2010

Flow Cytometric Analyses on the Activation, Proliferation, and Differentiation State of B Cells in Rainbow Trout (Oncorhynchus mykiss)

Maggie Jeanne Barr

College of William & Mary - Arts & Sciences

Follow this and additional works at: https://scholarworks.wm.edu/etd

Part of the Cell Biology Commons

Recommended Citation


This Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Dissertations, Theses, and Masters Projects by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
Flow Cytometric Analyses on the Activation, Proliferation, and Differentiation State of B Cells in Rainbow Trout (*Oncorhynchus mykiss*)

Maggie Jeanne Barr
Parkland, Florida

Bachelor of Science, Animal Biology, University of Florida 2007

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
January, 2010
This Thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Science

Maggie Jeanne Barr

Approved by the Committee, December 2009

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

Hamilton Professor Lizabeth Allison, Biology
College of William and Mary

Assistant Professor Oliver Kerscher, Biology
College of William and Mary

Visiting Assistant Professor Ashley Haines, Biology
College of William and Mary
Research approved by

Institutional Animal Care and Use Committee

Protocol number(s): IACUC-2009-03-03-5861-pxzwol
IACUC-2006-03-15-0-pxzwol

Date(s) of approval: June 15, 2009
June 1, 2006
Teleosts, including rainbow trout, provide an important animal model for immune system function as they lack bone marrow, but utilize the anterior kidney as their primary site for hematopoiesis. The activation, proliferation, and differentiation stages of trout B cells and the location of these processes within the immune tissues are not well defined. Expression patterns of B cell transcription factors can be used as markers to investigate the B cell stages. In this study, the transcription factors Pax-5 and Xbp-1, as well as RAG-1, Membrane IgM, Secreted IgM, Total IgM, and BrdU incorporation were used as markers in trout B cells. Pax-5 is expressed from pre-B through plasmablast stages, while Xbp-1 expression increases after B cell activation and is highest in plasma cells. RAG-1 is expressed in pro- and pre-B cells and activated B cells/plasmablasts during Ig gene rearrangement. BrdU incorporation is a marker for B cell proliferation and is seen in pre-B cells and plasmablasts. Membrane IgM is found on the cell surface from the resting B cell through plasmablast stages, while Secreted IgM is produced by plasmablasts and plasma cells. The expression patterns of these markers, when analyzed by flow cytometry, provide information about the B cell populations present in trout immune tissues. Trout immune cells from the spleen, blood, anterior and posterior kidney were LPS-activated in culture, fixed and permeabilized, and stained cells were analyzed by two-color flow cytometry. The results presented here suggest that the anterior kidney contains mostly developing B cells, as very few LPS-responsive mature B cells were seen through the lack of an increase in Xbp-1 or Total or Secreted IgM expression. In contrast to the anterior kidney, the spleen, blood, and posterior kidney were found to contain populations of resting B cells that become activated following LPS-stimulation. In addition, Xbp-1 can be used as a marker for B cell activation in these tissues. In spleen and blood B cells, Xbp-1 expression increases after activation, correlating with progression from the resting to activated B cell stage and eventual differentiation into plasmablasts and plasma cells. Lastly, BrdU proved a successful marker for B cell proliferation. Proliferating B cells were found in the spleen, anterior and posterior kidney, while no proliferation was detected in the blood. This study, for the first time, analyzed B cell responses to antigenic stimulation ex vivo as defined by the frequencies of the resting B, activated B, plasmablast, and plasma cell populations in the rainbow trout. As additional B cell markers are tested, future studies can provide further information about the complex B cell populations present in the immune tissues of the rainbow trout and other teleosts.
# Table of Contents

Acknowledgments iii  
List of Tables iv  
List of Figures v  
List of Abbreviations viii  

Chapter I. Literature Review 1  
1.1. The Mammalian Immune System 1  
1.1.1. Lymphopoiesis and B cell development 1  
1.1.2. B cell activation, proliferation, and differentiation 7  
1.2. The Teleost Immune System 11  
1.2.1. Immune tissues of teleosts 11  
1.2.2. Lymphopoiesis and B cell development in teleosts 13  
1.2.3. Teleost immunoglobulin classes 14  
1.2.4. B cell activation, proliferation, and differentiation in teleosts 15  
1.3. Transcription factors as B cell markers 18  
1.4. Cell proliferation markers 27  
1.5. Research aims, hypotheses, and significance 29  
1.5.1. Aim 1: To identify a marker for proliferating B cells using flow cytometry. 30  
1.5.2. Aim 2: To determine the B cell profiles of freshly isolated trout immune cells from anterior and posterior kidney, spleen, and blood using two-color flow cytometry. 31  
1.5.3. Aim 3: To determine B cell profiles of LPS-activated trout immune cells from anterior and posterior kidney, spleen, and blood using two-color flow cytometry. 32  
1.5.4. Significance 33  

Chapter II. Methods 35  
2.1. MPC11 cell line 35  
2.2. Mouse tissue collection 35  
2.3. Rainbow trout and facilities 36  
2.4. Trout tissue collection 36  
2.5. LPS activation 37  
2.6. BrdU incorporation 38  
2.7. PKH26 tracking dye 38  
2.8. Staining fresh trout cells with anti-IgM antibody 40  
2.9. Cell fixation and permeabilization 40  
2.10. One- and two-color flow cytometry 41  
2.11. Click-iT EdU flow cytometry 42  
2.12. BrdU one-color flow cytometry 43  
2.13. Antibodies 44  
2.14. ELISA 45
Chapter III. Results
3.1. Analysis of cell proliferation using one-color flow cytometry
   3.1.1. Cells used in the study
   3.1.2. Analysis of proliferation markers using one-color flow cytometry
3.2. Flow cytometric analysis of freshly isolated trout immune tissues
3.3. Analysis of ex vivo LPS-activation on trout immune tissues using two-color flow cytometry
   3.3.1. Spleen
   3.3.2. Blood (PBL)
   3.3.3. Anterior kidney
   3.3.4. Posterior kidney
3.4. Analysis of total IgM levels in supernatant from LPS-activated trout B cells

Chapter IV. Discussion
4.1. The transcription factor Xbp-1, together with Membrane IgM or Total IgM, provide highly reliable markers for B cell activation.
4.2. BrdU incorporation can be used to identify proliferating B cells in trout immune tissues.
4.3. The anterior kidney contains a low number of LPS-responsive mature B cells.
4.4. Unexpected low levels of Pax-5 expression in B cells of the anterior and posterior kidney.
4.5. A population of Pax-5+, Membrane IgM- cells suggests the presence of IgD+ or IgT+ B cells in the spleen and blood.
4.6. A novel Pax-5 low (+/-), Membrane IgM+ B cell population is found in the spleen.
4.7. LPS-induction of a RAG-1+, IgM-secreting B cell population in the spleen and blood.
4.8. Conclusions, significance, and future work.

References
Vita
Acknowledgments

I do not think I can adequately express my gratitude to everyone who helped to make this project a success. First and foremost, I would like to thank my graduate advisor and mentor, Dr. Patty Zwollo, for all of her guidance and support in pursuing my graduate education. She challenged me to become an independent thinker and to make this thesis project my own, and for that I am truly grateful. This project would not have been successful without her constant encouragement, patience, and friendship.

I am also grateful to the members of my thesis committee, Dr. Liz Allison, Dr. Oliver Kerscher, and Dr. Ashley Haines, who helped to facilitate my knowledge of immunology and molecular biology. Their continued encouragement and excitement about my project aided in its success more than they know.

I wish to thank the members of the Zwollo lab, both current and former. In particular, I would like to express my gratitude to Katrina Mott, who worked tirelessly with me on the flow cytometry experiments. The abundance of data collected in this project would not have been possible without the help I received from Katrina. I would also like to thank Raaj Talauliker, who has provided not only friendship and help in the lab, but who provided stimulating conversation about science, our thesis projects in particular.

I would like to thank Dr. Steve Kaattari for the gift of the Warrs 1-14 antibody and for providing our lab with rainbow trout and LPS on short notice. This project was funded primarily from the NIH in the form of a grant awarded to Dr. Zwollo. I am also grateful to the funding I received from the College of William and Mary Arts and Sciences Research Grants and to the support I received as a Teaching Assistant from the College of William and Mary.

Finally, I wish to thank my family and friends, for their constant support in all that I do. To my parents and brother, for their love and encouragement and for always reminding me that anything is possible. To the Biology graduate students, who shared a common passion for science and helped to enrich my knowledge on a daily basis. In particular, I would like to thank Allyson Jackson, Louise Lammons, and Lindsey Postaski, who provided not only friendship, but opened my eyes to the endless possibilities of science and life.
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B Cell Marker Expression Throughout B Cell Development, Activation, and Differentiation</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Fluorescent Markers Used With Flow Cytometry</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>Optimal B Cell Marker Combinations for Characterizing B Cell Activation and Differentiation Stages</td>
<td>123</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B Cell Development and Maturation in the Mammalian Immune System</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>The B Cell Receptor (BCR)</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>B Cell Activation, Proliferation, and Differentiation in the Mammalian Immune System</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Functional Interactions Between B Cell Markers</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>B Cell Development, Activation, and Differentiation in the Trout Immune System</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>BrdU Incorporation in MPC11 Cells</td>
<td>52</td>
</tr>
<tr>
<td>7</td>
<td>BrdU Incorporation in Mouse Spleen Cells</td>
<td>53</td>
</tr>
<tr>
<td>8</td>
<td>BrdU Incorporation in Trout Spleen Cells</td>
<td>54</td>
</tr>
<tr>
<td>9</td>
<td>c-myc Expression in Mouse Spleen Cells</td>
<td>57</td>
</tr>
<tr>
<td>10</td>
<td>c-myc Expression in Trout Anterior Kidney Cells</td>
<td>58</td>
</tr>
<tr>
<td>11</td>
<td>c-myc Expression in Trout Spleen Cells</td>
<td>59</td>
</tr>
<tr>
<td>12</td>
<td>PKH26 Intensity in MPC11 Cells: Contour Plots</td>
<td>61</td>
</tr>
<tr>
<td>13</td>
<td>PKH26 Intensity in MPC11 Cells: Histogram</td>
<td>62</td>
</tr>
<tr>
<td>14</td>
<td>PKH26 Intensity in Trout Anterior Kidney Cells</td>
<td>64</td>
</tr>
<tr>
<td>15</td>
<td>PKH26 Intensity in Trout Spleen Cells</td>
<td>65</td>
</tr>
<tr>
<td>16</td>
<td>Day 0 Trout Tissue Comparison: FSC/ SSC</td>
<td>69</td>
</tr>
<tr>
<td>17</td>
<td>Day 2 Trout Tissue Comparison: BrdU/ Membrane IgM</td>
<td>71</td>
</tr>
<tr>
<td>18</td>
<td>Day 0 Trout Tissue Comparison: Pax-5/ Membrane IgM</td>
<td>73</td>
</tr>
<tr>
<td>19</td>
<td>Day 0 Trout Tissue Comparison: RAG-1/ Membrane IgM</td>
<td>74</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>BrdU Incorporation and Membrane IgM Expression in Trout Anterior Kidney Cells</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>BrdU Incorporation and Secreted IgM Expression in Trout Posterior Kidney Cells</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>BrdU Incorporation and Membrane IgM Expression in Trout Posterior Kidney Cells</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>BrdU Incorporation and Pax-5 Expression in Trout Posterior Kidney Cells</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>BrdU Incorporation and Pax-5 or Membrane IgM Expression in Trout Posterior Kidney Cells: 2-Vertical Axis Line Graphs</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Xbp-1 and Membrane IgM Expression in Trout Posterior Kidney Cells</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Xbp-1 and Total IgM Expression in Trout Posterior Kidney Cells: 2-Vertical Axis Line Graphs</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>ELISA data</td>
<td></td>
</tr>
<tr>
<td>A-D</td>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anterior Kidney</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Posterior Kidney</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td></td>
</tr>
</tbody>
</table>
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC</td>
<td>antibody-secreting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>³HT</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>LC</td>
<td>light chain</td>
</tr>
<tr>
<td>LLPC</td>
<td>long-lived plasma cell</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCM</td>
<td>mouse complete medium</td>
</tr>
<tr>
<td>PB</td>
<td>plasmablast</td>
</tr>
<tr>
<td>PC</td>
<td>plasma cell</td>
</tr>
<tr>
<td>RSS</td>
<td>Recombination signal sequence</td>
</tr>
<tr>
<td>SLPC</td>
<td>short-lived plasma cell</td>
</tr>
<tr>
<td>TCM</td>
<td>trout complete medium</td>
</tr>
</tbody>
</table>
CHAPTER I

Literature Review

The immune system defends the body against invading pathogens such as bacteria, viruses, and fungi. Specialized cells make up the immune system and one of these is the B cell. The B cell is a unique cell that has a receptor on its membrane that is antigen-specific, the B Cell Receptor (BCR). When the BCR comes into contact with and binds to its specified antigen, the B cell becomes activated, proliferates, and differentiates into a plasma cell that secretes antibodies known as immunoglobulins (Ig). The secreted immunoglobulins recognize the same antigens as the BCR and travel throughout the body, clearing the infection.

1.1. The Mammalian Immune System

1.1.1. Lymphopoiesis and B cell development

In the immune system, there are two types of immune tissues: the primary and the secondary. The primary immune tissues are sites of cell development and maturation, while the secondary immune tissues are sites of antigen encounter and response. In the mammalian immune system, the bone marrow serves as the primary immune organ, providing a sterile environment for B cell development and maturation. The lymph nodes, spleen, and mucosa-associated lymphoid tissues (MALT) are the secondary immune
tissues where B cells interact with antigen, proliferate, and differentiate into immunoglobulin-secreting plasma cells.

The hematopoietic stem cell (HSC) gives rise to all of the cells of the immune system. HSCs are self-renewing, pluripotent cells that reside in the bone marrow of mammals [49, 105]. Depending on the environment and growth factors available, HSCs can follow either the lymphoid or myeloid developmental pathway. HSCs differentiate into either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP). The CLPs give rise to B and T cell progenitors or Natural Killer cells and the CMPs give rise to progenitors that eventually differentiate into macrophages, granulocytes (basophils, eosinophils, or neutrophils), platelets, erythrocytes (red blood cells), or dendritic cells [49]. B cell specific transcription factors and environmental cues will lead a CLP to B cell lineage commitment.

B cell development and maturation progresses through a series of successful site-specific Ig gene rearrangements (Figure 1 [9]). The immunoglobulin protein is made up of two heavy chains (HC) and two light chains (LC). The Ig HC and LC genes are comprised of variable (V), diversity (D), and joining (J) segments, which rearrange and combine with one another to form a functional Ig gene. The HC gene is made up of V, D, and J segments and the LC gene is made up of V and J segments (reviewed in [86]). The recombinases encoded by the Recombination Activating Genes-1 and -2 (RAG-1 and RAG-2) are required for the recombination of the V(D)J gene segments in the Ig genes. RAG-1 and RAG-2 are present in early B and T cells, where they initiate recombination
of the B cell receptor (BCR) HC and LC genes and the T cell receptor (TCR) genes, respectively. The RAG-1/2 complex initiates V(D)J recombination by recognizing, binding, and cleaving the DNA at specific sites known as recombination signal sequences (RSS). A double strand DNA break is formed by the RAG complex at the RSSs, initiating V(D)J recombination. The V(D)J segments are joined and the DNA is repaired, forming a complete Ig heavy chain or light chain gene [43, 74]. The RAG complex has not been found in non-lymphoid cell lines or tissues [67, 87]. However, CLPs have been shown to undergo D-J recombination on the HC locus, but retain their ability to differentiate into T cell progenitors [8].

**Bone Marrow**

<table>
<thead>
<tr>
<th>Lymphoid Progenitor</th>
<th>Early Pro-B Cell</th>
<th>Late Pro-B Cell</th>
<th>Pre-B Cell</th>
<th>Immature B Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pre-BCR</td>
<td>BCR</td>
</tr>
<tr>
<td><strong>D-J rearrangement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>V-DJ rearrangement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VDJ rearranged</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VDJ rearranged</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1. B cell development and maturation in the mammalian immune system.** The development of B cells from the earliest, uncommitted lymphoid progenitor cells to the terminally differentiated plasma cells. The stages of V(D)J recombination are indicated.

Progenitor B (pro-B) cells are the earliest distinctive cells of the B cell lineage (Figure 1). These cells develop in the bone marrow, an environment that provides the stromal cells necessary for their development and survival. Stromal cells provide the
growth factors and cytokines, such as IL-7, necessary to maintain the pro-B cells during this stage of development [33].

The first step in the Ig gene rearrangement occurs in the pro-B cell where the Ig HC gene is rearranged. The D and J segments are joined first, followed by the joining of the V segment to the D-J segment. Each HC gene has multiple V, D, and J segments that are randomly chosen for rearrangement, creating diversity in immunoglobulin specificity. The HC genes are located on both chromosomes in the cell. However, due to the process of allelic exclusion, only one HC allele undergoes recombination to produce the Ig HC protein [33, 69]. After VDJ recombination in the pro-B cells, the C_\mu gene segment, encoding IgM constant region, is joined to the immunoglobulin HC gene. The constant (C) segment determines the isoform of the immunoglobulin. Mammals have five different Ig isoforms: IgM, IgD, IgG, IgE, and IgA encoded by the C_\mu, C_\delta, C_\gamma, C_\epsilon, and C_\alpha gene segments. In pro-B cells, C_\mu is the only C segment capable of joining with the VDJ segment, thus producing a \mu HC gene. RNA polymerase II transcribes the \mu HC gene and this leads to the production of the \mu protein [46, 47].

After successful production of the \mu HC protein, the pre-BCR is assembled on the surface of pro-B cells (Figure 2). The pre-BCR consists of the rearranged \mu HC, the Ig\alpha/Ig\beta heterodimer, and the surrogate LC, composed of the V_{pre-B} and the \lambda.5 proteins [74]. The pre-BCR is an important checkpoint in early B cell development. Signaling through the pre-BCR promotes allelic exclusion, alerting the cell that the first Ig heavy chain locus has undergone successful VDJ recombination and preventing the second Ig
heavy chain locus from being processed [7, 46, 74]. The expression of the $\mu$ membrane Ig heavy chain protein is necessary for the progression of B cells beyond the pro-B cell stage [47]. The pre-BCR also signals a decrease in RAG-1 expression [29] and stimulates cell proliferation and differentiation of pre-B cells [74, 79].

Experiments done in pro-B cells with the loss of Ig$\alpha$, Ig$\beta$, or the entire complex show that Ig$\alpha$ and Ig$\beta$ are not required for VDJ recombination of the Ig heavy chain gene. However, pro-B cells lacking one or both parts of the Ig$\alpha$/Ig$\beta$ complex were arrested at the pro-B cell stage, showing that the signaling from the Ig$\alpha$/Ig$\beta$ complex is necessary for the pro-B cells to proliferate and differentiate to pre-B cells [71].

**Figure 2. The B cell receptor (BCR).** The stages of B Cell Receptor (BCR) formation are seen in Figure 2. The pro-B cell is seen on the left with the formation of the Ig$\alpha$/Ig$\beta$ (CD79a/CD79b) signaling complex. The pre-B cell is seen in the center of the figure. The two IgM heavy chain proteins are seen connected to each other and to the surrogate light chains by disulfide bonds. The two surrogate light chains are made up of the V$\text{pre-B}$ and the $\lambda 5$ proteins. The Ig$\alpha$/Ig$\beta$ complex is seen flanking the C region of the heavy chains. The long tails of the Ig$\alpha$/Ig$\beta$ complex extend from the cell membrane to the inside of the cell and act as the signal transducers of the pre-BCR and BCR. The immature, resting B cell is seen on the right. The surrogate LC has been replaced by either the $\kappa$ or $\lambda$ LC.

