax5 is not expressed as frequently in this tissue. Furthermore, the full length Pax5 protein is found in both trout blood and spleen, consistent with our finding that mature and activated B cells contain full length Pax5. The blots additionally reveal that the Pax5.E10 antibody recognizes both full length Pax5 and an additional isoform of smaller molecular weight in blood and spleen. This matches the high frequency of a Pax5.E10 population that lacks the Pax5 paired domain in these tissues. Finally, the blots demonstrate that Pax5.E6 is found consistently across tissues, thus matching the flow cytometry data.

Western blot analysis reveals the complexity of Pax5 patterns occurring across immune tissues and demonstrates that examining protein expression as a collective sum of the entire immune tissue protein expression leads to a poor understanding of Pax5 protein function. Our flow cytometric approach in conjunction with Pax5 antibody staining remedies this problem by identifying protein expression in individual B cell populations. Additionally, our approach has revealed multiple, Pax5 containing B cell populations regardless whether the mechanism resulting in these patterns is a consequence of alternative splicing or stage specific protein-protein interactions.

Therefore, our flow-cytometric technique has promising medical applications when assessing B cell populations in malignancies such lymphomas and myelomas.

Two-color flow and three-color cytometry is limited in its ability to identify all Pax5 isoforms. At this time, we have developed an approach to identify which specific Pax5 domains are present, but we still lack information for the Pax5 domains for which we do not possess antibodies, including the highly complex exon 7-9 region which undergoes extensive alternative splicing in humans, mouse, and trout [9, 12]. Therefore, we are still currently unable to determine if only one isoform is expressed per B cell population, or if multiple isoforms are expressed within a given population. However, one key advantage of our approach is that we may use the current antibodies to detect if the paired domain, exon 6 and exon 10 regions are absent from a B cell population. Thus, we are still able to garner important information on how key Pax5 domains are expressed and potentially used during B cell development and activation.

4.9 Future studies.

The primary goal of future studies will be to verify the presence of Pax5 isoforms in the populations revealed by flow cytometry. One obvious experiment is to transfect Pax5 isoforms isolated by RT-PCR into 293T cell lines and demonstrate that the flow cytometric patterns for these cells are similar to those identified in this study. However, this approach may not work, as the same caveat of interfering protein-protein interaction would persist. An alternative approach would be to separate early developing murine B cells via cell surface markers (not available for trout) and use RT-PCR to demonstrate

that isoforms containing the Pax5 C-terminus, but not paired domain, are more prevalent in these cells than in later developing stages. Although this experiment examines Pax5 at the RNA level and therefore may accurately demonstrate protein levels, it could still provide evidence that particular Pax5 isoforms are more present in any particular B cell developmental stage.

A second goal for future directions is to further characterize the unknown Pax5 expressing, IgM negative population in trout spleen. It has been postulated that this population is composed of non-conventional B cell types, such as phagocytic B cells or B-1 cells. One method to test this hypothesis is to culture trout immune cells in the presence of fluorescent beads and use two-color flow cytometry to identify if phagocytic cells are Pax5 positive and IgM negative. If our hypothesis is correct, we would predict that a subset of phagocytic B cells is present in trout spleen that are lacking in HCmu but positive for full length Pax5 expression.

Conclusion.

B cell development and activation are complex, dynamic, and remarkably conserved processes in vertebrates. The use of serological reagents and surface markers in mammalian models has enhanced our understanding of these processes by enabling us to demarcate distinct stages of the developmental pathway. Only recently has research opened our eyes to the unique implementation of adaptive immunity in teleosts. We now know that despite lacking the traditional primary immune organ, the bone marrow, teleosts follow a developmental pathway comparable to their mammalian counterparts.

They share similar B cell stages, secondary immune locations, and most importantly, expression of Pax5 isoforms.