Pre-B cells are also contained within the nutrient-rich environment of the bone marrow, but they do not require interaction with stromal cells for their growth and
survival [33, 99]. Rearrangement of the light chain gene occurs at this stage of B cell
development. The light chain gene is comprised of only V and J segments, and RAG-1
and RAG-2 are expressed in pre-B cells to allow for the V-J recombination to occur as it
did in the pro-B cells [67, 87]. RNA polymerase II transcribes the rearranged light chain
gene. Successful rearrangement of the light chain gene leads to the differentiation of the
pre-B cells to immature, resting B cells.

The rearranged V(D)J gene segments make up the variable region of the
immunoglobulin heavy chain and light chain proteins. The variable region is the site of
antigen recognition and is specific to one antigen. The constant region of the
immunoglobulin comes from the HC and provides the structure and signaling for the
immunoglobulin, as well as the Ig isoform and effector activities. As mentioned
previously, mammals have five different Ig isoforms encoded by the five different C gene
segments. The constant region is assembled through RNA splicing of the C segment to
the rearranged heavy chain gene and alternative splicing leads to the production of the
different Ig isotypes: IgM, IgD, IgG, IgE, and IgA (reviewed [86]).

Resting, immature B cells possess complete BCRs on their surface. The BCR is
composed of the complete IgM protein, including the HC and LC proteins, and the
Igα/Igβ heterodimer. The Igα/Igβ heterodimer communicates with the surface IgM when
antigen is bound. The Igα/Igβ heterodimer has a longer amino acid tail than the IgM
protein and is responsible for signaling to the B cell that antigen has been bound, setting
off the signaling cascade that leads to B cell activation [38]. Each B cell possesses
multiple BCRs on its surface. The binding site on each BCR is specific for one antigen, and all BCRs on the B cell are specific for the same antigen. While the resting B cells are in the bone marrow, they are not exposed to foreign antigen. If the BCR does interact with antigen in this environment, it would be self-antigen and negative selection would occur. The B cell and/or the BCR would be eliminated [45].

1.1.2. B cell activation, proliferation, and differentiation

Resting B cells travel to the peripheral lymphoid organs, such as the spleen or lymph nodes, by way of the blood or lymph fluid. Once in the periphery, the B cell is considered a mature, resting B cell. Mature, resting B cells have both membrane-bound IgM and IgD on their surface due to a change in the RNA processing that allows for the production of a chimeric molecule containing the Cμ and Cδ segments. IgD is a surface marker for mature, resting B cells, but it is never present in the secreted form and its function is unknown. The membrane IgM and IgD proteins on a B cell have the same antigenic specificity, but different effector activities. If the B cell encounters its specific antigen, the BCRs on its surface will bind the antigen and cross-link with one another. The cross-linking of the BCRs sets off a signaling cascade, activating the B cell and beginning the steps towards plasma cell differentiation (Figure 3).
Figure 3. B cell activation, proliferation, and differentiation in the mammalian immune system. This figure shows the maturation of B cells from the resting stage to the Ig-secreting plasma cell stage. Membrane-bound immunoglobulin is seen on the surface of resting B, activated B, and plasmablasts while Ig is secreted by plasmablasts and plasma cells. The plasmablast can differentiate into a memory B cell or a plasma cell.

Recognition and binding of the antigen by the BCRs initiates differentiation to plasmablasts and cell proliferation. Plasmablasts are larger than activated B cells as they increase in size before they divide. B cell proliferation at this stage creates clones that have the same antigenic specificity, thus creating a large number of B cells that recognize the invading antigen.

Recently, antibody-secreting cells (ASCs) have been defined in the mammalian immune system as plasmablasts (PB), short-lived plasma cells (SL), long-lived plasma cells (LL), and B-1 B cells (see below). Plasmablasts, as described above, are B cells that
proliferate and produce both membrane and secreted IgM and plasma cells are non-dividing, Ig secreting cells [56, 66, 97, 98].

As plasmablasts proliferate, they can differentiate into plasma cells or memory B cells. Plasma cells have no membrane-bound immunoglobulin and they do not proliferate. They are the effector cells of the B cell lineage, secreting immunoglobulins into the cytoplasm to travel throughout the body and remove antigen. The immunoglobulins secreted by plasma cells have the same antigenic specificity as the membrane-bound immunoglobulin of the earlier B cell stages [11].

Some plasmablasts rapidly proliferate upon antigenic stimulation and differentiate into SL plasma cells in the secondary lymphoid organs. These plasma cells survive for a few weeks, producing antibody to fight off the invading antigen [66]. Other plasmablasts proliferate and differentiate into LL plasma cells. LL plasma cells home back to the bone marrow where their life span increases from a few weeks to a few months. The bone marrow provides a safe, nutrient-rich environment where LL plasma cells may survive and maintain serum antibody levels for months [26, 57, 66].

In contrast to the short life span of plasma cells (SL plasma cells), memory B cells may survive for months to years. Memory B cells are produced in the peripheral lymphoid organs and may be stored in the lymph nodes or the spleen. Memory B cells do not secrete immunoglobulins, but they have membrane-bound immunoglobulin (BCR) of the same antigenic specificity as the B cells from which they arose. Upon future
stimulation with antigen, memory B cells can become activated and differentiate into Ig-secreting cells, thus providing a quicker, stronger response to the antigen [45].

The B cells discussed above have derived from the mammalian adult bone marrow and these cells are commonly referred to as B-2 cells. A subset of B cells, known as B-1 B cells, has recently been characterized. Studies suggest that B-1 cells originate in the fetal liver and are therefore termed fetal/neonatal B cells. B-1 B cells are found in the spleen and peritoneal and pleural cavities of mammals. B-1 cells express high levels of surface IgM and low levels of IgD. They secrete IgM and are believed to be responsible for the majority of the natural IgM produced in normal (without antigenic stimulation) animals. B-1 B cells have a similar phenotype to activated B cells (with the exception of a few surface markers) and are important to consider when looking at IgM production in peripheral immune organs (reviewed in [5, 34, 36]).

Following B cell activation, the process of class-switch recombination may occur, which allows for the production of the other Ig isoforms. Class switch recombination occurs through DNA rearrangement where one C gene segment replaces another. This allows plasma cells to secrete IgM, IgG, IgE, or IgA. The C segments determine the isotype and effector activity of the Ig. The Ig isoforms of a B cell all have the same antigenic specificity but different effector functions, giving the immunoglobulins more functional diversity (reviewed [86]).
1.2. The Teleost Immune System

1.2.1. Immune tissues of teleosts

Teleost fishes are members of the Osteichthyes (bony fish) superclass. The teleost infraclass contains both marine and freshwater fish, including the rainbow trout (Oncorhynchus mykiss), salmon (Salmo salar), zebrafish (Danio rerio), carp (Cyprinus carpio), cod (Gadus morhua), halibut (Hippoglossus hippoglossus), and pufferfish (Takifugu rubripes), among many others.

When compared to mammals, the anatomy of teleosts is unique in that they lack bone marrow and lymph nodes [107]. While the immune system provides comparable protection in mammals and teleosts, the timing and location of immune responses in teleosts have not yet been characterized, as they have in mammals. In the mammalian immune system, the bone marrow is the primary immune organ, while in teleosts, including rainbow trout, the anterior region of the kidney serves as the primary site for immune cell development and maturation. The spleen, and possibly the blood and posterior region of the kidney, serve as secondary immune organs in teleosts, where lymphocytes encounter and respond to antigen [93]. Teleost lymphocytes share the same basic morphology and structure of mammalian lymphocytes [25].

The trout kidney is located dorsally in the fish, along the ventral surface of the vertebral column, above the swim bladder. It is a narrow, bi-lobed (at the anterior end
organ that runs nearly the entire length of the trout body, from the braincase to the anterior region of the tail. The trout kidney can be separated into three regions, the anterior kidney (also called the head kidney or pronephros), the mid-kidney, and the posterior kidney. The exact functions of the mid-kidney are not clear at this point, but it does appear to contain both immune and renal tissue. The anterior kidney contains hematopoietic (immune), endocrine, and reticuloendothelial tissues, while the posterior contains mainly renal tissue and some immune tissue, making these regions of the kidney interesting in the understanding of the immune system of the trout [68].

The primary function of the anterior kidney is to act as a site for hematopoiesis [68, 107]. As a predominantly immune tissue, the anterior kidney is devoid of nephrons and has no renal function. The posterior kidney functions primarily in waste elimination, filtering the blood for excretion of metabolic waste through urine production [68]. While immune cells are in the minority in the posterior kidney, this tissue still provides immune function. The Zwollo lab has proposed that the posterior kidney is a secondary immune organ where B cells encounter antigen, become activated, and differentiate into plasma cells [108].

The spleen filters the blood and serves as a secondary immune site for lymphocyte maturation and antigen encounter [25]. The blood is a transport organ, bringing antigens and immune cells to the different immune tissues in the body, but we hypothesize that it may also contain its own distinct population of B cells and serve as a secondary immune tissue.
1.2.2. Lymphopoiesis and B cell development in teleosts

As a homologous tissue to the mammalian bone marrow, the teleost anterior kidney is a hematopoietic tissue and the site of immune cell production. Studies in carp (Cyprinus carpio) and zebrafish (Danio rerio) have shown that the teleost anterior kidney, much like the mammalian bone marrow, provides the nutrient-rich environment necessary for the development and survival of HSCs ([48, 101], respectively).

As in the mammalian bone marrow, HSCs in the teleost anterior kidney are believed to differentiate into the cells of the lymphoid and myeloid lineages (described in section 1.1.1 in mammals), but this has not yet been elucidated in teleosts. Stromal cells in the anterior kidney are believed to secrete the cytokines and growth factors necessary for the survival and maintenance of the immune cells. Lineage specific transcription factors and cues presumably lead to the development of the specific immune cells, such as the B cell. Some evidence for the existence of the earliest B cell progenitors (pro-B cells) in the anterior kidney has been documented in zebrafish (Danio rerio) [103] and carp (Cyprinus carpio) [40], where RAG-1 and RAG-2 expression were seen in the anterior kidney. These studies show that developing B cells exist in the anterior kidney, agreeing with the observation that it is the primary immune organ in teleosts. A RAG-1 mutation in zebrafish showed no V(D)J recombination, no Ig assembly, and no Ig secretion, indicating that RAG-1 expression is necessary for V(D)J recombination and Ig production in zebrafish, as it is in mammals [103]. Both studies show that developing B
cells are present in the anterior kidney and V(D)J recombination takes place in the cells here.

1.2.3. Teleost immunoglobulin classes

Immunoglobulins are produced by B cells in all jawed vertebrates through the process of V(D)J recombination. As mentioned previously, the effector activity of the immunoglobulin depends on the C isotype. In mammals, there are 5 isotypes (IgM, IgD, IgG, IgA, and IgE) while the number and class of isotypes vary in teleosts. IgM is the only isotype that is universal among all vertebrates, but its structure varies among species [23]. In both mammals and teleosts the membrane form is generally a monomeric structure consisting of two Ig heavy chains and two Ig light chains. The secreted form is generally a pentameric structure in mammals, while in teleosts the secreted form is generally a tetrameric structure (reviewed in [60]).

It was recently determined that teleosts, like mammals, produce IgD. Studies in channel catfish (Ictalurus punctatus) B cells found an Ig heavy chain that was not the well-characterized IgM heavy chain. The molecule was chimeric, expressing the μ exon of the IgM heavy chain and the next downstream exon. This new exon had the same characteristics as the mammalian δ exon of the IgD heavy chain and was co-expressed in some, but not all, of the catfish B cells, confirming the existence of a homolog to the mammalian IgD in teleosts [104]. This study found separate terminal exons for both membrane and secreted forms of IgD, which would differ from the mammalian with no
IgD secretion. A study in cod (*Gadus morhua*) found no IgD secretion and found less IgD than IgM on B cells [96].

In the rainbow trout (*Oncorhynchus mykiss*) a third Ig isotype, IgT, was recently characterized [32]. The cDNA of a unique isotype was found in trout B cells. The IgT gene was found to have its own D and J segments and separate terminal exons for membrane and secreted forms. IgT expression was seen in the spleen, anterior and mid-kidney with a small number of IgT expressing cells seen in the blood. IgM and IgT are both found in trout B cells throughout development; however, most B cells are IgM+, followed by IgD and then IgT [32]. Due to the lack of antibodies that recognize trout IgD and IgT, this study focused on the characterization of B cells that produce IgM.

### 1.2.4. B cell activation, proliferation, and differentiation in teleosts

A number of flow cytometric studies have been conducted in various teleost systems using monoclonal antibodies [21, 30, 41, 48, 59, 70, 72, 83, 84, 95, 96, 109]. In these studies, the majority of monoclonal antibodies used have been directed against the IgM HC/LC proteins, allowing for the identification of IgM+ B cells in trout (*Oncorhynchus mykiss*) [21, 83, 95, 109], Chinook salmon (*Oncorhynchus tshawytscha*) [59, 72], cod (*Gadus morhua*) [96], ginbuna carp (*Carassius auratus*) [48], and halibut (*Hippoglossus hippoglossus*) [30, 70].
Previous work from the Zwollo lab using flow cytometric analyses of purified B cells has shown low to undetectable levels of IgM secretion in freshly isolated spleen and blood B cells [109]. After LPS-activation, varying levels of IgM secretion were seen in these tissues by Day 7 in culture. In a study using B cells separated by cell density gradient centrifugation, the anterior kidney was found to contain the highest number of antibody-secreting cells (ASCs), with the majority of these cells most likely representing IgM secreting plasma cells. ASCs were found in the anterior and posterior kidney and the spleen, while few to no ASCs were found in the blood [108]. The results from these studies are in agreement with work done in both salmon [59, 72] and carp [50].

Studies by other groups in the salmon (Salmo salar) have used monoclonal antibodies and flow cytometry to look at membrane IgM and secreted IgM ([59, 72], respectively). Petterson et al. found plasma cells/ASCs in the spleen, blood, and anterior kidney of salmon, with the highest frequency of plasma cells seen in the spleen and very few seen in the blood [72]. Plasma cells have also been found in the spleen, blood, and anterior kidney of carp (Cyprinus carpio) [50].

Atlantic salmon (Salmo salar) anterior kidney, spleen, and blood cells that were LPS-activated and grown in vitro for 10 days, showed B cell activation as well as an increase in cell size. The resting B cell populations of the spleen and blood looked similar to one another. After LPS activation, the spleen showed an increase in the number of B cells and the amount of membrane IgM expression through Day 7. The anterior kidney B cell population did not show any change after LPS activation [59].
Studies done in cod (*Gadus morhua*) have shown that the anterior kidney, spleen, and blood all contain populations of Ig+ B cells. The blood has the lowest proportion of B cells relative to total cell number, while the spleen and anterior kidney contain high numbers of Ig+ B cells [80]. B cell distribution in immune tissues has been characterized in many teleosts based on the expression of membrane and secreted IgM. In studies with trout (*Oncorhynchus mykiss*) [75], cod (*Cyprinus carpio*) [89, 96], sea bass (*Dicentrarchus labrax*) [84], halibut (*Hippoglossus hippoglossus*) [70] and puffer fish (*Takifugu rubripes*) [60] B cells were seen scattered throughout the anterior kidney and spleen. In these same studies, B cells were also found clustered around blood vessels/vascular rich tissue within the anterior kidney and spleen where the cells may encounter antigen from the blood.

When characterizing B cell populations in rainbow trout, ASCs were found in the kidney, spleen, and blood [8]. Spleen and anterior kidney cells showed an ASC response (proliferation and Ig secretion) in the first day of culture in the absence of LPS. LPS-activation of these early responding cells repressed the ASC response in both the anterior kidney and the spleen, suggesting that previously activated B cells respond differently to LPS than developing/resting B cells. The lack of the ASC response may also be due to the inability of the activated B cells to respond to LPS ex vivo. Proliferating cells were seen in the anterior kidney, spleen, and blood based on 3HT uptake, and addition of hydroxyurea (HU), a proliferation blocker, terminated a majority of the proliferation in the spleen and anterior kidney with full termination in the blood. Due to the lack of
proliferation, there was no ASC development or Ig secretion seen in the blood, while a low level was seen in the anterior kidney and spleen, suggesting the presence of ASCs in these tissues. This study also looked at long-term cultures and found the anterior kidney to contain a population of long-lived plasma cells (ASCs) up to 35 weeks after immunization. These cells were resistant to HU and secreted Ig without antigenic stimulation. The anterior kidney provides the proper environment for the storage, survival, and maintenance of the long-lived plasma cells, which may home back to the anterior kidney after their production in the peripheral lymphoid organs [8].

1.3. Transcription factors as B cell markers

Transcription factors regulate commitment to and development along the B cell lineage pathway and have been extensively used as B cell markers [10]. The transcription factors c-myc, Pax-5, RAG-1, and Xbp-1 are expressed in both mammalian and teleost systems. These transcription factors, along with the proliferation markers bromodeoxyuridine (BrdU) and PKH26 tracking dye and the Membrane IgM and Secreted IgM proteins, can be used as markers for the stages of B cell development, activation, and differentiation (Table 1, Figure 4).

Pax-5. The paired-box containing-5 (Pax-5) gene is a member of the Pax family of transcription factors. Members of the Pax family, including Pax-2, Pax-5, and Pax-8, contain a highly conserved DNA-binding paired-domain. The paired-domain is a 128 amino acid conserved sequence found in all members of the Pax gene family. The target
DNA binding sites (cis-regulatory elements) are recognized by the amino- and carboxy-terminal subdomains of the paired-domain (reviewed in [13]) [16]. The paired domain of Pax-5 recognizes the cis-regulatory elements in the DNA of its target genes, such as Xbp-1 [1, 14, 16]. Binding to the target genes allows Pax-5 to activate or inhibit expression of these genes.

The B cell-specific activator protein (BSAP/Pax-5) is encoded by the Pax-5 gene [1, 14]. Pax-5 is associated with commitment to the B cell lineage and is found exclusively in B cells. A study in adult mice showed that a small population of CLPs and pre-pro-B cells started to express Pax-5, but it is possible that these cells were on the verge of committing to the B cell lineage [27]. Not only is Pax-5 expression necessary for B cell commitment, but its continued expression is required to maintain the B cell identity [19, 39]. BSAP/Pax-5 is expressed from the pro-B cell stage to the mature B/activated B cell stages and is not expressed in plasma cells [1]. During B cell development, Pax-5 represses B cell lineage inappropriate genes and myeloid lineage genes and activates B cell specific genes [19, 63, 88]. The expression of Ig genes is somewhat repressed by Pax-5 [92], as is the expression of Blimp-1 and Xbp-1 [62].
### B Cell Marker Expression Throughout B Cell Development, Activation, and Differentiation

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>BrdU</th>
<th>Pax5</th>
<th>RAG1</th>
<th>c-myc</th>
<th>Mem IgM HC</th>
<th>Mem IgM HC+LC</th>
<th>Cytoplasmic IgM HC</th>
<th>Sec IgM HC+LC</th>
<th>Total IgM</th>
<th>Xbp1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-B</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pre-BI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Large pre-BI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Small pre-BI</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Resting Mature B</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Activated B</td>
<td>-</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Plasmablast</td>
<td>+</td>
<td>+/-</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Plasma cell</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

*Table 1.* Table 1 shows the B cell markers and their expression patterns during B cell development, activation, and differentiation in the mammalian system. + = present in the cell type, - = absent in the cell type, +/- = low level of expression, ? = not known if present/absent in the cell type.
Pax-5 is expressed early on in B cell development and helps to maintain B cells in the B cell lineage and to progress past the pro-B cell stage [64]. Pax-5 is important for the development of B cells, as well as the normal development of the central nervous system and testes in adult mice [102]. A study using Pax-5 deficient mice showed that the B cells were arrested at the pro-B cell stage. There were no mature B cells (membrane IgM+) found in the spleen, lymph nodes, or bone marrow of these mice and there were no plasma cells present as determined by the lack of Ig secretion [102]. Pax-5 deficient pro-B cells have the ability to undergo D-J recombination on the Ig heavy chain gene at levels comparable to wild-type mice. However, these cells show a 50-fold decrease in the V-DJ rearrangement on the heavy chain gene, leading to difficulties in plasma cell production and Ig secretion [64].