Using our flow cytometric approach, we have discovered that isoform expression changes during B cell development and activation stages, and potentially begins at an earlier stage than previously assumed. By better understanding these complex developmental pathways, we open future avenues to characterizing immune system malignancies and furthermore, provide an approach to analyze aberrant molecular expression. We also pose important evolutionary questions. It is fascinating that teleosts and mammals share similar early developing B cell populations lacking in Pax5 paired domain expression and furthermore express these developing B cell populations in similar secondary immune tissues. How these similarities in Pax5 isoform expression evolved and where they diverge remain elusive. However, by identifying Pax5 isoforms in individual developing and activated B cell populations, our research brings us one step closer to understanding their biological validity and function in humoral immunity.

References

1. Kindt, T.J., Goldsby, R.A., Osborne, B.A. & Kuby, J. Kuby immunology (W.H. Freeman, New York, 2007).
2. Zwollo, P., Cole, S., Bromage, E. & Kaattari, S. (2005). B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. *J Immunol, 174*, 6608-16.
3. Bromage, E.S., Kaattari, I.M., Zwollo, P. & Kaattari, S.L. (2004). Plasmablast and plasma cell production and distribution in trout immune tissues. *J Immunol, 173*, 7317-23.
4. Kaattari, S.L. & Irwin, M.J. (1985). Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. *Dev Comp Immunol, 9*, 433-44.
5. Nutt, S.L., Urbanek, P., Rolink, A. & Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev, 11*, 476-91.
6. Nutt, S.L., Heavey, B., Rolink, A.G. & Busslinger, M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature, 401*, 556-62.
7. Urbanek, P., Wang, Z.Q., Fetka, I., Wagner, E.F. & Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell, 79*, 901-12.
8. Horcher, M., Souabni, A. & Busslinger, M. (2001). Pax5/BSAP Maintains the Identity of B Cells in Late B Lymphopoiesis. *Immunity, 14*, 779-790.
9. Zwollo, P. et al. (1997). The Pax-5 gene is alternatively spliced during B-cell development. *J Biol Chem, 272*, 10160-8.
10. Arseneau, J.R., Laflamme, M., Lewis, S.M., Maicas, E. & Ouellette, R.J. (2009). Multiple isoforms of PAX5 are expressed in both lymphomas and normal B-cells. *Br J Haematol, 147*, 328-38.
11. Borson, N.D., Lacy, M.Q. & Wettstein, P.J. (2006). Expression of mRNA for a newly identified Pax5 exon is reduced in multiple myeloma. *Mamm Genome, 17*, 248-56.
12. Robichaud, G.A., Nardini, M., Laflamme, M., Cuperlovic-Culf, M. & Ouellette, R.J. (2004). Human Pax-5 C-terminal isoforms possess distinct transactivation properties and are differentially modulated in normal and malignant B cells. *J Biol Chem, 279*, 49956-63.
13. Zwollo, P. (2011). Dissecting teleost B cell differentiation using transcription factors. *Dev Comp Immunol, 35*, 898-905.
14. Fange, R. (1986). Lymphoid organs in sturgeons (Acipenseridae). *Vet Immunol Immunopathol, 12*, 153-61.
15. Zon, L.I. (1995). Developmental biology of hematopoiesis. *Blood, 86*, 2876-91.
16. Barr, M., Mott, K. & Zwollo, P. (2011). Defining terminally differentiating B cell populations in rainbow trout immune tissues using the transcription factor XbpI. *Fish Shellfish Immunol, 31*, 727-35.
17. Meseguer, J., Lopez-Ruiz, A. & Garcia-Ayala, A. (1995). Reticulo-endothelial stroma of the head-kidney from the seawater teleost gilthead seabream (Sparus aurata L.): an ultrastructural and cytochemical study. *Anat Rec, 241*, 303-9.