Pax-5 deficient pro-B cells have been found to lack commitment to the B cell lineage, but maintain their ability to proliferate [64]. In the absence of Pax-5 and the cytokine IL-7, pro-B cells have the potential to develop into any of the hematopoietic precursors [10, 58, 65]. Pax-5 deficient pro-B cells in vitro in the absence of IL-7 will differentiate into macrophages in the presence of the myeloid cytokine macrophage-colony stimulating factor (m-csf) [58]. In the absence of Pax-5 but in the presence of T cell growth factors, pro-B cells will differentiate into pro-T cells, both in vitro and in vivo [58, 78]. The reversion of pro-B cells to other hematopoietic precursors in the absence of Pax-5 shows the necessity of Pax-5 expression for commitment to the B cell lineage in early B cell progenitors.
The conditioned loss of Pax-5 expression in mature B cells causes them to lose their B cell identity through the decreased expression of IgD and other B cell specific genes and the loss of B cell surface proteins [39]. The re-expression of Pax-5 in the Pax-5-/- pro-B and mature B cells restores their B cell identity and leads to continued B cell development and differentiation into Ig-secreting plasma cells [65].

Pax-5 gradually decreases in mature B cells and its expression is lost upon plasma cell differentiation and Blimp-1 expression [10, 94]. In a study using a constructed Pax-5 deficient DT40 chicken B cell line (membrane IgM+) an up-regulation of Blimp-1 and Xbp-1 expression was seen, along with an increase in IgM secretion, which are all characteristic changes associated with plasma cell differentiation [62]. As B cells move closer to plasma cell differentiation, Pax-5 levels decrease in response to rising Blimp-1 levels. As Pax-5 expression is lost, B cells begin to express plasma cell genes and secrete immunoglobulin.

**RAG-1.** As described previously, the Recombination Activating Genes-1 and -2 (RAG-1 and RAG-2) encode the recombinase that initiates V(D)J recombination. In mammals, RAG-1 and RAG-2 are present in pro- and pre-B and T cells, where they initiate recombination of the B cell receptor (BCR) heavy chain and light chain genes and the T cell receptor (TCR) genes. The RAG complex has not been detected in non-lymphoid cell lines or tissues [67, 87]. Studies done with RAG-/- mice show that the lymphocytes are blocked at the progenitor B and T cell stages (reviewed in [3]).
RAG-1 and RAG-2 expression has been documented in zebrafish [103] and carp [40], where expression was seen in the anterior kidney. The location of RAG-expressing cells in the anterior kidney shows that there are developing B cells in this tissue, just as there are in the bone marrow of mammals. Zebrafish with a RAG-1 mutation showed the importance of V(D)J recombination and RAG expression for the progression of B cell development in teleosts [103].

Studies in mammals have shown that immature B cells in the bone marrow do indeed express low levels of RAG-1 and RAG-2 [29, 61, 106]. Low, residual levels of RAG-1 and RAG-2 expression are seen in immature B cells in the bone marrow, but as membrane IgM levels increase on these cells, RAG expression decreases [106]. A study done by Nagaoka, et al. [61] found that immature B cells in the bone marrow expressing low levels of RAG-1 and RAG-2 travel to the spleen after immunization. They suggest that the migration of these immature RAG-expressing B cells to the spleen after immunization may explain the RAG-1 and RAG-2 expression seen in peripheral lymphoid organs [61]. RAG-1 and RAG-2 expression was seen in B cells in the mouse spleen and lymph nodes after immunization [37, 69] and in mature B cells lines after antigenic stimulation [17, 37]. These studies suggest that immature and mature B cells express RAG-1 and RAG-2, but this point remains a source of debate. RAG expression may be seen in the immature and mature B cell stages as these cells are undergoing receptor editing. If the BCR on an immature B cell comes into contact and binds antigen, then the B cell will either undergo cell death or edit its BCR (reviewed in [34]).
c-myc. c-myc is a protein that belongs to the myc family of basic helix-loop-helix/leucine zipper transcription factors. The c-myc protein must dimerize with its partner max in order to bind to the DNA of its target genes ([6, 73] reviewed in [52]). c-myc is known to be involved with cell growth, proliferation, differentiation, and/or apoptosis [24].

Expression of c-myc is seen at high levels in pro- and pre-B cells but expression decreases as the cells differentiate into immature, resting (IgM+) B cells [54]. Levels of c-myc expression increase again after antigenic stimulation as B cells are proliferating. As B cells leave the cell cycle and differentiate into plasma cells or memory B cells, c-myc expression is lost [51, 54]. Studies have shown that c-myc is not only related to cell division, but to the cell growth that occurs before cell division [15, 31, 42]. As a cell prepares to divide, it increases in size and mass with an increase in protein synthesis [15]. c-myc may play a role in both cell growth and division, but some studies show that c-myc only regulates cell growth, not division [31, 42].

While its role is not completely understood, c-myc is known to be expressed at high levels in proliferating B cells: pro- and pre-B cells and plasmablasts. As the cells differentiate into immature, resting B cells or Ig secreting plasma cells, c-myc expression is lost [51]. The expression patterns of c-myc allow for its use as a marker to identify proliferating cells.

Blimp-1. B-lymphocyte induced maturation protein-1 (Blimp-1) is a transcriptional regulator required for plasma cell differentiation [53, 90, 91]. One of the genes that
Blimp-1 regulates c-myc. Blimp-1 directly represses c-myc gene expression, terminating proliferation and causing B cells to exit the cell cycle [55]. It is shown that repression of c-myc and cessation of proliferation is not enough to induce plasma cell differentiation. Blimp-1 must be expressed and allowed to regulate genes other than c-myc to allow for plasma cell differentiation [54]. Blimp-1 also binds directly to the Pax-5 promoter and represses Pax-5 expression. Through this action, Blimp-1 indirectly allows for the expression of Xbp-1 and for plasma cell differentiation to occur [53, 77, 90].

Xbp-1. X-box binding protein-1 (Xbp-1) is a transcription factor with a basic DNA binding region associated with a leucine-zipper [77]. The expression of Xbp-1 is associated with endoplasmic reticulum (ER) stress and plasma cell differentiation. As B cells are preparing to differentiate into Ig-secreting plasma cells, there is remodeling of the secretory apparatus, including the ER. The inositol-requiring endonuclease (IRE-1) is activated by stress in the ER. At this point, Xbp-1 is expressed in its active form. IRE-1 removes 26 nucleotides from the Xbp-1 mRNA, allowing for the expression of the more stable, active form of Xbp-1 [12].

As mentioned above, Xbp-1 is important in plasma cell differentiation and has an inverse relationship with Pax-5. As Pax-5 levels decrease, Xbp-1 levels increase in B cells [77]. Studies in Xbp-1 deficient mice showed that these mice still expressed Blimp-1, but Blimp-1 deficient mice did not express Xbp-1 or other plasma cell genes [90]. This shows that Xbp-1 is located downstream of Blimp-1 and needs Blimp-1 repression of
Pax-5 to allow for its expression. Xbp-1 is important in promoting plasma cell differentiation, both through phenotypic and functional changes and through plasma cell gene activation. In B cells, Xbp-1 induces phenotypic changes that are characteristic of plasma cell differentiation. There is an increase in cell size and organelle mass, along with an increase in endoplasmic reticulum function and protein synthesis [91]. The changes occur as the B cells prepare to differentiate into plasma cells and secrete antibody (Ig).

Low levels of Xbp-1 expression are seen in activated B cells as Pax-5 levels decrease and the cells prepare for and begin to secrete low levels of immunoglobulin. Xbp-1 expression is at its peak in plasmablasts and plasma cells (Table 1). A study by Reimold et al. [77] suggests that Xbp-1 is also expressed in early B cell progenitors, the pre-pro-B cell, when Pax-5 levels are low. As Pax-5 levels increase, Xbp-1 expression is lost in the pre-B cells, where Pax-5 levels are at their highest [77]. The level of Xbp-1 expression in early developing B cells is yet to be fully understood; however, its expression in later stages signifies preparation for plasma cell differentiation and immunoglobulin secretion.
1.4. Cell proliferation markers

The use of fluorescently-labeled markers has been important in detecting cell proliferation, especially in lymphocytes. One of the more commonly used markers is the radioactive marker tritiated thymidine (\(^3\)HT). This marker is incorporated into the DNA of replicating cells and allows for the detection of the proliferative response of an entire cell population to antigenic stimulation. Tritiated thymidine does not allow for the identification of the effects on individual cells (reviewed in [81]). This method has been
used in both mammalian and teleost systems [8, 85], but is used less frequently now due to the difficulty of disposing of the radioactive waste.

Recently, two of the more commonly used proliferation markers have been the PKH26 tracking dye and BrdU incorporation. PKH26 tracking dye is a long-chain aliphatic fluorescent dye [28]. The PKH26 dye is added to cells in culture and becomes integrated in the lipid membrane. As the cells divide, the dye is portioned equally to each daughter cell, reducing the dye intensity by half with every division. When analyzed with flow cytometry, the cells with the highest dye intensity are the cells that are likely to divide and the cells with the low intensity are cells that have divided. The PKH26 tracking dye allows for the cumulative measurement of cell proliferation in a population over time [2].

Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated into the DNA of proliferating cells [35]. BrdU provides information about the proliferating cells at one time point, on a single-cell level [2]. Studies with mouse and human peripheral blood lymphocytes (PBL) have been successful in using BrdU to look at B cell proliferation with flow cytometry [35, 81]. The anti-BrdU antibody or Click-iT EdU dye recognize and bind to the BrdU in DNA. We predicted that BrdU incorporation would occur at the pre-B cell and plasmablast stages when the cells were dividing (Table 1).
1.5. Research aims, hypotheses, and significance

Based on the limited knowledge of the teleost immune system, this study was designed to further explore B cell development, activation, and differentiation in the rainbow trout (*Oncorhynchus mykiss*). The focus of this thesis research was to further characterize the B cells populations in the immune tissues of the rainbow trout and determine a B cell profile for each tissue.

The hypotheses for this study are represented in the model seen in Figure 4 [108]. This model shows the proposed movement of B cells throughout the kidney, spleen, and blood of the rainbow trout. B cell development and maturation is illustrated as occurring in the anterior kidney. The mature, resting B cells of the anterior kidney then move to the posterior region of the kidney or travel via the blood to the spleen. In these peripheral lymphoid organs, the resting B cells encounter antigen and become activated, proliferate, and differentiate into plasma cells. The plasma cells may remain in the periphery or they may home back to the anterior kidney for storage as long-lived plasma cells.
1.5.1. Aim 1: To identify a marker for proliferating trout B cells using flow cytometry.

Very few studies have been conducted on B cell proliferation in the rainbow trout. Previous studies used $^3$HT to look at cell proliferation in the anterior kidney, spleen, and blood of the trout with success [8, 108]. However, this approach does not allow for the concomitant identification of the proliferating cells as B cells. Therefore, we wished to identify proliferation markers for flow cytometry that would allow for the use of multiple markers per cell and enabling the identification of proliferating B cells. Three different proliferation markers, BrdU, c-myc, and PKH26 tracking dye, were tested.
1.5.2. Aim 2: To determine the B cell profiles of freshly isolated trout immune cells from the anterior and posterior kidney, spleen, and blood using two-color flow cytometry.

This study aimed to identify the types of B cell populations present in freshly isolated immune tissues of the rainbow trout. Two-color flow cytometry using combinations of the fluorescent markers BrdU, Pax-5, RAG-1, Membrane IgM, Secreted IgM, Total IgM, and Xbp-1 was used to analyze the B cell populations within the trout spleen, blood, and anterior and posterior kidney.

Since the teleost anterior kidney is functionally homologous to the mammalian bone marrow, we predicted that we would see B cell development occurring in the anterior kidney of the trout. We hypothesized that in the absence of immunization, the anterior kidney contains a diverse population of immune cells, including populations of developing B cells with the phenotype: BrdU+ or −/Pax-5+/RAG-1+ or −/Membrane IgM−/Secreted IgM−/Total IgM−/Xbp-1+, as well as a small population of ASCs.

There is little known about the B cell profile of the posterior kidney and it may contain a mixture of B cells at various stages of activation. We hypothesized that, prior to LPS-activation, the majority of the lymphocytes in the posterior kidney are either resting, mature B cells, activated B cells, or ASCs.
As secondary immune organs, we hypothesized that the majority of lymphocytes in the spleen and blood prior to LPS-activation are resting, mature B cells with the phenotype: BrdU−/Pax-5+/Membrane IgM+/RAG-1−/Secreted IgM−/Total IgM+/Xbp-1+.

1.5.3. Aim 3: To determine the B cell profiles of LPS-activated trout immune cells from the anterior and posterior kidney, spleen, and blood using two-color flow cytometry.

In order to identify the changes seen in the B cell populations in the immune tissues of the rainbow trout after antigenic stimulation, immune cells from the trout anterior and posterior kidney, spleen, and blood were LPS-activated and cultured ex vivo. Two-color flow cytometry using combinations of the markers BrdU, Pax-5, RAG-1, Membrane IgM, Secreted IgM, Total IgM, and Xbp-1 was used to analyze the B cell populations in the trout immune tissues after LPS-activation.

Developing B cells are insensitive to LPS, therefore we hypothesized that after stimulation with LPS we would continue to see a large population of developing B cells in the anterior kidney with the phenotype: BrdU+/Pax-5+/RAG-1+/Membrane IgM−/Secreted IgM−/Total IgM−/Xbp-1+. As a storage site for ASCs both before and after LPS-activation, we hypothesized that we would see IgM secreting, high Xbp-1 expressing cells in addition to the developing B cells in the anterior kidney.
After LPS-activation, we hypothesized that the population of resting B cells in the spleen, blood, and posterior kidney would decrease in frequency and in turn, the frequency of the activated B cell, plasmablast, and plasma cell populations in these tissues would increase.

To monitor B cell activation, supernatants from LPS-activated trout anterior and posterior kidney, spleen, and blood B cells were analyzed for total IgM levels using ELISAs. We hypothesized that we would see higher IgM production in the anterior kidney, posterior kidney, and spleen. IgM secretion has been documented in the spleen and anterior kidney [8] and as a secondary immune organ, we expected the posterior to secrete IgM as well. We expected to see lower levels of IgM secretion in the blood as this tissue produces low numbers of plasma cells.

1.5.4. Significance

The results from this study have the potential to provide new understanding of the development, maintenance, and functions of the immune system of rainbow trout and other teleosts. The patterns of B cell populations within each trout immune tissue would allow for the development of a “B cell profile” for each immune site. This could then be applied to disease studies, or to other teleosts systems, such as the salmon or carp.

Using various combinations of B cell markers, two-color flow cytometry can provide information on the B cell populations present within the spleen, blood, and
kidney of the trout. With this method we can identify which B cell populations are present in the immune tissues before and after activation/antigenic stimulation. By comparing the staining patterns of the markers before and after activation/antigenic stimulation, we can begin to characterize B cell responses to pathogens.

Many teleosts, including salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are commercially grown as aquaculture species. Fish grown in this environment are susceptible to various diseases, especially at the juvenile stage. As the aquaculture industry continues to grow, the need to maintain fish health is important. Understanding the teleost immune system will allow for the development of vaccines to protect the fish, without having to treat them with antibiotics or drugs.
CHAPTER II

Methods

2.1. MPC11 cell line

The murine plasmacytoma line MPC11 was purchased through the American Type Culture Collection (ATCC) and grown according to ATCC guidelines. The cells were maintained at a concentration of 1-5x10^5 cells/ml in Mouse Complete Medium (MCM) consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; BioWhitaker, Inc.), 2 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and 50 µM beta–mercaptoethanol.

2.2. Mouse tissue collection

Three-month old male Y0 84b mice were a gift from Dr. Eric Bradley. Mice were euthanized through asphyxiation with CO_2, followed by cervical dislocation, in accordance with IACUC protocol. The spleen was removed and placed into a Petri dish (Falcon, BD Biosciences) filled with 2 ml HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM Na_2HPO_4•2H_2O, and 20 mM Hepes at pH 7.05). The tissue was repeatedly aspirated using a 10 ml syringe and forced through a 40 nm nylon cell strainer (Falcon, BD Biosciences) to create a single-cell suspension. The cells were centrifuged for 10 min at 1300 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 10 ml HBSS. The cells were centrifuged for 5 min at 1000 rpm at 4°C.
This procedure was repeated two times. The supernatant was removed and the pellet was resuspended in 5 ml MCM at a concentration of $10^6$ cells/ml.

### 2.3. Rainbow trout and facilities

Out-bred rainbow trout (200-500 grams) were purchased from Casta Line Trout Farms (Goshen, VA). Fish were maintained in a 100-gallon tank with a re-circulating system employing biologically-filtered well water. Fresh water exchange was approximately 10% per day. Water temperature was maintained at 12°C. Fish were fed dry, floating pellets (Aqua Max Grower 600, Purina) once a day.

### 2.4. Trout tissue collection

Trout were anesthetized with ethyl-3-Aminobenzoate methanesulfate salt (Sigma) in accordance with our IACUC protocol. Blood (2-5 ml) was drawn from the caudal vein and placed into heparinized tubes. The spleen, anterior kidney and posterior kidney were collected and placed into Petri dishes filled with 2 ml HBSS. The tissue was repeatedly aspirated using a 10 ml syringe and then forced through a 40 nm nylon cell strainer (Falcon, BD Biosciences) to create a cell suspension for each tissue. The cells were centrifuged for 10 min at 1300 rpm at 4°C and the supernatant was removed. The cells were resuspended in 10 ml HBSS and centrifuged for 10 min at 1300 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 10 ml HBSS. In a separate 50 ml Falcon tube, 10 ml of Histopaque (1077, Sigma Aldrich) was added. The cell
suspension was layered onto the Histopaque and the cells were centrifuged for 30-45 min at 2100 rpm at 4°C. The supernatant, including the cell layer, was removed and transferred to a new 50 ml Falcon tube. The volume was increased to 45 ml with HBSS and the cells were centrifuged for 10 min at 1300 rpm at 4°C. The supernatant was removed and the cells were resuspended in 10 ml HBSS. Cells were centrifuged for 10 min at 1300 rpm at 4°C and the supernatant was removed. The pellet was resuspended in 5 ml trout complete medium (TCM). The cells were counted using a hemacytometer (Fisher Scientific) and cell concentrations were determined for each tissue.

2.5. LPS activation

**Fresh trout cells:** Cells were resuspended in trout complete medium (TCM) consisting of RPMI 1640 with 10 mM L-glutamine, 10% fetal calf serum (FCS), 50 g/ml gentamicin, 50 M 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (10 g/ml; Sigma-Aldrich) at a concentration of $10^7$ cells per 1 ml of medium. The B cell mitogen lipopolysaccharide (LPS, 055:B5 from *E. coli*, Sigma) was added to the cells at 100 µg/ml to activate the B-lymphocytes. The cell suspension was transferred to a 24-well culture plate (Corning, BD Biosciences) at $2 \times 10^7$ cells (2 ml) per well. The cells were grown at 18°C in the presence of blood gas (10% CO$_2$, 10% O$_2$, 80% N$_2$). Anterior kidney cells were collected everyday for 7 days and posterior kidney cells were collected everyday for 5 days. Spleen and blood cells were collected every other day over a 10-day period. Cells were fed every other day with one-tenth of the culture volume of a 10x tissue culture cocktail containing 500 µg/m gentamycin, 10x essential amino acids (aas),
10x non-essential aas, 70 mM L-glutamine, 70 mg/ml dextrose, 10x nucleosides, and 33% FBS.

**Fresh mouse spleen cells:** Cells were resuspended in MCM at a concentration of $10^7$ cells/ml. The B cell mitogen LPS was added to the cells at 20 µg/ml to activate the mouse B-lymphocytes. The cell suspension was transferred to a 24-well culture plate (Corning, BD Biosciences) at $10^7$ cells/ml and 1ml per well. The mouse spleen cells were grown at 37°C in a CO$_2$ incubator (5.0% CO$_2$) and were collected every 24 hr over a 4-day period. Cells were fed every other day with MCM at one-fifth of the culture volume.