18. Hansen, J.D., Strassburger, P. & Du Pasquier, L. (1997). Conservation of a master hematopoietic switch gene during vertebrate evolution: isolation and characterization of Ikaros from teleost and amphibian species. *Eur J Immunol, 27*, 3049-58.
19. Zaccone, G. Fish defenses (Science Publishers, Enfield, NH, 2009).
20. Hansen, J.D. & Kaattari, S.L. (1995). The recombination activation gene 1 (RAG1) of rainbow trout (Oncorhynchus mykiss): cloning, expression, and phylogenetic analysis. *Immunogenetics, 42*, 188-95.
21. Civin, C.I. et al. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol, 133*, 157-65.
22. Mohty, M. & Ho, A.D. (2011). In and out of the niche: perspectives in mobilization of hematopoietic stem cells. *Exp Hematol, 39*, 723-9.
23. Honjo, T., Alt, F.W. & Neuberger, M.S. Molecular biology of B cells (Elsevier, Amsterdam ; Boston, 2004).
24. Kondo, M., Weissman, I.L. & Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell, 91*, 661-72.
25. Tsapogas, P. et al. (2011). IL-7 mediates Ebf-1-dependent lineage restriction in early lymphoid progenitors. *Blood, 118*, 1283-90.
26. Roessler, S. et al. (2007). Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol, 27*, 579-94.
27. Liberg, D., Smale, S.T. & Merkenschlager, M. (2003). Upstream of Ikaros. *Trends Immunol, 24*, 567-70.
28. Georgopoulos, K. et al. (1994). The Ikaros gene is required for the development of all lymphoid lineages. *Cell, 79*, 143-56.
29. Pongubala, J.M. et al. (2008). Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat Immunol, 9*, 203-15.
30. Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N. & Kincade, P.W. (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity, 17*, 117-30.
31. Northrup, D.L. & Allman, D. (2008). Transcriptional regulation of early B cell development. *Immunol Res, 42*, 106-17.
32. Zwollo, P., Mott, K. & Barr, M. (2010). Comparative analyses of B cell populations in trout kidney and mouse bone marrow: establishing "B cell signatures". *Dev Comp Immunol, 34*, 1291-9.
33. Hoffmann, R., Seidl, T., Neeb, M., Rolink, A. & Melchers, F. (2002). Changes in gene expression profiles in developing B cells of murine bone marrow. *Genome Res, 12*, 98-111.
34. Lu, L.S. et al. (2002). Identification of a germ-line pro-B cell subset that distinguishes the fetal/neonatal from the adult B cell development pathway. *Proc Natl Acad Sci U S A, 99*, 3007-12.
35. Dragone, L.L., Barth, R.K., Sitar, K.L., Disbrow, G.L. & Frelinger, J.G. (1995). Disregulation of leukosialin (CD43, Ly48, sialophorin) expression in the B-cell lineage of transgenic mice increases splenic B-cell number and survival. *Proc Natl Acad Sci U S A, 92*, 626-30.

36. Rolink, A.G., Schaniel, C., Busslinger, M., Nutt, S.L. & Melchers, F. (2000). Fidelity and infidelity in commitment to B-lymphocyte lineage development. *Immunol Rev, 175*, 104-11.
37. Jung, D., Giallourakis, C., Mostoslavsky, R. & Alt, F.W. (2006). Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu Rev Immunol, 24*, 541-70.
38. Lutz, J. et al. (2011). Pro-B cells sense productive immunoglobulin heavy chain rearrangement irrespective of polypeptide production. *Proc Natl Acad Sci U S A, 108*, 10644-9.
39. Kikuchi, K., Lai, A.Y., Hsu, C.-L. & Kondo, M. (2005). IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *The Journal of Experimental Medicine, 201*, 1197-1203.
40. Hardy, R.R., Carmack, C.E., Shinton, S.A., Kemp, J.D. & Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med, 173*, 1213-25.
41. Kitamura, D. et al. (1992). A critical role of Î»5 protein in B cell development. *Cell, 69*, 823-831.
42. Espeli, M., Mancini, S.J., Breton, C., Poirier, F. & Schiff, C. (2009). Impaired B-cell development at the pre-BII-cell stage in galectin-1-deficient mice due to inefficient pre-BII/stromal cell interactions. *Blood, 113*, 5878-86.
43. King, L.B. & Monroe, J.G. (2000). Immunobiology of the immature B cell: plasticity in the B-cell antigen receptor-induced response fine tunes negative selection. *Immunol Rev, 176*, 86-104.
44. Tang, J., Scott, G. & Ryan, D.H. (1993). Subpopulations of bone marrow fibroblasts support VLA-4-mediated migration of B-cell precursors. *Blood, 82*, 3415-23.
45. Ye, J., Bromage, E., Kaattari, I. & Kaattari, S. (2011). Transduction of binding affinity by B lymphocytes: a new dimension in immunological regulation. *Dev Comp Immunol, 35*, 982-90.
46. Hu, M.-C. et al. (2007). XBP-1, a key regulator of unfolded protein response, activates transcription of IGF1 and Akt phosphorylation in zebrafish embryonic cell line. *Biochemical and Biophysical Research Communications, 359*, 778-783.
47. Gass, J.N., Gifford, N.M. & Brewer, J.W. (2002). Activation of an Unfolded Protein Response during Differentiation of Antibody-secreting B Cells. *Journal of Biological Chemistry, 277*, 49047-49054.
48. Tirosh, B., Iwakoshi, N.N., Glimcher, L.H. & Ploegh, H.L. (2005). XBP-1 specifically promotes IgM synthesis and secretion, but is dispensable for degradation of glycoproteins in primary B cells. *The Journal of Experimental Medicine, 202*, 505-516.
49. Reimold, A.M. et al. (1996). Transcription factor B cell lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J Exp Med, 183*, 393-401.
50. Kaattari, S.L., Irwin, M.J., Yui, M.A., Tripp, R.A. & Parkins, J.S. (1986). Primary in vitro stimulation of antibody production by rainbow trout lymphocytes. *Vet Immunol Immunopathol, 12*, 29-38.
51. Minges Wols, H.A. in eLS (John Wiley & Sons, Ltd, 2001).

52. Ramirez-Gomez, F. et al. (2011). Discovery and characterization of secretory IgD in rainbow trout: secretory IgD is produced through a novel splicing mechanism. *J Immunol, 188*, 1341-9.
53. Wilson, M. et al. (1997). A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proc Natl Acad Sci U S A, 94*, 4593-7.
54. Zhang, Y.-A., Salinas, I. & Oriol Sunyer, J. (2011). Recent findings on the structure and function of teleost IgT. *Fish & Shellfish Immunology, 31*, 627-634.
55. O'Connor, B.P., Cascalho, M. & Noelle, R.J. (2002). Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. *J Exp Med, 195*, 737-45.
56. Ye, J., Kaattari, I. & Kaattari, S. (2011). Plasmablasts and plasma cells: reconsidering teleost immune system organization. *Dev Comp Immunol, 35*, 1273-81.
57. Kaattari, S., Bromage, E. & Kaattari, I. (2005). Analysis of long-lived plasma cell production and regulation: Implications for vaccine design for aquaculture. *Aquaculture, 246*, 1-9.
58. Arkoosh, M.R. & Kaattari, S.L. (1991). Development of immunological memory in rainbow trout (Oncorhynchus mykiss). I. An immunochemical and cellular analysis of the B cell response. *Dev Comp Immunol, 15*, 279-93.
59. Adams, B. et al. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev, 6*, 1589-607.
60. Schebesta, A. et al. (2007). Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. *Immunity, 27*, 49-63.
61. Delogu, A. et al. (2006). Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity, 24*, 269-81.
62. McManus, S. et al. (2011). The transcription factor Pax5 regulates its target genes by recruiting chromatin-modifying proteins in committed B cells. *EMBO J, 30*, 2388-2404.
63. Holmes, M.L., Carotta, S., Corcoran, L.M. & Nutt, S.L. (2006). Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment. *Genes Dev, 20*, 933-8.
64. Czerny, T., Schaffner, G. & Busslinger, M. (1993). DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev, 7*, 2048-61.
65. Xu, W., Rould, M.A., Jun, S., Desplan, C. & Pabo, C.O. (1995). Crystal structure of a paired domain-DNA complex at 2.5 A resolution reveals structural basis for Pax developmental mutations. *Cell, 80*, 639-50.
66. Czerny, T. & Busslinger, M. (1995). DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). *Mol Cell Biol, 15*, 2858-71.
67. Robson, E.J., He, S.J. & Eccles, M.R. (2006). A PANorama of PAX genes in cancer and development. *Nat Rev Cancer, 6*, 52-62.
68. Eberhard, D. & Busslinger, M. (1999). The partial homeodomain of the transcription factor Pax-5 (BSAP) is an interaction motif for the retinoblastoma and TATA-binding proteins. *Cancer Res, 59*, 1716s-1724s; discussion 1724s-1725s.
69. Robert A, W. (1995). The retinoblastoma protein and cell cycle control. *Cell, 81*, 323-330.