### 2.6. BrdU incorporation

Bromodeoxyuridine (BrdU kit, Invitrogen) was added to cells in culture at a final concentration of 3µg/ml. Trout cells were incubated with BrdU in culture for 18 hr, mouse spleen cells were incubated for 3 hr, and MPC11 cells were incubated for 16 hr before they were collected and fixed (see section 2.9).

### 2.7. PKH26 tracking dye

Cells were collected in 15ml Falcon tubes to be stained with PKH26 tracking dye (Red Fluorescent Cell Linker Mini Kit, Sigma). The cells were centrifuged for 5 min at 15000 rpm at 25°C and the supernatant was removed. The pellet was resuspended in 1ml trout medium without serum for trout cells and mouse medium without serum for mouse
spleen and MPC11 cells. The cells were centrifuged as above. The cell pellet was resuspended in 1 ml Diluent C (Red Fluorescent Cell Linker Mini Kit, Sigma). Immediately prior to staining, a 4 µM PKH26 dye reagent was prepared by diluting 4 µl of 1 mM PKH26 dye in 1ml of Diluent C. The cells were rapidly added to the 1 ml of staining solution and resuspended. The cells were incubated with the dye for 3 min at 25°C with periodic inversion of the tube. After the 3 min incubation, 2 ml PBS containing 1% Bovine Serum Albumin (BSA) was added to the cells and they were incubated for 1 min at 25°C. The cells were then diluted with 4 ml TCM for trout cells or MCM for mouse spleen and MPC11 cells and were centrifuged for 10 min at 1500 rpm at 25°C. The supernatant was removed and the cells were resuspended in 1 ml TCM for trout cells or complete MCM for mouse spleen and MPC11 cells and then were transferred to a new tube for washing. The 1 ml of cell suspension was resuspended in 9 ml TCM for trout cells or 9 ml MCM for mouse spleen or MPC11 cells. The cells were centrifuged for 5 min at 1500 rpm at 25°C and the supernatant was removed. This was repeated for a total of three washes. After the third wash, all of the supernatant was removed and the cells were resuspended in 2 ml TCM for trout cells or MCM for mouse spleen and MPC11 cells. The cells were grown in culture for 24 hr and collected at 24-hour intervals for a total of 96 hr or 4 days. For starvation experiments, the cells were not fed during this time in culture.
2.8. Staining fresh trout cells with anti-IgM antibody

Fresh trout cells were counted and half of the cells were taken to be “pre-stained” with a monoclonal mouse anti-trout total IgM antibody (Warrs 1-14 [20]). The cells were centrifuged for 5 min at 1000 rpm at 4°C. The supernatant (containing secreted Ig) was saved and stored at -20°C for use in enzyme-linked immunosorbent assays (ELISA). The pellet was resuspended in 10 ml Staining Buffer (PBS+ 2% FBS+ 1 mM EDTA) and centrifuged for 5 min at 1000 rpm at 4°C. The supernatant was removed and the pellet resuspended in 1.5 ml staining buffer. The mouse anti-trout total IgM 555 antibody (Warrs 1-14) was added to the cells at 1 µg/1.5ml. The cells were incubated for 35 min at 4°C on a rotating platform, protected from light. After the incubation, the cells were centrifuged for 5 min at 1000 rpm at 4°C and the supernatant was removed. The pellet was resuspended in 1 ml staining buffer and spun for 5 min at 1000 rpm at 4°C. This was repeated for a total of 2 washes. The cells were then fixed and permeabilized (see section 2.9).

2.9. Cell fixation and permeabilization

Cells were centrifuged for 5 min at 1000 rpm at 4°C. The supernatant (containing secreted Ig) was saved and stored at -20°C for use in ELISA assays. The pellet was resuspended in 1 ml PBS (1.9 mM NaH₂PO₄•H₂O, 8.1 mM Na₂HPO₄• 7H₂O, 137 mM NaCl, and 2.6 mM KCl, pH 7.4) containing 0.02% NaN₃ and cells were centrifuged for 5 min at 1000 rpm at 4°C. The supernatant was removed and the pellet resuspended in 1ml
1% Paraformaldehyde (16% PF solution, E-M grade; Electron Microscopy Sciences) in PBS and incubated on ice for 15 min. Next the cells were centrifuged for 5 min at 1000 rpm at 4°C. The supernatant was removed and the pellet resuspended in 1 ml PBS containing 0.02% NaN\textsubscript{3}. The cells were centrifuged for 5 min at 1000 rpm at 4°C and the supernatant removed. The cell pellet was vortexed until the cells were in suspension and 1 ml of 80% ice-cold methanol was added drop-wise to the cells while vortexing. The cells were stored at -20°C for a minimum of 16 hr and remained stable at -20°C for up to 6 weeks.

2.10. One- and two-color flow cytometry

Immediately before use, fixed and 80% methanol-permeabilized cells were removed from -20°C storage and 1 ml ice-cold PBS + 0.02% NaN\textsubscript{3} was added to the 1 ml of 80% cell suspension. The cells were centrifuged for 5 min at 1500 rpm at 4°C and the supernatant was removed. Cells were re-fixed as described but for 10 min instead of 15 min. Next, the cells were resuspended in 1 ml permeabilizing solution (BD perm wash, BD-Biosciences) containing 2% FBS and counted. A concentration of (0.5-1x10\textsuperscript{6} cells x # of samples) was used. Cells were centrifuged for 5 min at 1500 rpm at 4°C. The supernatant was removed and the cells were resuspended in perm wash+ 5% FBS in a volume of (# of samples + 1) x 45 µl. The cells were incubated for 15 minutes on a nutator at 4°C. Fluorescent antibodies were diluted to 10x stock solutions at a concentration of 0.15 µg/ml in perm wash+ 5% FBS. From each freshly diluted antibody solution 5 µl was added to the 45 µl cell sample and gently resuspended. The cells were
incubated with the fluorescent antibodies for 90 min at 4°C on a rotating platform, protected from light. After the 90 min incubation, 1 ml perm wash+ 2% FBS was added to each sample. The cells were incubated for 10 min at 4°C on the nutator, protected from light. After the 10-min incubation, the cells were centrifuged for 3 min at 2000 rpm at 4°C and the supernatant was removed. This wash and 10 min incubation was repeated once more. The pellet was resuspended in 200 μl perm wash+ 2% FBS and the cell suspension was transferred to a 96 well round-bottom plate (Fisher). The samples were immediately analyzed by flow cytometry (BD FACSArray, BD-Biosciences). For each sample, 50,000 cells were acquired. From this mixture of cells, a gate was placed around the lymphoid population to exclude red blood cells and debris from analysis.

Flow data were analyzed using the WinMDI software, version 2.8.

2.11. Click-iT EdU flow cytometry

Mouse or trout cells were fixed as described above, and incubated in the presence of fluorescent antibodies as before. After the 90 min incubation in the presence of fluorescent antibodies, 1ml perm wash+ 2% FBS was added to each sample and the cells were resuspended. The cells were centrifuged for 3 min at 2000 rpm at 4°C and the supernatant was removed. While the cells were spinning, the Click-iT EdU reaction buffer additive and Click-iT EdU reaction cocktail (Invitrogen) were prepared as follows: First, the 1x Click-iT EdU buffer additive was prepared by diluting the 10x solution 1:10 in deionized water. Second, the fluorescent dye azide was diluted 1:8 in perm wash+ 5% FBS. Finally, the Click-iT EdU Reaction Cocktail (438 μl reaction buffer, 10 μl CuSO₄,)
2.5 μl Alexa 647 fluorescent dye azide, 50 μl Click-iT reaction buffer additive) was prepared for each sample. After the spin, each cell pellet was resuspended in 500 μl of the Click-iT Reaction Cocktail and the cells were incubated for 30 min at 25°C (room temperature), protected from light. After the 30-min incubation, the cells were washed twice as above. The pellet was resuspended in 200 μl perm wash+ 2% FBS and the cell suspension was transferred to a 96 well round-bottom plate (Fisher). The samples were immediately analyzed by flow cytometry (BD FACSArray, BD-Biosciences), as described previously (see section 2.10).

2.12. BrdU one-color flow cytometry

Immediately before use, MPC11 cells were removed from -20°C storage and resuspended in 500 μl ice-cold PBS with 0.02% NaN₃. Cells were centrifuged for 2 min at 3000 rpm at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml permeabilizing solution (BD perm wash, BD-Biosciences) and counted. A concentration of (10⁶ cells x # of samples) was used. The cells were centrifuged as before and the supernatant was removed. The pellet was resuspended in 100 μl BD Cytoperm Plus Buffer (BrdU kit, BD Biosciences) and incubated on ice for 10 min. The cells were centrifuged as before. The pellet was resuspended in 1 ml permeabilizing solution and centrifuged as before. The cells were resuspended in 100 μl BD Cytofix/Cytoperm Buffer (BrdU kit, BD Biosciences) and incubated on ice for 5 min. The cells were centrifuged as before. The pellet was resuspended in 1 ml permeabilizing solution and centrifuged as before. The DNase solution was prepared by diluting the DNase (BrdU kit, BD
Biosciences) 1:3 in PBS. The pellet was resuspended in 100 µl ice-cold DNase solution and incubated for 1 hr at 37°C. The cells were centrifuged as before and resuspended in 1 ml permeabilizing solution. The cells were centrifuged as before. This process was repeated for a total of two washes. The supernatant was removed and the cells were resuspended in perm wash+ 5% FBS in a volume of (# of samples + 1) x 45 µl. The cells were incubated for 15 min on a rotating platform at 4°C. Fluorescent antibodies were diluted to 10x stock solutions at a concentration of 0.15 mg/ml in perm wash+ 5% FBS. From each freshly diluted antibody solution 5 µl was added to each sample and gently resuspended. The cells were incubated with the fluorescent antibodies for 20 min at room temperature on the nutator, protected from light. After the 20-min incubation, 1ml permeabilizing solution was added to each sample and the cells were resuspended. The cells were centrifuged as before and this wash was repeated twice. The supernatant was removed and the pellet was resuspended in 200 µl staining buffer (PBS containing 3% FBS and 0.09% NaN₃). The cell suspension was transferred to a 96-well round bottom plate (Fisher) and the samples were immediately analyzed by flow cytometry (BD FACSArray, BD-Biosciences), as previously described (see section 2.10).

### 2.13. Antibodies

The c-myc, Pax-5, RAG-1, Secreted IgM, Total IgM (Warrs), and Xbp-1 antibodies (Table 2) were conjugated to Alexa 555 or Alexa 647 using protein-labeling kits (Molecular Probes, Invitrogen). Antibodies were labeled according to manufacturers instructions. Frozen aliquots were stored at -80°C.
**Fluorescent Markers Used With Flow Cytometry**

<table>
<thead>
<tr>
<th>Source</th>
<th>Name</th>
<th>Target Peptide</th>
<th>Type of Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purchased (Invitrogen)</td>
<td>Click-iT EdU</td>
<td>BrdU</td>
<td>N/A*</td>
</tr>
<tr>
<td>Purchased (Invitrogen)</td>
<td>BrdU</td>
<td>BrdU</td>
<td>mouse monoclonal IgG</td>
</tr>
<tr>
<td>Purchased (Santa Cruz Biotechnology Inc.)</td>
<td>c-myc (N-262)</td>
<td>c-myc aa 1-262</td>
<td>rabbit anti-human polyclonal IgG</td>
</tr>
<tr>
<td>In-house [110]</td>
<td>Pax-5 (ED-1)</td>
<td>Trout BSAP/Pax-5 148aa of paired domain</td>
<td>polyclonal anti-paired domain IgG (ED-1)</td>
</tr>
<tr>
<td>Purchased (Santa Cruz Biotechnology Inc.)</td>
<td>RAG-1 (H-300)</td>
<td>RAG-1 aa 744-1043</td>
<td>rabbit anti-human polyclonal IgG</td>
</tr>
<tr>
<td>In-house (Gen-script) [109]</td>
<td>Secreted IgM</td>
<td>Trout Secreted IgM WLVDDEPVERTSSS</td>
<td>polyclonal rabbit-antiserum Secreted IgG</td>
</tr>
<tr>
<td>Gift from Dr. Steve Kaattari [20]</td>
<td>Warrs (1-14)</td>
<td>Trout Membrane and Secreted IgM</td>
<td>mouse anti-trout Ig HC monoclonal IgG</td>
</tr>
<tr>
<td>In-house (Gen-script)</td>
<td>t-Xbp-1</td>
<td>Trout Xbp-1 SGYERSPSPFSDMSS PLCSEGSWDDVFANEL</td>
<td>rabbit anti-trout IgG</td>
</tr>
</tbody>
</table>

Table 2. Fluorescent antibodies used in thesis work. Table 2 presents the antibodies used, their source, full name, target peptide, and type of antibody. *Click-iT EdU is not an antibody. This is a chemical reaction resulting in the binding of the Click-iT dye azide to the BrdU in the DNA.

## 2.14. ELISA

A 96 well microtiter plate (Falcon, BD Biosciences) was coated with 50μl (2μg/ml) of the monoclonal mouse anti-trout total IgM antibody (Warrs 1-14 [20]). The Warrs antibody was diluted at 1:500 in PBS containing 0.05% NaN₃. The plates were incubated with the primary antibody at 4°C overnight.
Next, the plate was washed with TBST Buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). This was repeated for a total of three washes.

300 µl of Blocking Buffer was added to each well and the plate was incubated for 1 hr at room temperature while gently shaking. After the incubation, the plate was washed three times with TBST Buffer. Trout supernatants were diluted 1:4 in PBS with 0.05% NaN₃. Fifty microliters of the sample was added to each well. Each sample was performed in triplicate. As a negative control TCM was diluted 1:4 in PBS with 0.05% NaN₃. As a positive control, trout serum was used either undiluted or at a dilution of 1:10 or 1:100 in PBS with 0.05% NaN₃, and 50 µl was added to the wells. The plate was incubated for 1 hr at room temperature while gently shaking. After the incubation, the plate was washed three times with TBST Buffer as before. The secondary antibody, mouse-anti-trout IgM (Warrs 1-14) with a biotin label, was diluted at 1 µg/ml in Blocking Buffer and 50 µl was added to each well. The plate was incubated for 1 hr at room temperature while gently shaking. After the incubation, the plate was washed three times as before. A streptavidin-HRPO solution was prepared by diluting the streptavidin-HRPO (1 mg/ml) in Blocking Buffer and 50 µl was added to each well. The plate was incubated for 1 hr at 25°C while gently shaking. After the incubation, the plate was washed 5 times with TBST as before. The ABTS developer [8.4 ml Citrate Buffer, 100 µl 10% ABTS (2-2’ Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt–98%; Sigma), 5 µl 30% H₂O₂ (Sigma-Aldrich)] was prepared and 50 µl was immediately added to each well. Plate was read by a microplate reader (Model 550, BioRad) with a filter at a wavelength of 405nm after 5 and 10 min. ELISA data analyzed using Microsoft Excel.
3.1. Analysis of cell proliferation using one-color flow cytometry

The ultimate goal of this proliferation study was to find a marker to identify proliferating B cells in the rainbow trout and use this marker for further studies of this organism. This study aimed to test three separate markers for detecting cell proliferation: BrdU incorporation, c-myc, and PKH26 tracking dye. Each marker was analyzed with one-color flow cytometry for cell proliferation. Before the frequencies of the stained cell populations were determined, dot plots were made and the lymphocyte population gated. The forward-scatter (FSC) and side-scatter (SSC) properties of the cells are used to determine the location of the lymphocyte population from the total tissue cell mixture. FSC provides information about the size of the cell and the SSC provides information about the internal complexity of the cells. For this study, the proliferation markers were analyzed against cell size (FSC) and shown as contour plots.

Contour plots display the density of the cell population. In contour plots, the individual events of a dot plot are replaced by contour lines and resemble a topographic map. As the contours become more compact, the higher the density of cells at that point, expressing that intensity of the marker. Contour lines are shown as log algorithms with intervals of 50% [93].
We hypothesized that we would see varying populations of proliferating cells in each of our systems using the three different proliferation markers with one-color flow cytometry. No B cell specific markers were used in conjunction with the proliferation markers and we therefore were unable to distinguish proliferating B cells from proliferating T or other non-B cells in this experiment.

The timing of proliferation and location of proliferating B cells in trout immune tissues are poorly understood, hence, the initial goal of this thesis work was to find usable proliferation markers for the trout immune system. In mammals, B cell proliferation occurs at two time points during the life of a B cell: during B cell development in pro- and pre-B cells and again as plasmablasts. The three proliferation markers were tested in three different systems: the MPC11 plasmacytoma line, mouse spleen cells, and trout anterior kidney and spleen cells. Mouse spleen cells and trout anterior kidney and spleen cells were placed in culture and activated with the B cell mitogen Lipopolysaccharide (LPS) to activate the B cells and induce proliferation.

3.1.1. Cells used in the study

**MPC11 cell line.** The well-defined murine plasmacytoma cell line MPC11 is known to incessantly divide every 16-20 hours, making it an ideal model for testing proliferation markers. This cell line does not require activation with LPS for proliferation to occur and any proliferation we detected with the proliferation markers was B cell proliferation.
BrdU incorporation and PKH26 tracking were tested in this system for detection of B cell proliferation.

**Mouse spleen cells.** The mouse is a well-accepted and well-defined model organism for immunology studies. Previous work (See Chapter I) has shown that populations of proliferating B cells are present in the mouse spleen after stimulation, thus making the mouse a model system for use in this study. The proliferation markers BrdU and c-myc were tested in this model. Detection of proliferation with these markers in the mouse was used to show that we could use these methods to detect proliferation in the trout.

**Trout anterior kidney cells.** The anterior kidney of the rainbow trout is hypothesized to contain a large population of developing B and T cells, including proliferating pre-B and T cells. There may also be a smaller population of plasmablasts in this tissue. To identify the populations of proliferating cells in the anterior kidney, the cells were analyzed with one-color flow cytometry using the proliferation markers BrdU, c-myc, and PKH26 tracking dye.

**Trout spleen cells.** The trout spleen is hypothesized to be a site of antigen encounter and activation for B cells. We would expect to see a population of proliferating plasmablasts in this tissue. The samples in this study were a mixture of immune cells and may contain proliferating T cells as well as B cells. Trout spleen cells were analyzed with one-color flow cytometry using BrdU incorporation, c-myc, and PKH26 tracking dye as markers for cell proliferation.
3.1.2. Analysis of proliferation markers using one-color flow cytometry

**BrdU incorporation.** Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated into newly synthesized DNA of proliferating cells. In one method of detection, an anti-BrdU antibody binds to the BrdU in the DNA, identifying the proliferating B and T cells. A newer method, used here in the mouse and trout cells, is the Click-iT EdU method. This method uses a chemical reaction to bind a fluorescent dye azide to the BrdU in the DNA. The MPC11 cells, mouse spleen cells, and trout anterior kidney and spleen cells were incubated with BrdU in culture before collection. The cells were then fixed and permeabilized. MPC11 cells were stained with the fluorescent anti-BrdU antibody while the mouse and trout cells were incubated with the Click-iT dye reagent. All cells were analyzed by one-color flow cytometry for the proliferating cells.

Figure 6 depicts the contour plots of the MPC11 cells with BrdU incorporation and FSC (cell size) as the parameters. The cells were grown in culture for four days with BrdU added at the last 16 hours of incubation. Once a day, $10^6$ cells were collected, fixed, and permeabilized. There is a population of cells with high proliferation occurring on Days 0 and 3 and a population of cells with low proliferation on Days 2 and 4. The lower proliferation on Days 2 and 4 may be due to the cells being fed on Day 2 and Day 4 in culture, as fresh nutrients are likely to boost proliferation in cells.
Using normal mouse splenic B cells after LPS induction, a population of proliferating cells was observed on each of the five days measured (Day 0-4). As shown in the contour plots in Figure 7A, the proliferating cells are relatively large cells (high FSC), supporting the assumption that they most likely represent plasmablasts or proliferating T cells. This population increased over the 4-day culture period, with 15.8% of cells proliferating on Day 4, as seen in the graph depicted in Figure 7B.