70. Eberhard, D., Jimenez, G., Heavey, B. & Busslinger, M. (2000). Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J, 19*, 2292-2303.
71. Dorfler, P. & Busslinger, M. (1996). C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2 and Pax-8. *EMBO J, 15*, 1971-82.
72. Short, S. & Holland, L.Z. (2008). The evolution of alternative splicing in the Pax family: the view from the Basal chordate amphioxus. *J Mol Evol, 66*, 605-20.
73. Kozmik, Z., Kurzbauer, R., Dorfler, P. & Busslinger, M. (1993). Alternative splicing of Pax-8 gene transcripts is developmentally regulated and generates isoforms with different transactivation properties. *Mol Cell Biol, 13*, 6024-35.
74. Kozmik, Z., Czerny, T. & Busslinger, M. (1997). Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. *EMBO J, 16*, 6793-803.
75. Mackereth, M.D., Kwak, S.J., Fritz, A. & Riley, B.B. (2005). Zebrafish pax8 is required for otic placode induction and plays a redundant role with Pax2 genes in the maintenance of the otic placode. *Development, 132*, 371-82.
76. Zwollo, P., Haines, A., Rosato, P. & Gumulak-Smith, J. (2008). Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. *Dev Comp Immunol, 32*, 1482-96.
77. Heller, N. & Brändli, A.W. (1999). Xenopus Pax-2/5/8 orthologues: Novel insights into Pax Gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. *Developmental Genetics, 24*, 208-219.
78. Cobaleda, C., Schebesta, A., Delogu, A. & Busslinger, M. (2007). Pax5: the guardian of B cell identity and function. *Nat Immunol, 8*, 463-70.
79. Singh, M. & Birshtein, B.K. (1993). NF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3' alpha enhancer at early stages of B-cell differentiation. *Mol Cell Biol, 13*, 3611-22.
80. Neurath, M.F., Strober, W. & Wakatsuki, Y. (1994). The murine Ig 3' alpha enhancer is a target site with repressor function for the B cell lineage-specific transcription factor BSAP (NF-HB, S alpha-BP). *J Immunol, 153*, 730-42.
81. Rinkenberger, J.L., Wallin, J.J., Johnson, K.W. & Koshland, M.E. (1996). An interleukin-2 signal relieves BSAP (Pax5)-mediated repression of the immunoglobulin J chain gene. *Immunity, 5*, 377-86.
82. Lin, K.I., Angelin-Duclos, C., Kuo, T.C. & Calame, K. (2002). Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol Cell Biol, 22*, 4771-80.
83. Hagman, J. & Lukin, K. (2007). "Hands-on" regulation of B cell development by the transcription factor Pax5. *Immunity, 27*, 8-10.
84. Lowen, M., Scott, G. & Zwollo, P. (2001). Functional analyses of two alternative isoforms of the transcription factor Pax-5. *J Biol Chem, 276*, 42565-74.
85. Anspach, J., Poulsen, G., Kaattari, I., Pollock, R. & Zwollo, P. (2001). Reduction in DNA binding activity of the transcription factor Pax-5a in B lymphocytes of aged mice. *J Immunol, 166*, 2617-26.