Using trout splenic cells, we saw a population of proliferating cells present on Days 1-7 after LPS activation (Fig. 8). The proliferating cells are largest on Day 1 and show high BrdU incorporation. The cells then decrease in size and show a range of BrdU incorporation, from intermediate to high. By Day 6, the cells have increased in size again and still show a range of BrdU incorporation. These cells are most likely plasmablasts or proliferating T cells.

In summary, in each of the three systems tested here (MPC11 cells, mouse spleen cells, and trout spleen cells) we detected dividing cell populations using the BrdU proliferation marker.
Figure 6. BrdU incorporation in MPC11 cells. One-color flow cytometry showing BrdU incorporation and cell size (FSC) on fixed and permeabilized MPC11 cells over a 4-day LPS-activation period, Days 0-4 (D0-D4). Cell populations on the right side of each graph represent BrdU+ proliferating cells. Numbers indicate percent cells in each region.
Figure 7. BrdU incorporation in mouse spleen cells. Analysis of BrdU incorporation in fixed and permeabilized mouse spleen cells over a 4-day LPS-activation period. A. Contour plots of one-color flow cytometry looking at BrdU incorporation and cell size (FSC). Cells on the right side of the line represent BrdU+ proliferating cells. B. Line graph of the proliferating (BrdU+) cell population. One representative experiment seen here.
Figure 8. BrdU incorporation in trout spleen cells. One-color flow cytometry looking at BrdU incorporation and cell size (FSC) on fixed and permeabilized trout spleen cells over a 7-day LPS-activation period. Proliferating cells are seen on the right side of each graph. Numbers represent percent cells in each region. One representative experiment. C= Control, D= Day.
c-myec. As discussed in the introduction, the protein c-myc is expressed during cell proliferation, and should therefore provide a good proliferation marker for our studies. Mouse spleen cells and trout anterior kidney and spleen cells were stained with the fluorescent anti-c-myc antibody and analyzed for proliferation by one-color flow cytometry. Proliferation was detected in both tissues with this marker, but no B cell-specific markers were used and we were unable to identify proliferating B from proliferating T cells.

When looking at c-myc expression in mouse spleen cells, we did not see any change in the population during the activation period. At all time points during the culture period, we saw two distinct populations: one with intermediate FSC and one with high FSC (Figure 9). We did not see a change in c-myc intensity in the population over the 4-day LPS-activation period as compared to Day 0.

Similar results to those of the mouse spleen were seen when looking at c-myc expression in the anterior kidney. In Figure 10 we see a population of cells with low FSC and a population of cells with intermediate FSC. Only very low c-myc expression was detected in these cells over the 6-day LPS-activation period.

The relationship between c-myc staining intensity and cell size (FSC) in trout spleen cells is examined in Figure 11. As was seen in the mouse spleen and trout anterior kidney, we saw in the trout spleen two distinct cell populations of different sizes, one with low/intermediate FSC and one with high FSC. On Day 0 there is a population of
larger cells that show a slightly higher staining intensity for c-myc. This population is most likely plasmablasts or proliferating T cells. The c-myc intensity in this population showed no significant change by Day 7 after LPS-activation.

We did not see c-myc expression in the mouse spleen or trout anterior kidney cells before or after LPS-activation (Figures 9-11). However, a population of cells showing a slightly higher c-myc staining intensity was seen in the trout spleen cells on Day 0 before LPS-activation. These cells have a high FSC and may represent plasmablasts or proliferating T cells. There was no c-myc expression detected on Day 7 in the trout spleen cells, so we did not see any c-myc expression in either the mouse or the trout after LPS-activation.
Figure 9. c-myc expression in mouse spleen cells. One-color flow cytometry using c-myc and cell size (FSC) on fixed and permeabilized mouse spleen cells over a 4-day LPS-activation period. One representative experiment shown here.
Figure 10. c-myc expression in trout anterior kidney cells. One-color flow cytometry looking at c-myc and cell size (FSC) on fixed and permeabilized trout anterior kidney cells over a 5-day LPS-activation period. One representative experiment shown here. C = Control, D = Day.
Trout Spleen
c-myc vs. FSC
Day 0 and Day 7

Figure 11. c-myc expression in trout spleen cells. One-color flow cytometry looking at c-myc and cell size (FSC) on freshly isolated (D0) and LPS-activated (D7) fixed and permeabilized trout spleen cells. One representative experiment shown here.
**PKH26 tracking dye.** PKH26 tracking dye is a method for tracking cell proliferation through dye intensity in a cell population. The dye penetrates and attaches to lipids in the cell membrane. As the cells divide, the amount of dye per cell decreases. MPC11 and trout anterior kidney and spleen cells were stained with the PKH26 dye and placed in culture. The cells were collected every 24 hours and analyzed by one-color flow cytometry for cell proliferation. The earliest cells (Day 0 or Day 1, depending on experiment) are expected to contain the most dye and have the highest intensity for PKH26 since they have not yet divided. As the cells proliferate, the amount of dye in each cell and the intensity of PKH26 will decrease. The proliferating cells seen in each tissue may be either B or T cells, as no B cell-specific marker used to allow for the differentiation between B and T cells.

The top panel of Figure 12 shows the MPC11 control cells that were not stained with PKH26 dye. The Day 0 cells are expected to have the highest intensity of PKH26 dye since each cell contains more dye than it would if it had divided. There is not a noticeable change in PKH26 intensity between Day 2 and Day 3, but by Day 4 there is a cell population that contains a lower intensity of PKH26 and more proliferating cells. A histogram of the cells from Day 0, 2, and 3 is seen in Figure 13. The histogram depicts the results seen in the contour plots, with Day 0 showing the highest PKH26 intensity and Day 2 and 3 cells decreasing in intensity. The histogram shows separation of the peaks for each day and clearly shows that the cells were proliferating between Day 0 and Day 3.
Figure 12. PKH26 intensity in mouse spleen cells: contour plots. Analysis of cell proliferation in fixed and permeabilized MPC11 cells using PKH26 tracking dye. Contour plots from one-color flow cytometry using PKH26 tracking dye and cell size (FSC). One representative experiment shown here. C= Control, D= Day.
MPC11
PKH26 vs. Events
Days 0, 2, 3

Key:

Black Dotted: Day 0  Red: Day 2  Green: Day 3

Figure 13. PKH26 intensity in MPC11 cells: histogram. Analysis of cell proliferation in fixed and permeabilized MPC11 cells using PKH26 tracking dye. Histograms of PKH26 tracking dye on Day 0 (Dotted Black Line), Day 2 (Red Line), and Day 3 (Green Line). One representative experiment shown here.
Anterior kidney cells were stained with PKH26 tracking dye, LPS-activated, and placed in culture. The cells were collected every 24 hours for six days. Figure 14 shows the contour plots for the PKH26 staining intensity and cell size (FSC). There are three distinct populations of different cell size and PKH26 intensity seen by Day 3 in culture, but there is no clear pattern seen in proliferation in terms of percent proliferating cells. The cells with the highest PKH26 intensity are also the largest cells, suggesting that they are plasmablasts or proliferating T cells. The cells with the lowest PKH26 intensity are also the smallest cells, suggesting that these cells are no longer dividing. These cells may be pre-B II cells, which do not proliferate and are smaller than the earlier B cells (pro-B and pre-B I cells).

Trout spleen cells were collected and analyzed using one-color flow cytometry as described above for the anterior kidney cells (Figure 15). There are no major differences seen in PKH26 staining intensity over the 6-day culture period. On Days 1-6 we see a large population of cells with a high PKH26 intensity. These cells are most likely cells that are preparing to divide, however, we do not see a large amount of proliferation over the 6-day activation period. We also see a population of small cells that have a low PKH26 intensity that appear by Day 1-2. This population of cells is no longer proliferating and seems to decrease after Day 2 in culture.
Figure 14. PKH26 intensity in trout anterior kidney cells. One-color flow cytometry using PKH26 tracking dye and cell size (FSC) on fixed and permeabilized trout anterior kidney cells over a 6-day LPS-activation period. Lines separate populations based on PKH26 staining intensity. One representative experiment shown here.
Figure 15. PKH26 intensity in trout spleen cells. One-color flow cytometry using PKH26 tracking dye and cell size (FSC) on fixed and permeabilized trout spleen cells over a 6-day LPS-activation period. One representative experiment shown here.
The PKH26 tracking dye gives us the ability to track cell proliferation over time. As the cells divide, there is a decrease in the PKH26 intensity per cell and we saw this in both the MPC11 cells and the trout anterior kidney and spleen cells. However, the patterns of proliferation are not clear in this study, with variation in intensity seen from one day to the next.

BrdU incorporation analyzed through one-color flow cytometry revealed proliferating cells in the MPC11 cell line, mouse spleen cells, and trout anterior kidney and spleen cells. While small populations of proliferating cells were seen using the PKH26 tracking dye and c-Myc with one-color flow cytometry in all systems, the populations were not clearly separated from the population of non-proliferating cells. The BrdU incorporation method showed strong separation between the proliferating and non-proliferating populations in the MPC11, mouse spleen, and trout anterior kidney and spleen cells, making it an ideal marker for tracking proliferation in these systems and for further use in investigating B cell proliferation in the trout immune tissues.
3.2. Flow cytometric analysis of freshly isolated trout immune tissues

Based on previous work from our lab [108, 109] we hypothesized that the majority of lymphocytes in the trout spleen and blood before LPS-activation are resting, mature B cells with the phenotype: BrdU-/Pax-5+/Membrane IgM+/RAG-1-/Secreted IgM-/Total IgM+/Xbp-1++. In contrast, this work suggests that the kidney is a complex organ, with multiple B cell populations present in freshly isolated samples from both the anterior and posterior regions [108, 109]. The anterior kidney is the primary immune organ and site for B cell development and maturation [107]. We hypothesized that fresh cells from the anterior kidney would contain a population of developing B cells with the phenotype: BrdU+/Pax-5+/RAG-1+/Membrane IgM-/Secreted IgM-/Total IgM-/Xbp-1++. In addition, we hypothesized that the anterior kidney provides a storage site for antibody secreting cells (ASC) with the phenotype: BrdU+ or ~/Pax-5+ or ~/RAG-1~/Membrane IgM+ or ~/Secreted IgM++/Total IgM+ or ++/ Xbp-1+ or ++. The posterior kidney is thought to be a secondary immune organ, providing a site for antigen interaction for B cells. We hypothesized that freshly isolated cells from the posterior kidney would contain a mixture of resting and activated B cells as well as a small population of Ig secreting cells.

In order to analyze the profiles of B cell populations in the spleen, blood, and kidney of the rainbow trout, fresh, unstimulated trout cells were harvested, fixed and permeabilized, and stained with fluorescent antibodies. Analysis was done using two-color flow cytometry. Combinations of BrdU incorporation and Membrane IgM, Pax-5, RAG-1, Secreted IgM, Total IgM, and Xbp-1 fluorescent antibodies were used.
When determining the relative frequency of each population we can begin to define the “B cell profiles” of freshly isolated trout spleen, blood, and kidney cells.

**FSC and SSC.** As described earlier (see section 3.1), dotplots were made in order to correctly gate the lymphocyte population located within our heterogeneous cell samples. Figure 16A shows the dot plots, with FSC and SSC as the parameters, for the spleen, blood, anterior kidney, and posterior kidney for fresh Day 0 cells. Each dot on the dot plot represents one cell. Figure 16B shows the same dot plots with a gate around a population that contains the lymphocyte population. Mature lymphocyte populations typically have low FSC and low SSC and this is an abundant population in the spleen. The lymphocyte population in the blood consists of two separate populations. Both have low SSC and low FSC, but one population has slightly higher FSC than the other. These populations are seen in Figure 16A as two circles in the lymphoid population of the blood dot plot. The lymphocyte population in both the anterior and posterior kidney is made up of cells of different size and complexity as seen by the increase in FSC and SSC of the population. The population spans from cells with low FSC and low SSC to cells with high FSC and high SSC. This would be expected in the anterior and posterior kidney since we hypothesized that they both are made up of a mixture of B cell populations. The black arrows in Figure 16B point out the cellular debris in each sample, which has very low FSC (size), and is not included in the gate. A population with low FSC and high SSC represent red blood cells, which were also excluded from analysis (Fig. 16B, red arrows).
Figure 16. **Day 0 trout tissue comparison: FSC/SSC.** Analysis of cell size (FSC) and internal complexity (SSC). A. Dot plots of fixed and permeabilized Day 0 cells from trout spleen, blood, anterior and posterior kidney. Refer to text for circles in blood dot plot. B. Dot plots with the lymphocyte population gated. Black arrows: cellular debris. Red arrows: red blood cells. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish in each experiments.
**Proliferation.** In order to determine the frequency of proliferating B cells in the trout immune tissues, we looked at BrdU incorporation and membrane IgM expression in Day 2 cells. The ratio of resting IgM+ B cells (BrdU-, mem IgM+) to plasmablasts (BrdU+, mem IgM+) can be seen in Figure 17. Day 2 cells were used in this analysis because BrdU incorporation cannot be measured in fresh Day 0 cells. The cells need to be grown in the presence of BrdU for at least 18 hours for detectable BrdU incorporation to occur. We chose to collect samples once a day for the anterior and posterior kidney and every other day for the spleen and blood. Because of this collection pattern, we did not have Day 1 samples for each tissue and had to use Day 2 samples, which we collected for each tissue, for our analysis of BrdU incorporation. The largest population of proliferating IgM+ B cells is seen in the anterior kidney (1.2%) with smaller populations seen in the spleen and posterior kidney (<1%). These cells are most likely plasmablasts. Very low levels of proliferation are detected in the blood any time during the 10-day LPS-activation period of the blood. The spleen and blood contain the highest number of resting B cells (41% and 21%, respectively), which is hypothesized to be the majority of the B cell population in these tissues on Day 0.

**Pax-5 and Membrane IgM.** Pax-5 expression is seen in pro- and pre-B cells and throughout B cell maturation, but it is not expressed in plasma cells. The staining patterns for Pax-5 and membrane IgM provide information about the presence of (im)mature, resting B and early activated B cells in each of the tissues. A
**BrdU/Membrane IgM**

**Day 2 Tissue Comparison**

**Figure 17. Day 2 trout tissue comparison: BrdU incorporation/Membrane IgM.** Two-color flow cytometry using BrdU incorporation and Membrane IgM on fixed and permeabilized Day 2 cells from the trout spleen, blood, anterior, and posterior kidney. Box A contains the IgM+ resting B cells and Box B contains the IgM+ plasma blasts. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish in each.
representative experiment of the relationship between Pax-5 and membrane IgM staining intensity in fresh Day 0 cells from each tissue is seen in Figure 18. The highest percentage of Pax-5+, membrane IgM+ cells is seen in the spleen (33%) and the blood (21%). While smaller populations, with low levels of Pax-5, were seen in the anterior (13%) and posterior kidney (5.5%). These cells are most likely resting B or early activated B cells. We expect to see high percentages of these cells in the spleen and blood on Day 0, based on previous work from our lab [108]. The highest Pax-5 and membrane IgM staining intensities are seen in the blood lymphocytes, suggesting that there are higher levels of Pax-5 and Membrane IgM per cell. This population of Pax-5+, Membrane IgM+ cells does not change throughout the anterior or posterior kidney culture period, as Pax-5 levels are low in this tissue (further discussed in Chapter IV), but interesting patterns develop at later time-points in the spleen and blood as discussed later.

**RAG-1 and Membrane IgM.** RAG-1 is involved in Ig gene rearrangement. In mammals, RAG-1 is expressed in pro-B, pre-B, and some immature and activated B cell populations [67, 87]. Figure 19 shows the co-expression of RAG-1 and membrane IgM in Day 0 cells for each tissue. The top right panel in each contour plot shows the cells that are expressing RAG-1 and membrane IgM. This population is most clearly seen in the spleen, and most likely represents activated B cells/plasmablasts. We do not see a similar pattern in the Day 0 cells from the blood or the kidney. This population is less than 1% in each tissue and does not change in the spleen, blood, or kidney after LPS activation and will be further discussed below.
Pax-5/Membrane IgM
Day 0 Fresh Tissue Comparison

Figure 18. Day 0 trout tissue comparison: Pax-5/Membrane IgM. Two-color flow cytometry using Pax-5 and Membrane IgM on fixed and permeabilized Day 0 cells from the trout spleen, blood, anterior, and posterior kidney. Top left: IgM+/Pax5- cells; Top right: IgM+/Pax-5+ cells; Bottom right: IgM-/Pax-5+ cells; Bottom left: IgM-/Pax-5- cells. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish.
Figure 19. Day 0 trout tissue comparison: RAG-1/Membrane IgM. Two-color flow cytometry using RAG-1 and Membrane IgM on fixed and permeabilized Day 0 cells from the trout spleen, blood, anterior, and posterior kidney. Top left: Mem IgM+/RAG1- cells; Top right: Mem IgM+/RAG1+ cells; Bottom right: Mem IgM-/RAG1+ cells; Bottom left: Mem IgM-/RAG1- cells. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish in each experiment.
**Secreted IgM and Membrane IgM.** The expression patterns of secreted IgM and membrane IgM revealed the presence or absence of antibody secreting cells (ASC), either plasmablasts or plasma cells, in each tissue on Day 0. The right side of each contour plot in Figure 20 shows the cells that are secreting IgM. There is a very low frequency of IgM secretion in the spleen or blood in fresh Day 0 cells (<1%). The anterior and posterior kidney have a higher frequency of IgM secreting cells on Day 0, considering that much fewer B cells reside in these tissues. Interestingly, the blood contains a unique population of IgM secreting, Membrane IgM− cells on Day 0 (see box).

**Xbp-1 and Membrane IgM.** Xbp-1 is expressed in early developing B cells (pro- and pre-B) and late activated B cells, plasmablasts, and plasma cells, with the highest levels of expression seen in plasma cells [76, 77, 91]. The co-expression of Xbp-1 and Membrane IgM identified populations of resting B and activated B cells/plasmablasts. In Figure 21 the staining patterns of Xbp-1 and membrane IgM in fresh Day 0 cells are shown for each tissue. Cells expressing intermediate levels of Xbp-1 and high levels of membrane IgM are most likely mature, resting B cells (Xbp-1 +/−, Membrane IgM+) while cells with higher Xbp-1 expression are most likely activated B cells/plasmablasts (Xbp-1+, Membrane IgM+). A small population of activated B cells is seen in each tissue on Day 0 (see arrow). The main cell population that is Xbp-1+/− and Membrane IgM+ is most likely mature, resting B cells, and we see a large number of these cells in both the spleen and blood, as expected. There is a
Figure 20. Day 0 trout tissue comparison: Secreted IgM/Membrane IgM. Two-color flow cytometry using Secreted IgM and Membrane IgM on fixed and permeabilized Day 0 cells from the trout spleen, blood, anterior, and posterior kidney. Left side: Sec IgM- cells; Right side: Sec IgM+ cells. Refer to text for box on blood contour. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish in each experiments.
Figure 21. Day 0 trout tissue comparison: Xbp-1/Membrane IgM. Two-color flow cytometry using Xbp-1 and Membrane IgM on fixed and permeabilized Day 0 cells from the trout spleen, blood, anterior, and posterior kidney. Arrows point to activated B cells (Xbp-1+/Membrane IgM+). Refer to text for box on blood contour. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish in each experiments.
small population of cells in the blood that are Xbp-1 ++, Membrane IgM− (see box), which suggests that these are most likely plasma cells.