86. O'Brien, P., Morin, P., Jr., Ouellette, R.J. & Robichaud, G.A. (2011). The Pax-5 gene: a pluripotent regulator of B-cell differentiation and cancer disease. *Cancer Res, 71*, 7345-50.
87. Heltemes-Harris, L.M. et al. (2011). Ebf1 or Pax5 haploinsufficiency synergizes with STAT5 activation to initiate acute lymphoblastic leukemia. *J Exp Med, 208*, 1135-49.
88. Nebral, K., Krehan, D. & Strehl, S. (2011). Expression of PAX5 splice variants: a phenomenon of stress-induced, illegitimate splicing? *Br J Haematol, 155*, 277-80.
89. Gorlov, I.P. & Saunders, G.F. (2002). A method for isolating alternatively spliced isoforms: isolation of murine Pax6 isoforms. *Anal Biochem, 308*, 401-4.
90. Jaroszeski, M. & Radcliff, G. (1999). Fundamentals of flow cytometry. *Molecular Biotechnology, 11*, 37-53.
91. Zwollo, P., Rao, S., Wallin, J.J., Gackstetter, E.R. & Koshland, M.E. (1998). The transcription factor NF-kappaB/p50 interacts with the blk gene during B cell activation. *J Biol Chem, 273*, 18647-55.
92. DeLuca, D., Wilson, M. & Warr, G.W. (1983). Lymphocyte heterogeneity in the trout, Salmo gairdneri, defined with monoclonal antibodies to IgM. *Eur J Immunol, 13*, 546-51.
93. Nematollahi, A., Decostere, A., Pasmans, F. & Haesebrouck, F. (2003). Flavobacterium psychrophilum infections in salmonid fish. *Journal of Fish Diseases, 26*, 563-574.
94. Leeds, T.D. et al. (2010). Response to selection for bacterial cold water disease resistance in rainbow trout. *Journal of Animal Science, 88*, 1936-1946.
95. Mitchell, T. (1993). Increased hematopoiesis in mice soon after infection by Friend murine leukemia virus. *Journal of Virology, 67*, 3665-3670.
96. Cotterell, S.E.J., Engwerda, C.R. & Kaye, P.M. (2000). Enhanced Hematopoietic Activity Accompanies Parasite Expansion in the Spleen and Bone Marrow of Mice Infected with Leishmania donovani. *Infection and Immunity, 68*, 1840-1848.
97. MacNamara, K.C., Racine, R., Chatterjee, M., Borjesson, D. & Winslow, G.M. (2009). Diminished Hematopoietic Activity Associated with Alterations in Innate and Adaptive Immunity in a Mouse Model of Human Monocytic Ehrlichiosis. *Infection and Immunity, 77*, 4061-4069.
98. Hadidi, S., Glenney, G.W., Welch, T.J., Silverstein, J.T. & Wiens, G.D. (2008). Spleen size predicts resistance of rainbow trout to Flavobacterium psychrophilum challenge. *J Immunol, 180*, 4156-65.
99. Li, Z., Perez-Casellas, L.A., Savic, A., Song, C. & Dovat, S. (2011). Ikaros isoforms: The saga continues. *World J Biol Chem, 2*, 140-5.
100. Ingram, R.M. et al. (2011). Differential regulation of sense and antisense promoter activity at the Csf1R locus in B cells by the transcription factor PAX5. *Experimental hematology, 39*, 730-740.e2.
101. Dor, F.J.M.F. et al. (2006). Primitive hematopoietic cell populations reside in the spleen: Studies in the pig, baboon, and human. *Experimental hematology, 34*, 1573-1582.
102. Gao, J. et al. (2012). A novel function of murine B1 cells: Active phagocytic and microbicidal abilities. *European Journal of Immunology,* n/a-n/a.
103. Li, J. et al. (2006). B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol, 7*, 1116-1124.