**Xbp-1 and Total IgM.** The co-expression of Xbp-1 and Total IgM identified populations of resting B cells and activated B cells/plasmablasts and plasma cells. Figure 22 shows the relationship between Xbp-1 and Total IgM staining intensity in Day 0 cells from the spleen, blood, and kidney. The top box in each contour plot contains the IgM secreting B cells (Xbp-1++, total IgM++) while the lower box contains the resting IgM+ B cells, which express lower levels of both Xbp-1 and total IgM (Xbp-1+/-, total IgM+). The spleen and blood have the highest percentage of resting IgM+ B cells on Day 0 (42% and 20%, respectively), while the anterior kidney has the highest percentage of IgM secreting B cells on Day 0 (6.5%). The anterior kidney has the lowest ratio of resting B to plasma cells on Day 0 (1.75) followed by the posterior kidney (2.48), spleen (16), and blood with the highest ratio (40). In agreement with our hypothesis, we have show that there is a higher number of IgM secreting cells present in the kidney on Day 0.

Based on the data presented in this section we can see that the B lymphocyte population in the spleen and blood on Day 0 consists mainly of resting B cells, in agreement with previous work from our lab. The levels of cell proliferation detected in fresh cells from the spleen and blood are very low, as expected. The anterior and posterior kidney, in agreement with our hypothesis, contain a mixture of B cell populations. The anterior kidney contains the largest population of proliferating B
Figure 22. Day 0 trout tissue comparison: Xbp-1/Total IgM. Two-color flow cytometry using Xbp-1 and Total IgM on fixed and permeabilized Day 0 cells from the trout spleen, blood, anterior, and posterior kidney. The top box contains the IgM secreting B cells (ASCs) and the bottom box contains the mature, resting IgM+ B cells. Cells from one representative experiment shown here. Experiments repeated at least three times total. Cells pooled from 3-5 fish in each experiment.
cells among the four tissues. Pax-5 levels are low in both the anterior and posterior kidney, and both tissues were the sites of highest IgM secretion with low numbers of resting B cells, as expected for unstimulated anterior and posterior kidney cells.

3.3. Analysis of \textit{ex vivo} LPS-activation on trout immune tissues using two-color flow cytometry

Total trout immune cells were stimulated with LPS in culture and analyzed by two-color flow cytometry as before. Combinations of BrdU incorporation, Membrane IgM, Pax-5, RAG-1, Secreted IgM, Total IgM, and Xbp-1 fluorescent antibodies were used.

3.3.1. Spleen

Earlier work suggests that the spleen acts as a secondary lymphoid organ in the trout, providing a site where B cells encounter antigen and become activated \cite{8, 107, 108, 109}. Hence, we expect to see changing frequencies of activated B cells, plasmablasts and plasma cells after LPS-activation in this tissue. We hypothesized that as a result of LPS-activation, we would see a decrease in the frequency of resting B cells and an increase in the frequency of activated B cells, plasmablasts, and/or plasma cells.
**Pax-5 and Membrane IgM.** As described in the introduction (Chapter I), Pax-5 is first expressed in early developing B cells (pro-B). Its expression is seen throughout B cell development and maturation and is lost in plasma cells [1]. The combination of Pax-5 and Membrane IgM provided us with the frequency of resting or activated B cells during LPS activation. Figure 23 shows the relationship between Pax-5 and Membrane IgM staining intensity in Day 0, Day 4, and Day 8 spleen cells from four separate experiments. The Day 0 cells show a different pattern in each experiment with two cell populations in the 1/28/09 experiment, three populations in the 2/19/09 experiments, and four populations in the 3/25/09 experiment. Each experiment shows a different pattern without stimulation, but after LPS activation, similar populations are seen in all experiments, with variation as to which day those populations are induced.

Figure 23 shows the contour plots for Pax-5 and Membrane IgM expression with a schematic for interpreting the contour plots. The Pax-5 low (+/-), Membrane IgM+ cells are in Box A, the Pax-5+, Membrane IgM+ cells are in Box B, and the Pax-5+, Membrane IgM- cells are in Box C. The Pax-5 low (+/-) and Membrane IgM+ population (Figure 23, Box A) varies greatly between experiments. These cells are most likely activated IgM+ B cells, but may also be resting IgM+ B cells. This population is present on Day 0 in the 1/28/09 experiment, but then lost by Day 4. The same population is not seen on Day 0 in the 2/19/09 experiments, but is induced by Day 8. In the 3/25/09 experiment, this population is seen on Day 0, Day 4, and Day 8. This population of activated IgM+ B cells varies but is induced in all experiments by
Figure 23. Pax-5 and Membrane IgM expression in trout spleen cells: contour plots. Two-color flow cytometry using Pax-5 and Membrane IgM on fixed and permeabilized spleen cells over a 10-day LPS-activation period. Three representative days shown here: Day 0, Day 4, and Day 8. A. Contour plots from four independent experiments. B. Schematic of cell populations in part A. Box A: Pax-5 low (+/-)Mem IgM+ cells; Box B: Pax-5+/Mem IgM+ cells; Box C: Pax-5+/Mem IgM- cells.
Figure 24. Pax-5 and Membrane IgM expression in trout spleen cells: line graphs. Analysis of Pax-5 and Membrane IgM expression in spleen cells over a 10-day LPS-activation period. A. Line graph of Pax-5 low (+/-)/Mem IgM+ cells. B. Line graph of resting IgM+ B cells (Pax-5+/Mem IgM+). C. Line graph of IgD+ or IgT+ B cells or pro-B cells (Pax-5+/Mem IgM-). Results shown from four or five independent experiments. Cells pooled from 3-5 fish in each experiment.
Day 2, with a peak seen between Day 6 and Day 8 as shown in Figure 24A.

A large population of Pax-5+, Membrane IgM+ cells is seen in each experiment on Day 0, Day 4, and Day 8 (Figure 23, Box B). These are most likely resting B cells. This population increases slightly from Day 0-2 (22-32%) followed by a gradual decrease after Day 4 in culture from 20-25% at Day 4 to 7-12% on Day 10 (Figure 24B).

Finally, a minor population of Pax-5+, Membrane IgM- cells is seen in each experiment (Figure 23, Box C). These cells may be IgD+ or IgT+ B cells or possibly, but less likely, pro-B cells. There is a general decrease in this population over the 10-day LPS-activation period from 9-14% on Day 0 to 2-4% on Day 10 (Fig. 24C).

RAG-1 and Secreted IgM. The co-expression of RAG-1 and Secreted IgM identified a population of IgM secreting activated B cells or plasmablasts. Figure 25 shows two representative experiments of the relationship between the RAG-1 and secreted IgM staining intensity in Day 0 and Day 8 spleen cells. This combination was repeated for a total of six experiments. This figure shows that LPS induces a splenic B cell population that expresses RAG-1 and secretes IgM. The cells within this population show increased levels of both secreted IgM and RAG-1 by Day 8 with variation seen between experiments. In the 3/25/09 experiment, there is an increase in the number of cells with this phenotype, from 2.73% on Day 0 to 14% on Day 8 (see boxed region)
Figure 25. RAG-1 and Secreted IgM expression in trout spleen cells. Two-color flow cytometry using RAG-1 and Secreted IgM on fixed and permeabilized spleen cells over a 10-day LPS-activation period. Day 0 and Day 8 shown here. Results from 2 representative experiments. Experiment repeated five times. Box: Induced RAG+/Sec IgM+ population. Arrow: RAG+/Sec IgM- population. C= Control, D= Day.
and in the 5/22/09 experiment there is an increase from 1.27% on Day 0 to 10% on Day 8 (see boxed region). These cells are most likely plasmablasts or late activated B cells. Also of interest is a population that is present on Day 0 and Day 8 in both experiments that is RAG-1+ but secreted IgM− (arrow).

**Proliferation.** The plasmablast population in the spleen was best defined by looking at BrdU incorporation. When combined with Pax-5, we were able to measure the frequency of proliferating B cells in this tissue. These cells are most likely plasmablasts but may also be pre-B cells. This population peaks between Day 4 and Day 6 in culture (Fig. 26A). A population of Membrane IgM+ plasmablasts (Fig. 26B) and IgM secreting plasmablasts (Fig. 26C) are also seen to peak between Day 4 and Day 6. All three populations of proliferating B cells decrease after Day 6 in the 10-day LPS-activation period. The 1/28/09 experiment shows a different expression pattern from the other three experiments with BrdU incorporation and Pax-5 or Secreted IgM expression. Throughout this work we observed that this experiment displayed patterns different from all other experiments regardless of marker combination. This suggests that the fish used in this experiment may have been immune-challenged prior to our using them, and therefore we would expect to see different expression patterns.

**Xbp-1 and Membrane IgM.** The staining patterns of Xbp-1 and Membrane IgM allowed for the identification of resting B cells and activated B cells/plasmablasts and plasma cells. Figure 27 examines these patterns in spleen cells over a 10-day LPS-
Figure 26. BrdU incorporation and Pax-5/Membrane IgM/Secreted IgM expression in trout spleen cells. Analysis of B cell proliferation in fixed and permeabilized spleen cells over a 10-day LPS-activation period. A. BrdU+/Pax-5+ proliferating B cells. B. BrdU+/Mem IgM+ plasmablasts. C. BrdU+/Sec IgM+ plasmablasts. Results from five or six independent experiments. Cells pooled from 3-5 fish in each experiment.
activation period. There is a clear decrease seen in the frequency of the resting B cell population (Xbp-1+/−, Membrane IgM+) from Day 0-10 (Fig. 27A), while the frequency of activated B cells/plasmablasts (Xbp-1+, Membrane IgM+) peaks around Day 6 in culture then decreases slightly through Day 10 (Fig. 27B). Figure 27C shows a two vertical axis graph of the B cell populations seen in parts A and B of Figure 27. This graph allows for the visualization that while the resting B cell population is decreasing in frequency, the activated B cell/plasmablast population begins to increase in frequency and peak at Day 6 in culture. The 5/22/09 experiment does not exhibit this same pattern, and this is addressed in Chapter IV.

**Xbp-1 and Total IgM.** The relationship between the Xbp-1 and total IgM staining patterns in LPS-activated spleen cells is shown in Figure 28. The resting B cell population (Xbp-1+/−, Total IgM+) decreases over the 10-day LPS-activation period (Fig. 28A). The number of activated B cells (Xbp-1+, Total IgM+) peaks between Day 2 and Day 4 and decreases through Day 10 (Fig. 28B) while the plasmablast/plasma cell (Xbp-1++, Total IgM++) population clearly increases through Day 10 (Fig. 28C). Figure 28D shows the two vertical axis graph for the populations seen in Figure 28 parts B and C. The relationship between the activated B cells and the plasmablast/plasma cell populations is clear, with the activated B cell population decreasing in frequency over time while the plasmablast/plasma cell population increases in frequency over time.
Figure 27. Xbp-1 and Membrane IgM expression in trout cells. Two-color flow cytometry using Xbp-1 and Membrane IgM on fixed and permeabilized spleen cells over a 10-day LPS activation period. A. IgM+ resting B cells (Xbp-1+/−/Mem IgM+). B. IgM+ activated B cells or plasmablasts (Xbp-1+/Mem IgM+). Results shown from four independent experiments. Repeated five times total. Cells pooled from 3-5 fish in each experiment.
Figure 27C. Xbp-1 and Membrane IgM expression in trout spleen cells: two-vertical axis graphs. Analysis of Xbp-1 and Mem IgM in spleen B cells. Two-vertical axis graphs shown for four independent experiments. Repeated a total of five times. Cells pooled from 3-5 fish in each experiment. Solid line: resting B cells (Xbp-1+/−/Mem IgM+). Dashed Line: Activated B cells or plasmablasts (Xbp-1+/Mem IgM+).
Figure 28. Xbp-1 and Total IgM expression in trout spleen cells. Two-color flow cytometry using Xbp-1 and Total IgM on fixed and permeabilized spleen cells over a 10-day LPS activation period. A. IgM+ resting B cells (Xbp-1+/-/Total IgM+). B. IgM+ activated B cells (Xbp-1+/Total IgM+). C. Plasmablasts or plasma cells (Xbp-1++/Total IgM++). Three representative experiments shown. Repeated five times total. Cells pooled from 3-5 fish in each experiment.
Figure 28D. Xbp-1 and Total IgM expression in trout spleen cells: two-vertical axis graphs. Analysis of Xbp-1 and Total IgM in spleen B cells. Two-vertical axis graphs shown for three independent experiments. Experiment repeated four times. Solid line: resting B cells (Xbp-1+/−/Total IgM+). Dashed line: Plasmablasts or plasma cells (Xbp-1++/Total IgM+). Results shown from three representative experiments. Repeated five times total. Cells pooled from 3-5 fish in each experiment.
The data presented in this section identified changes in populations of resting B cells, activated B cells, plasmablasts, and plasma cells in the spleen after LPS activation. The resting B cell population decreases over the 10-day activation period, while the populations of activated B cells, plasmablasts, and plasma cells increase. We have shown that the majority of B cells in the trout spleen are resting B cells that become activated after stimulation with LPS. Small populations of plasmablasts and plasma cells were seen at later time points in the 10-day LPS activation period. The antibody combinations of RAG-1/Membrane IgM and Secreted IgM/Membrane IgM were also investigated, but no clear changes were seen in the B cell populations after LPS activation. Further investigation with these antibody combinations is necessary to gain more information about the B cell populations in the trout spleen after LPS activation.

3.3.2. Blood (PBL)

Teleost blood may act as a “transport tissue”, bringing immune cells from one tissue to another. Because of this function, the blood likely contains a mixture of immune cells, including various types of B cells at different stages of development and activation. Previous work from our lab suggests that the blood contains resting and activated B cells and plasmablasts, with few to no plasma cells [108]. Here we hypothesized that after LPS activation in culture, resting B cells in the blood become activated early on but that this population may die in culture with new populations of
activated B cells rising from the resting B cells over time. We examined BrdU incorporation in three separate experiments. No detectable levels of BrdU incorporation were seen in any of the immune cells in the blood (data not shown), suggesting that no proliferating cells exist or survive in the blood cultures. To better understand the types of B cells found in the blood, cells were analyzed by two-color flow cytometry.

**RAG-1 and Secreted IgM.** In order to investigate the presence of activated B cells in the blood, levels of RAG-1 and Secreted IgM were measured. Figure 29 examines the staining patterns of RAG-1 and secreted IgM in Day 0 and Day 8 blood B cells in three separate experiments. When looking at the fresh Day 0 cells, we see very few RAG-1+, IgM secreting cells, suggesting that very few of these cells are present in healthy trout blood. By Day 8 in culture there is induction of a RAG-1 expressing, IgM secreting population of B cells (see boxed region). These cells are most likely late activated B cells or pre-plasma cells. Of particular interest is a RAG-1+, Secreted IgM- population present in two of the experiments (arrow). This population is also seen in the spleen with this antibody combination.

**Secreted IgM and Membrane IgM.** A population of B cells that produces both membrane and secreted IgM was seen in the blood. This population of activated B or B-1 cells (not plasmablasts, as no cells incorporate BrdU in the blood) peaks between Day 2 and Day 4 and then decreases through Day 10 (Fig. 30A). The blood also contains a population of IgM secreting cells that gradually decreases over the 10-day
Figure 29. RAG-1 and Secreted IgM expression in trout blood cells. Two-color flow cytometry using RAG-1 and Secreted IgM on fixed and permeabilized blood (PBL) cells over a 10-day LPS-activation period. Day 0 and Day 8 shown here. Results from three representative experiments. Experiment repeated five times. Box: Induced RAG+/Sec IgM+ population. Arrow: RAG+/Sec IgM- population.
Figure 30. Secreted IgM and Membrane IgM expression in trout blood cells. Two-color flow cytometry using Secreted IgM and Membrane IgM on fixed and permeabilized blood cells over a 10-day LPS activation period. A. Activated B or B-1 cells (Sec IgM+/Mem IgM+). B. IgM secreting B cells (Sec IgM+/Mem IgM-). Results from three representative experiments. Repeated five times total. Cells pooled from 3-5 fish in each experiment.
LPS-activation period (Fig. 30B). Figure 30C shows the contour plots of the secreted IgM+, membrane IgM+ population to visually see the shift in the amount of IgM secretion per cell. There appears to be an increase in membrane IgM intensity followed by an increase in secreted IgM intensity, with more IgM secreted over time.

**Pax-5 and Membrane IgM.** The combination of Pax-5 and Membrane IgM identified resting B or early activated B cells in our samples. Figure 31 depicts the relationship between Pax-5 and membrane IgM staining intensities in blood B cells over a 10-day LPS-activation period. As expected, the main B cell population consists of resting B cells (Pax-5+, membrane IgM+) which peaks between Day 2 and Day 4 (Fig. 31A) while a moderate population of membrane IgD+, membrane IgT+, or possibly pro-B cells (Pax-5+, Membrane IgM-) is seen to generally decrease after Day 2 of the LPS-activation period (Fig. 31B). There are no resting B cells of any isotype present on Day 0 in the 1/28/09 experiment. There is induction of a small population of membrane IgM+ resting B cells by Day 2 in culture but it then decreases. By Day 10 we see very few resting B cells independent of isotype (IgM, IgD, or IgT) and it may be that these cells become activated by Day 10 in culture.

**Xbp-1 and Membrane IgM.** The co-expression of Xbp-1 and membrane IgM in a cell population allows for the identification of a population of activated B cells as cells increase their Xbp-1 levels after activation. Figure 32A visually shows the frequency of a membrane IgM+ population that increases between Day 0 and Day 8. There is an increase in Xbp-1 intensity over the 10-day period, suggesting that these
Figure 31. Pax-5 and Membrane IgM expression in trout blood cells.
Two-color flow cytometry using Pax-5 and Membrane IgM on fixed and permeabilized blood cells over a 10-day LPS activation period. A. IgM+ resting B cells (Pax-5+/Mem IgM+). B. IgD+ or IgT+ or pro-B cells (Pax-5+/Mem IgM-). Three representative experiments shown. Repeated a total of five times. Cells pooled from 3-5 fish in each experiment.
cells become activated in culture. We see a shift from IgM+ resting B cells to IgM+ activated B cells between Day 2 and Day 4 in culture based on the increase in Xbp-1 intensity. Figure 32B shows the population of IgM+, Xbp-1 +/- resting B cells peaking between Day 0 and Day 2 and then gradually decreasing through Day 10 in culture, while the IgM+, Xbp-1+ activated B cells population is seen to peak between Day 2 and Day 4 in culture (Fig. 32C), but this population varies between experiments.

**Xbp-1 and Total IgM.** A population of resting B cells (Xbp-1+/-, Total IgM+) gradually decreases over the 10-day LPS-activation period (Fig. 33A), presumably because such cells become activated and shift from Xbp-1+/- to Xbp-1+ during this time. Figure 33B shows a population of activated B/pre-plasma cells (Xbp-1++, total IgM+). This population peaks on different days depending on the fish, but it shows that LPS activated the cells in culture. Similar patterns for these populations are also seen in the spleen. Figure 33C shows the 2 vertical axis graph of the populations seen in Figure 33 parts A and B, and the patterns between the resting B and activated B cell populations is clearly seen.

In agreement with our hypothesis, we saw that the majority of the B cell population in the blood consists of resting B cells that become activated after stimulation with LPS. We have shown that the number of resting B cells decreases while the number of activated B cells increases over the 10-day LPS activation period. As mentioned above, we did not see any detectable levels of BrdU
Blood (PBL)
Xbp-1/ Membrane IgM
Day 0, 4, and 8

A.

C

D0

D4

D8

Figure 32. Xbp-1 and Membrane IgM expression in trout blood cells: contour plots. Two-color flow cytometry using Xbp-1 and Membrane IgM on fixed and permeabilized blood cells over a 10-day LPS activation period. A. Contour plots. One representative experiment shown. Experiment repeated 3 times. Cells pooled from 3-5 fish in each experiment.
Figure 32. Xbp-1 and Membrane IgM expression in trout blood cells: line graphs. Two-color flow cytometry using Xbp-1 and Membrane IgM on fixed and permeabilized blood cells over a 10-day LPS-activation period. B. IgM+ resting B cells (Xbp-1+/−/Mem IgM+). C. IgM+ ASCs (Xbp-1++/Mem IgM+). Results from three representative experiments. Repeated five times. Cells pooled from 3-5 fish in each experiment.
Figure 33. Xbp-1 and Total IgM expression in trout blood cells. Two-color flow cytometry using Xbp-1 and Total IgM on fixed and permeabilized blood cells over a 10-day LPS-activation period. A. IgM+ resting B cells (Xbp-1+/−/Total IgM+). B. IgM+ activated B or pre-plasma cells (Xbp-1++/Mem IgM+). Results from three representative experiments. Repeated five times. Cells pooled from 3-5 fish in each experiment.
C. Blood (PBL): Xbp-1 and Total IgM Markers:

1/28/09

Days Post-Activation

Percent Resting B Cells
Xbp-1+/−/Total IgM+
(Solid Line)

Percent Activated B Cells
Xbp-1+/+/Total IgM+
(Dashed Line)

3/25/09

Days Post-Activation

Percent Resting B Cells
Xbp-1+/−/Total IgM+
(Solid Line)

Percent Activated B Cells
Xbp-1+/+/Total IgM+
(Dashed Line)

5/22/09

Days Post-Activation

Percent Resting B Cells
Xbp-1+/−/Total IgM+
(Solid Line)

Percent Activated B Cells
Xbp-1+/+/Total IgM+
(Dashed Line)

Figure 33C. Xbp-1 and Total IgM expression in trout blood cells: two-vertical axis graphs. Two-color flow cytometry using Xbp-1 and Total IgM in blood B cells over a 10-day LPS-activation period. Two-vertical axis graphs shown for three independent experiments. Repeated five times. Cells pooled from 3-5 fish in each experiment. Solid line: resting B cells (Xbp-1+/−/Total IgM+). Dashed Line: Late activated B or pre-plasma cells (Xbp-1+/+/Total IgM+).
incorporation in the blood lymphocytes, suggesting that proliferating cells are not
induced or do not survive in culture. A small population of IgM secreting cells was
seen in the blood, suggesting that there are very low numbers of plasma cells in this
tissue as seen in previous work from our lab. The lack of proliferation seen in this
study is unexpected, considering the presence of activated B cell and pre-plasma cell/
IgM-secreting cell populations (for further discussion see Chapter IV). The RAG-1
and Membrane IgM antibody combination was also investigated, but no change was
seen in the population after activation with LPS and further work needs to be done
with this combination in the blood.

3.3.3 Anterior Kidney

The anterior kidney serves as the primary immune tissue of the rainbow trout
[107] and we expected to see a large population of developing B cells characterized
as Pax-5+/RAG-1+/Membrane IgM−/Secreted IgM−/Total IgM−/Xbp-1+ present in
this tissue. In addition, the anterior kidney is thought to store antibody-secreting cells
(ASC) and we predicted that we would see IgM secreting and Xbp-1++ cells here
after LPS activation. Developing B cells are insensitive to LPS activation and if the
main population of B cells is developing B cells, then we would expect to see low
levels of activated B cells and ASCs in the anterior kidney. The anterior kidney may
also contain a population of developing non-B immune cells among the populations
of B cells.
Figure 34. BrdU incorporation and Membrane IgM expression in trout anterior kidney cells. Analysis of BrdU incorporation and Membrane IgM staining patterns in fixed and permeabilized anterior kidney cells. Contour plot of two-color flow cytometry. The non-proliferating Membrane IgM+ cells are in Box A and the Membrane IgM+ plasmablasts are in Box B, with corresponding line graphs. Results from three independent experiments. Cells pooled from 3-5 fish in each experiment.
**Proliferation.** The anterior kidney is hypothesized to contain a population of proliferating, developing B cells (pre-B). Figure 34 shows the relationship between BrdU incorporation and membrane IgM staining intensity and the contour plot for the population of proliferating B cells. The number of proliferating mature B cells (BrdU+, membrane IgM+; Figure 34, Box B) peaks between Day 4 and Day 5, while the frequency of non-proliferating mature B cells (BrdU-, membrane IgM+; Figure 34, Box A) remains fairly constant with a slight peak around Day 4.

Analysis with two-color flow cytometry identified a population of proliferating, developing B cells in the anterior kidney after LPS activation. Pax-5, Secreted IgM, and Xbp-1 levels were low in this tissue. The combinations of RAG-1/Membrane IgM and RAG-1/Secreted IgM showed no change in the cell populations over the 7-day LPS activation period. When looking at the co-expression of Xbp-1 and Total IgM, we saw a population that is high for both markers and would most likely represent plasma cells (ASC), but there was variation among experiments and the population will need to be further investigated. Based on the markers we used in this study, it appears that the B cell populations do not change in the anterior kidney after LPS activation. These are preliminary results and further investigation of the anterior kidney with these markers and other developing B cell markers is necessary to begin to identify the B cell profile of the anterior kidney.
3.3.4. Posterior Kidney

The posterior region of the kidney contains both immune and renal cells. This tissue functions mainly as a renal tissue, aiding in the removal of metabolic waste from the fish [68]. Immune cells are in the minority in the posterior kidney and there is little known about the B cell profile of this tissue. It has been hypothesized the posterior kidney may act as a secondary immune organ and that populations of activated B cells, plasmablasts, and plasma cells are found in this tissue, but there may also be populations of developing B cells.

**Proliferation.** The incorporation of BrdU into cells allowed for the identification of proliferating cells. Figure 35 shows the relationship between BrdU incorporation and secreted IgM staining intensity in Day 1 posterior kidney cells. The B cells that are secreting IgM and not proliferating are gated and such cells are most likely plasma cells (Box A). From this antibody combination, we can see that there are very few (<1%) IgM secreting plasma cells in the posterior kidney. We can also see that there are less than 1% IgM secreting plasmablasts on Day 1 in the posterior kidney (Box B).

Proliferating B cells were also observed through the co-expression of BrdU incorporation and Membrane IgM. Figure 36 shows the Day 2 contour plot highlighting the Membrane IgM+, non-proliferating B cells (Box A) and the Membrane IgM+, proliferating plasmablasts (Box B), with corresponding line graphs.
Figure 35. BrdU incorporation and Secreted IgM expression in trout posterior kidney cells. Two-color flow cytometry using BrdU incorporation and Secreted IgM markers in Day 1 fixed and permeabilized posterior kidney cells. Contour plot of Day 1 cells. Box A: non-proliferating IgM secreting cells. Box B: proliferating, IgM secreting cells. Results from one representative experiment. Experiment repeated three times. Cells pooled from 3-5 fish in each experiment. C= Control, D= Day.
Figure 36. BrdU incorporation and Membrane IgM expression in trout posterior kidney cells. Analysis of BrdU incorporation and Membrane IgM staining patterns in fixed and permeabilized posterior kidney cells. Contour plot of two-color flow cytometry. The non-proliferating Membrane IgM+ cells are in Box A and the Membrane IgM+ plasmablasts are in Box B, with corresponding line graphs. Results from three independent experiments. Cells pooled from 3-5 fish in each experiment.
A population of proliferating B cells was also detected using the Pax-5 and BrdU incorporation markers. Figure 37 shows a contour plot with a gate around the proliferating B cells (BrdU+, Pax-5+). This population decreases over the 5-day LPS-activation period.

Figure 38 compares the BrdU+/Pax-5+ (Solid Line) and BrdU+/Membrane IgM+ (Dashed Line) populations for each experiment in a two-vertical axis graph. In the 2/19/09 experiments, the number of proliferating B cells (BrdU+/Pax-5+) decreases while the number of plasmablasts (BrdU+/Membrane IgM+) increases. The populations in the 1/28/09 experiment exhibit an opposite pattern, with the BrdU+/Membrane IgM+ plasmablast population starting out high and then decreasing, while the BrdU+/Pax-5+ proliferating B cell population starts out low and increases through Day 4 of the 4-day LPS-activation period.

**Xbp-1 and Membrane IgM.** The combination of Xbp-1 and Membrane IgM allowed for the identification of resting B and activated B cells. Figure 39 shows the relationship between Xbp-1 and membrane IgM staining intensity over the 5-day culture period. Between 3-6% of posterior kidney cells express high Xbp-1 and are
Figure 37. BrdU incorporation and Pax-5 expression in trout posterior kidney cells. Two-color flow cytometry using BrdU incorporation and Pax-5 antibodies in Day 2 fixed and permeabilized posterior kidney cells. The box gates the Pax-5+ proliferating B cells. Results from one representative experiment. Experiment repeated three times. Cells pooled from 3-5 fish in each experiment. C= Control, D= Day.
Figure 38. BrdU incorporation and Pax-5/Membrane IgM expression in trout posterior kidney cells: two-vertical axis graphs. Analysis of Pax-5+ proliferating B cells and Membrane IgM+ proliferating B cells in the posterior kidney over a 5-day LPS-activation period. Line graphs representing three independent experiments. Cells pooled from 3-5 fish in each experiment. Solid line: BrdU+/Pax-5+. Dashed Line: BrdU+/Membrane IgM+.
Figure 39. Xbp-1 and Membrane IgM expression in trout posterior kidney cells. Analysis of Xbp-1+/Membrane IgM+ activated B cells/plasmablasts in fixed and permeabilized posterior kidney cells over a 5-day LPS-activation period. Results from four independent experiments. Total of six experiments. Cells pooled from 3-5 fish in each experiment.
The Membrane IgM+, non-proliferating B cell population, approximately 5-8% of all cells, does not appear to change over time while the population of Membrane IgM+ plasmablasts doubles between Day 3 and Day 4 of the 5-day LPS-activation period, increasing from <1% to approximately 1.5-2% of the total B cell population.

Membrane IgM+. This population of activated B cells/ plasmablasts (Xbp-1+, membrane IgM+) increases overall with a subtle peak in the population around Day 2 to 3 in culture.

**Xbp-1 and Total IgM.** The relationship between the staining patterns of Xbp-1 and Total IgM allowed for the identification of resting B and activated B cells based on a shift in Xbp-1 levels after activation. Figure 40 examines the relationship between Xbp-1 and Total IgM in the posterior kidney over a 5-day LPS-activation period. The contour plot for Day 0 Xbp-1/Total IgM cells highlights the resting B and activated B cell populations (Figure 40A, see boxes). Figure 40B looks at the two vertical axis graphs for the Xbp-1, Total IgM expression in the posterior kidney. A population of resting B cells (Xbp-1+/-, Total IgM+, solid line) is shown to increase in two experiments, while in a third experiment it decreases over time. The population of activated B cells (Xbp-1+, Total IgM+, dashed line) increases over time in all three experiments.

The data presented above identified populations of resting B and activated B cells, plasmablasts, and plasma cells in the posterior kidney. As discussed earlier, there is little known about the B cell profile of the posterior kidney, but in agreement
Figure 40. Xbp-1 and Total IgM expression in trout posterior kidney cells. Analysis of fixed and permeabilized posterior kidney cells over a 6-day LPS-activation period using Xbp-1 and Total IgM. A. Contour plot of two-color flow cytometry. Top box contains activated B cells and bottom box contains resting B cells. B. Line graphs for three independent experiments. Solid Line: Resting B cells (Xbp-1+/Total IgM+). Dashed Line: Activated B cells (Xbp-1+/Total IgM+). Experiment repeated four times. Cells pooled from 3-5 fish in each experiment.
with our hypothesis, we saw resting B cells that became activated B cells after stimulation with LPS. We saw small populations of plasmablasts and plasma cells, but at very low levels (0.5-3%). The combinations of RAG-1/Membrane IgM and RAG-1/Secreted IgM expression were analyzed but there was no change in the populations. Immune cells are the minority in the posterior kidney and we are just beginning to identify what types of B cells are present in this tissue. Preliminary results were presented in this section and further investigation of this tissue is necessary to identify the B cell populations present in the posterior kidney after LPS activation.

3.4. Analysis of Total IgM levels in supernatant from LPS-activated trout B cells

Based on previous work from our lab, we hypothesized that the anterior and posterior kidney would have the highest levels of IgM secretion, while the spleen would have lower levels and the blood would have little to no IgM secretion [108, 109]. In the spleen, blood, and kidney, low levels of IgM secretion were detected through two-color flow cytometry. Trout B cells were activated with LPS as part of the flow cytometry analysis, grown in culture for 24 (kidney) or 48 (spleen and blood) hours, and the supernatants were collected. To independently detect LPS-induced IgM secretion, ELISAs were done on the supernatants from the spleen, blood, and kidney B cells.
The supernatants from the spleen B cells showed an increase in detectable levels of IgM secretion upon cell culturing in the presence of LPS (Figure 41A). The anterior kidney showed variation between experiments, but in all experiments the amount of detectable IgM was lowest on Day 3 and Day 6 of the 7-day LPS-activation period (Figure 41B). In the posterior kidney, an increase in the detectable levels of IgM is seen after Day 1. On Day 3 in all experiments, the levels drop and then increase again through Day 4 or 5 (Figure 41C). In the blood, the detectable IgM levels increase from Day 2-10 of the LPS-activation period (Figure 41D). We saw low levels of increase in IgM secretion in each tissue over the culture period, which agrees with the low levels of IgM secretion seen in the two-color flow cytometry analysis.
Detectable IgM Secretion in Spleen B Cell Supernatants:

1/28/09

Days Post-Activation

2/19/09

Days Post-Activation

5/22/09

Days Post-Activation

Figure 41A. ELISA data from LPS-activated trout spleen cells. Analysis of detectable IgM secretion in supernatants from LPS-activated spleen cells. Three independent experiments shown. Repeated a total of 4 times. Samples ran in quadruplicate for each day in each experiment.
Detectable IgM Secretion from Anterior Kidney B Cell Supernatants:

1/28/09

![Graph showing Absorption over Days Post-Activation for 1/28/09](image)

2/19/09

![Graph showing Absorption over Days Post-Activation for 2/19/09](image)

5/22/09

![Graph showing Absorption over Days Post-Activation for 5/22/09](image)

Figure 40B. ELISA data from LPS-activated trout anterior kidney cells. Analysis of detectable IgM secretion in supernatants from LPS-activated anterior kidney cells. Three independent experiments shown. Repeated a total of 4 times. Samples ran in quadruplicate for each day in each experiment.
Detectable IgM Secretion from Posterior Kidney B Cell Supernatants:

**1/28/09**

![Graph for 1/28/09]

**2/19/09**

![Graph for 2/19/09]

**5/22/09**

![Graph for 5/22/09]

**Figure 40C. ELISA data from LPS-activated trout posterior kidney cells.** Analysis of detectable IgM secretion in supernatants from LPS-activated posterior kidney cells. Three independent experiments shown. Repeated a total of 4 times. Samples ran in quadruplicate for each day in each experiment.
Detectable IgM Secretion in Blood (PBL) B Cell Supernatants:

1/28/09

![Graph for 1/28/09 showing relative absorption over days post-activation.]

3/27/09

![Graph for 3/27/09 showing relative absorption over days post-activation.]

5/22/09

![Graph for 5/22/09 showing relative absorption over days post-activation.]

Figure 40D. ELISA data from LPS-activated trout blood cells. Analysis of detectable IgM secretion in supernatants from LPS-activated blood cells. Three independent experiments shown. Repeated a total of 4 times. Samples ran in quadruplicate for each day in each experiment.
As described by Zapata et al [107], trout lack bone marrow and typical lymph nodes and utilize the kidney, spleen, and blood for B lymphocyte development, activation, and differentiation. Through this thesis project, our aim was to identify B cell populations within the trout anterior and posterior kidney, spleen, and blood ex vivo in freshly isolated and LPS-activated cells. In agreement with our hypothesis, the majority of the lymphocyte population in freshly isolated spleen cells consisted of resting B cells. After LPS-activation, we saw a decrease in the frequency of resting B cells accompanied by an increase in the frequency of activated B cells, plasmablasts, or plasma cells. The blood, like the spleen, was found to contain a large population of resting B cells in freshly isolated cells. As hypothesized, following LPS-activation the frequency of resting B cells decreased as the frequency of activated B cells increased. No proliferation was detected in this tissue, but a small population of low-IgM secreting ASCs was found in the blood both before and after LPS-activation. The anterior kidney was found to contain primarily a population of ASCs and developing B cells, with few resting B cells seen. However, the anterior kidney is a complex tissue containing various immune cells at different stages of development, and populations of B cells were difficult to detect in this tissue. The posterior kidney was found to contain a population of resting B cells that decreased in frequency after LPS-activation while a population of activated B cells increased in frequency.
B cells were analyzed with two-color flow cytometry using combinations of BrdU incorporation, Pax-5, RAG-1, Membrane IgM, Secreted IgM, Total IgM, and Xbp-1 markers. After testing various combinations of these markers, we determined that BrdU, Membrane IgM, Total IgM, and Xbp-1 provide the most information about the B cell populations in the immune tissues of the rainbow trout. The expression levels of these markers in resting B cells, activated B cells, plasmablasts, and plasma cells are summarized in Table 3.

**Optimal B cell marker combinations for characterizing B cell activation and differentiation stages**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>BrdU Incorporation</th>
<th>Membrane IgM</th>
<th>Total IgM</th>
<th>Xbp-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting B</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>Activated B</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Plasmablast</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Plasma Cell</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3. Summary of the most informative B cell markers combinations from this thesis research. The BrdU, Membrane IgM, Total IgM, and Xbp-1 markers were the most successful in providing information about the B cell populations present in the trout immune tissues before and after LPS-activation. This table and the following Discussion focus mainly on the frequencies of the resting B cell, activated B cell, plasmablast, and plasma cell populations, as characterized by the phenotypes presented in Table 3.

4.1. The transcription factor Xbp-1, together with Membrane IgM or Total IgM, provide highly reliable markers for B cell activation.

Xbp-1 is expressed in early developing B cells and during the terminal stages of B cell differentiation, with the highest levels of Xbp-1 expression seen in plasma cells [77, 91]. In this thesis research, we used Xbp-1 intensity in B cell populations, in combination
with either Membrane IgM or Total IgM expression, to measure B cell responses in vivo. Spleen, blood, and posterior kidney cultures stimulated with LPS showed an increase in the levels of Xbp-1 protein per cell, and this phenotypic change correlates with B cell activation. We propose that resting B cells are Xbp-1+/- / Membrane IgM+/ Total IgM+, activated B cells are Xbp-1+/ Membrane IgM+/ Total IgM+, plasmablasts are Xbp-1+/ Membrane IgM+/ Total IgM++, and plasma cells are Xbp-1++/ Membrane IgM−/ Total IgM++. As resting B cells become activated B cells, we see an increase in the amount of Xbp-1 protein per cell. The Xbp-1 expression levels increase further as the cells differentiate into ASCs.

In contrast, B cells in the anterior kidney showed no change in the levels of Xbp-1/Membrane IgM or Xbp-1/Total IgM expression before or after LPS-activation. This suggests that the majority of the B cell population in the anterior kidney consists of early developing B cells, which are insensitive to LPS. Interestingly, there is a population of Xbp-1++, Total IgM++ ASCs present in the anterior kidney in fresh Day 0 cells. However, this population remains unchanged throughout the 7-day LPS-activation period, suggesting that plasmablasts and plasma cells are insensitive to LPS, but do not undergo cell death during culture. In agreement with these data, ELISA data from LPS-activated anterior kidney cells showed no consistent change in the amount of IgM produced by these cells over the culture period.

In freshly isolated cells from the spleen and blood, the great majority of the B cell population consisted of resting B cells based on the Xbp-1+/-, Membrane IgM+ or Xbp-
1+/-, Total IgM+ phenotype. After LPS-activation, there was an increase in the amount of Xbp-1 protein per cell, as the cells progressed from resting B cells to activated B cells (Xbp-1+, Membrane IgM+ or Xbp-1+, Total IgM+). A population of ASCs (Xbp-1++, Total IgM++) that was present at a low frequency (0.8-2%) in freshly isolated spleen cells continued to increase in frequency after LPS-stimulation. This population only reached a frequency of 3-5% by Day 10 of the culture period. This suggests that B cell activation in the trout is a longer process as compared to mammals, whose plasma cell population is induced by Day 4 after antigenic stimulation and reaches a frequency of 30% at this time [91, 100]. When grown in culture, mouse cells receive one-fifth the amount of LPS that trout cells receive. In addition, trout cells are maintained in culture at 18°C, while mouse cells are grown at 37°C. The cooler temperature may, in part, account for the difference in the timing of activation between the trout and mammalian system.

In a previous study from our lab [109], as well as in this research, we saw very low frequencies of both plasmablasts and plasma cells in the trout immune tissues, in agreement with the possibility that B cell activation may take longer in the trout.

The increase in the frequency of the ASC population is in agreement with ELISA data from the LPS-activated spleen cells. The ELISA data showed an increase in IgM production over the 10-day culture period in further support of the increasing ASC population in the spleen. In addition, PCR data from our lab support these data as well. They show an increase in the secreted IgM to membrane IgM RNA transcript ratio over the LPS-activation period (Day 0-7), hence at the RNA level, splenic B cells are
producing more secreted IgM transcripts than IgM transcripts after LPS-activation (Raaj Talauliker, unpublished data).

In the blood, we saw a general decrease in the frequency of the resting B cell population over the 10-day LPS-activation period. Accompanying this was induction of an ASC population (Xbp-1++, Membrane IgM+/−) by Day 2 following LPS stimulation. This population peaked on Day 4 and decreased through Day 10 of the culture period, suggesting that these cells may be dying in culture, as observed through other antibody combinations in this study. However, a unique population of IgM-secreting, Membrane IgM− cells was observed in freshly isolated blood cells, suggesting the presence of plasma cells in this tissue. This population is highest on Day 0 and decreases in frequency following LPS-activation, but this population is never completely lost over the 10-day culture period. The loss of cells in this population suggests that these plasma cells are undergoing cell death after LPS-activation. It is possible that a few of these plasma cells survive the LPS and maintain the population or it may be that new plasma cells are produced that take their place.

In partial agreement with the data presented here, ELISA data from LPS-activated blood B cells showed an increase in IgM production over the 10-Day culture period. While we did not see an increase in the plasma cell/ASC population during the culture period, the accumulation of the antibody from the low IgM-secreting cells may be the reason for the increase in antibody production seen through the ELISA data. PCR data from our lab have shown no significant increase in the secreted IgM to Membrane IgM
RNA transcript ratio over the LPS-activation period (Day 0-7; Raaj Talauliker, data not published). This suggests that B cell activation may be a slow process in blood cultures. Future experiments in trout blood with these markers and apoptosis markers are required to better understand the ASC population in this tissue.

In agreement with previous work with trout immune tissues, this study found resting B cells, activated B cells, and ASCs in the spleen and blood of the trout [8, 109]. However, this study has, for the first time, enabled the detailed analysis of B cell responses as defined by the frequency of activated B cell, plasmablast, and plasma cell populations upon LPS-induction through flow cytometry. Combinations of Xbp-1 and Membrane IgM or Total IgM, based on the phenotypes presented here, can be used to assess the immune status of fish.

4.2. BrdU incorporation can be used to identify proliferating B cells in trout immune tissues.

The rate of proliferation and the frequency of proliferating B cells in the trout immune tissues are unknown. Previous work has shown that the trout spleen, anterior kidney, and blood contain populations of ASCs that are sensitive to the DNA replication inhibitor hydroxyurea (HU), suggesting that these cells are plasmablasts [8]. An earlier study from our lab [108] used a percoll-gradient to identify plasmablasts (P60 layer) in the trout immune tissues. The kidney (anterior and posterior regions) was found to contain the largest number of proliferating cells, while low levels of proliferating cells
were seen in the P60 layer of the spleen and blood. The anterior kidney was the only tissue to show significant proliferation in the P70 layer, which would include proliferating early B cells, as expected in this hematopoietic tissue [108].

Bromage et al [8] looked at total cell proliferation in the spleen, blood, and anterior kidney, while Zwollo et al [108] looked at percoll-purified cells. Neither study included a B cell specific marker to determine if the proliferating cells were B cells. This thesis work used BrdU incorporation in combination with the B cell-specific markers Membrane IgM, Secreted IgM, and Pax-5 to identify proliferating B cells in LPS-activated spleen, blood, and kidney cell populations. Through flow cytometric analyses, frequencies of proliferating B cells were determined for each of the trout immune tissues.

This study found proliferating B cells in the spleen, anterior kidney, and posterior kidney of the trout. Proliferating B cells were found in the spleen using all three marker combinations. The population of proliferating B cells that are Pax-5+ are most likely plasmablasts, but may also be pro- or pre-B cells. As a secondary immune organ, we expect the spleen to contain mostly resting B or activated B cells, plasmablasts, and plasma cells [8, 108, 109]. Therefore, it is less likely that the proliferating B cells in the spleen are developing B cells. This population peaks between Day 4 and Day 6 at about 1%. We see variation among the experiments with this combination. This could be due to the variation in Pax-5 expression in this tissue (see sections 4.2 and 4.3). The variation in Pax-5 levels could be attributed to alternative splicing of Pax-5, which our lab has found in the spleen (Raaj Talauliker, data not published). If alternative isoforms of Pax-5 are
present in the splenic B cells, then our Pax-5 (ED-1) antibody may not bind all of the Pax-5 expressed in those cells.

Populations of Membrane IgM+, IgM-secreting plasmablasts are found in the spleen. These populations peak between Day 4 and Day 6, just as the Pax-5+ proliferating cells did. The plasmablast population in the spleen is very small, with less than 2% of plasmablasts in the spleen in all cases.

The anterior kidney contained the highest frequency of proliferating B cells. The BrdU incorporation/Membrane IgM combination identified a population of proliferating IgM+ cells that peaks between Day 4 and Day 6. These cells are most likely plasmablasts, but could also be pre-B cells. The anterior kidney contains a population of ASCs, including plasmablasts, that is seen with and without LPS stimulation [8, 108]. Further investigation with tri-color flow cytometry including BrdU incorporation, Membrane IgM, and Secreted IgM or an early B cell marker, such as EBF, would allow for the distinction between plasmablasts and pre-B cells.

Low levels of Pax-5 and Secreted IgM were detected in the anterior kidney, and in combination with BrdU incorporation, we found very low frequencies of Pax-5+ proliferating cells and IgM secreting plasmablasts (<1% for both combinations) over the 7-day LPS-activation period (data not shown). As discussed above, the low levels of Pax-5 may be attributed to alternative splicing. BrdU incorporation is strong in anterior
kidney cells and further investigation with different Pax-5 antibodies may provide more information about the proliferating B cell population in the anterior kidney of the trout.

A population of plasmablasts was found in the posterior kidney using all three combinations. This is in agreement with previous work from our lab that found plasmablasts through percoll-density experiments [108]. The data from this research indicates that there are <1% IgM-secreting plasmablasts and Pax-5+ proliferating B cells, while there is a slightly higher frequency of Membrane IgM+ plasmablasts (1.5-2%) in the posterior kidney.

BrdU incorporation was a successful marker for cell proliferation in trout spleen, anterior and posterior kidney. The combination of BrdU incorporation with Membrane IgM proved to be the best for identifying proliferating B cells in these immune tissues. Proliferating B cells were identified in the spleen, anterior kidney, and posterior kidney at low frequencies. Trout B cells may proliferate slower than we expect and we could be missing the proliferative phase or there may be a low frequency of proliferating B cells in response to antigenic stimulation in the trout immune tissues.

The low-levels of Secreted IgM seen through flow cytometry in this study may be attributed to the loss of the Secreted IgM protein from the cell. As the cells are fixed and permeabilized, the protein may diffuse out of the cells and be lost. Future studies using Golgi-stop to prevent the Secreted IgM protein from leaving the Golgi apparatus, keeping
it in the cell, may allow for better results and provide more information on the ASC populations, including plasmablasts, in the trout immune tissues.

BrdU incorporation was analyzed in LPS-activated blood cells as well. There was little to no proliferation detected (0.1%) in this tissue during the 10-day culture period. This is in agreement with recent work from our lab that found low levels of activated B cells and plasmablasts in the blood before and after LPS-activation [109]. However, plasmablasts were found in the trout blood in two previous studies [8, 108]. Both studies looked at total immune cell populations. Bromage et al [8] looked at total cell proliferation in the blood using hydroxyurea (HU), while Zwollo et al [108] looked at percoll-purified cells.

The data from this study disagree with the blood proliferation data presented by Bromage et al [8] and Zwollo et al [108]. In the current study, we found few to no plasmablasts in the blood during the 10-day LPS-activation period. This study used BrdU incorporation in combination with the B cell-specific markers Membrane IgM, Secreted IgM, and Pax-5 to identify proliferating B cells in a heterogeneous mixture of immune cells. It is possible that there were too few plasmablasts/proliferating cells present in the blood to be detected with this method. It is also possible that, as discussed above, the blood cells may be dying in culture due to LPS-activation or lack of cytokines necessary for survival. In order to determine if there are proliferating B cells present in the blood, future studies with BrdU incorporation need to be conducted. Apoptosis studies should also be considered to determine if the blood B cells are dying before they are able to
proliferate, which would help to explain the lack of plasmablasts and the few plasma cells seen in this tissue.

4.3. The anterior kidney contains a low number of LPS-responsive mature B cells.

The anterior kidney of the rainbow trout is a complex organ with a heterogeneous immune cell population. As the primary site of hematopoiesis, the anterior kidney is expected to contain early developing B cells. Studies have reported that a population of ASCs exists in the anterior kidney as well [8, 108, 109]. Developing B cells are insensitive to LPS-activation. Therefore, if the majority of B cells in the anterior kidney are developing B cells, then there will be little to no change in the population after LPS-activation.

It has been well documented that RAG-1 is expressed in developing B cells in mammalian bone marrow [67, 87] and as the site for B cell development we expect to see RAG-1 expressing cells in the anterior kidney of the rainbow trout. RAG-1 expression has been documented in the anterior kidney of both the zebrafish (Danio rerio) [103] and the carp (Cyprinus carpio) [40], suggesting that developing B cells are present in the anterior kidney of these fish. Developing B cells have not been investigated or well defined in the trout immune tissues. Future experiments using two-color flow cytometry with RAG-1 and other early B cell markers such as Pax-5, EBF, and Ikaros, would provide insight into the developing B cell populations in the trout anterior kidney.
In this study, as well as in previous work from our lab [109], it was found that the anterior kidney contains very few resting, mature B cells. This would be in agreement with the anterior kidney containing mostly developing B cells, as discussed above. Resting, mature B cells (IgM+) are classified as Pax-5+/Membrane IgM+/Secreted IgM−/Total IgM+/Xbp-1+/-.. Through flow cytometric analyses, we found 1%< resting B cells in the anterior kidney, based on this phenotype. The previous study from our lab analyzed purified Membrane IgM+ B cell populations from the anterior kidney, finding no resting, mature B cells [109].

It has been proposed that as the developing B cells in the anterior kidney reach maturity, they leave the anterior kidney for the posterior kidney or the blood and eventually travel to the spleen ([108], also see Figure 4 in Chapter 1). The results from this thesis research support this hypothesis in that few resting B cells are found in the anterior kidney while the posterior kidney, blood, and spleen contain high numbers of resting, mature B cells.

4.4. Unexpected low levels of Pax-5 expression in B cells of the anterior and posterior kidney.

In mammals, Pax-5 is expressed from the pre-B cell through plasmablast stages, where levels begin to decrease after activation [1]. Surprisingly, in the trout kidney, we observed very low levels of Pax-5 expression, which was also observed in earlier RT-PCR experiments from our lab [109]. We hypothesize that the anterior kidney B
lymphocyte population consists mainly of developing B cells, while the B lymphocyte population in the posterior kidney would contain mostly resting and activated B cells. If this were the case, we would expect to see higher levels of Pax-5 expression in both regions of the kidney.

The low levels of Pax-5 expression in the trout kidney may be due to alternative splicing. Work done in our lab has found extensive alternative splicing of Pax-5 in the trout, with different isoforms present depending on the developmental and activation state of the B cells (P. Zwollo and R. Talauliker, data not shown). If alternative isoforms of Pax-5 are present in the kidney after activation, then our Pax-5 (ED-1) paired-domain antibody may not bind to the isoforms, leaving their presence undetected. Further investigation with antibodies directed against the Pax-5 isoforms found in the kidney is necessary to understand the Pax-5 expression patterns in the trout anterior and posterior kidney.

4.5. A population of Pax-5+, Membrane IgM− cells suggests the presence of IgD+ or IgT+ B cells in the spleen and blood.

A previous study from our lab, which used density gradients to separate B cell populations, found that the majority of the freshly isolated spleen cells were present in the high-density P70 layer (resting B cells), while very few cells were present in the intermediate density P60 (activated B cells or early plasmablasts) or low-density P50 (late plasmablasts or plasma cells) layers [108]. Much like the spleen, prior to LPS-
activation, the majority of blood B cells are present in the P70 layer [108]. In agreement with the percoll-density study, flow cytometric and qPCR data from our lab have shown that freshly isolated B cells from the spleen are mostly in a resting state [109]. This work has found the majority of freshly isolated B cells from the spleen, as well as the blood, are resting B cells, characterized as Pax-5+, Membrane IgM+.

After LPS-activation, the frequency of the resting B cell population decreases in both the spleen and the blood. We saw variation in the number of Pax-5+, Membrane IgM+ resting B cells in our experiments, but this population consistently decreases over the 10-day LPS-activation period in all experiments. This correlates with previous findings that B cells lose their Pax-5 and Membrane IgM expression after LPS-activation [109].

Interestingly, a portion of these resting B cells is characterized as Pax-5+, Membrane IgM− through flow cytometric analyses, suggesting that these cells may be IgD+, IgT+, or, less likely, pro-B cells. In the previous study [109], this population of resting B cells, characterized as Pax-5+, Total IgM− was highest in frequency on Day 0 and then decreased after LPS-activation, in agreement with the data from the current study. Freshly isolated blood B cells show a similar population of Pax-5+, Membrane IgM− cells that decrease in frequency after Day 2 of the LPS-activation period. In both the spleen and the blood, the cells are most likely resting IgD+ or IgT+ cells that become activated B cells after stimulation. Future studies with IgD or IgT antibodies, when they
become available, are necessary to determine if the Pax-5+, Membrane IgM- population consists of resting B cells of a different Ig isoform or if they are pro-B cells.

In the blood, it is likely that the Pax-5+, Membrane IgM- cells become activated B cells after LPS-stimulation. However, it is possible that these resting B cells may undergo cell death in culture. As this research and previous work has shown, the blood contains very few plasmablasts and plasma cells, suggesting that the B cells in this tissue do not progress past the activation stage [108, 109]. We see a large portion of the blood B cell population die during the culture period (data not shown). The cells may undergo apoptosis due to LPS-activation or due to lack of cytokines necessary to sustain the B cells in culture. The lack of plasmablasts and low levels of IgM secretion suggest that the blood B cells are dying after LPS-activation.

The combination of Pax-5 and Membrane IgM markers, when analyzed by two-color flow cytometry, provide information about the activation state of B cells as we characterize resting B cells as Pax-5+, Membrane IgM+, activated B cells as Pax-5+, Membrane IgM++, plasmablasts as Pax-5+/−, Membrane IgM+, and plasma cells as Pax-5−, Membrane IgM−. The changes in the expression levels of Pax-5 and Membrane IgM correlate with the progression through the later B cell stages where we see Pax-5 and Membrane IgM expression decrease as the cells differentiate into plasma cells.

The information provided by the Pax-5 and Membrane IgM expression patterns may help to assess the health of fish. In teleosts, the spleen becomes enlarged during an
infection, providing an indicator as to the immune state of the fish. Analyzing splenic B cells through flow cytometry may provide a tool to determine immune activation prior to the enlargement of the spleen. The data provided by the Pax-5 and Membrane IgM markers in this study show that, by looking at the relative levels of Pax-5 low (+/-)/Membrane IgM+ cells, Pax-5+/Membrane IgM+ cells, and Pax-5+/Membrane IgM− cells in freshly isolated spleen cells, the activation state of the fish immune system could be assessed.

4.6. A novel Pax-5 low (+/-), Membrane IgM+ B cell population is found in the spleen.

The co-expression of Pax-5 and Membrane IgM, as analyzed by flow cytometry, identifies populations of either resting B cells or activated B cells/plasmablasts, within a cell population. A population of Pax-5 low (+/-), Membrane IgM+ cells was found in freshly isolated and LPS-activated spleen cells. There was variation as to the frequency of this population between three independent experiments. The B cell stage of these Pax-5 low, Membrane IgM+ cells is not clear. These cells may be activated B cells that are already decreasing their Pax-5 expression levels. In mammals, Pax-5 is expressed from the pre-B cell through mature B cell stages and activated B cells begin to lose their Pax-5 expression [1]. As a site of B cell activation, we expect to see lower levels of Pax-5 expression in splenic B cells. This novel population, found exclusively in the spleen, suggests that splenic B cells may become activated faster than B cells in the blood or kidney, if these cells are indeed activated B cells.
It is possible that our Pax-5 (ED-1) antibody is not binding all of the Pax-5 expressed in the spleen. Our lab has found extensive alternative splicing of Pax-5 in the spleen (data not shown), which could explain the presence of B cells (Membrane IgM+) with little to no Pax-5 expression. These cells may be expressing higher levels of Pax-5, making them resting B cells instead of activated B cells. This is why it is unclear at this point what type of B cells these Pax-5 low (+/-), Membrane IgM+ cells are and future experiments with Pax-5 antibodies that recognize the alternatively spliced isoforms are necessary.

4.7. LPS-induction of a RAG-1+, IgM-secreting B cell population in the spleen and blood.

The Recombination Activating Genes (RAG-1 and RAG-2) are expressed in pro- and pre-B cells as the Ig genes undergo V(D)J recombination [29, 67, 87]. Low levels of RAG expression have also been found in immature, resting B cells, but as membrane IgM levels decrease in these cells, RAG expression decreases [106]. RAG expression has been documented in peripheral lymphoid organs after antigenic stimulation, suggesting that mature B cells also express RAG [37, 69]. It has also been shown that immature B cells expressing RAG travel to the spleen after antigenic stimulation, accounting for the RAG expression seen in peripheral organs [61]. In this thesis research, RAG-1 was used in combination with Secreted IgM in order to distinguish between developing B cells and activated B cells.
Flow cytometric analysis of RAG-1 and Secreted IgM staining revealed interesting expression patterns in both the spleen and the blood. Surprisingly, there is induction of a RAG-1 expressing, IgM secreting B cell population after LPS-activation in both tissues. There is variation between experiments in the number of cells with this phenotype, but this population increases in both RAG-1 and Secreted IgM levels over the 10-day culture period. This population most likely consists of activated B cells or plasmablasts. As this study was conducted \textit{ex vivo} and the spleen and blood are secondary immune organs, it is likely that we are seeing expression of RAG-1 in mature, activated B cells, in agreement with mammalian studies [37, 69]. The re-expression of RAG-1 in these cells may accompany class switch recombination after activation in these cells. It has been shown that in addition to IgM, trout B cells produce IgT and IgD ([32, 104], respectively). The re-expression of RAG-1 in the activated B cells/ASCs of the spleen and blood suggests that these cells are undergoing the process of class-switch recombination. At this point there are no antibodies available for trout IgD or IgT, but future work with these antibodies and PCR would provide information on class-switch recombination in these cells.

\textbf{4.8. Conclusions, significance, and future work.}

The data from this thesis work provide information on the development, activation, proliferation, and differentiation of B cells in trout immune tissues. Previous work from our lab has looked at these processes in purified B cell populations [108, 109],
while this study analyzed individual cells from trout spleen, blood, and kidney _ex vivo_.

The flow cytometric analyses in this thesis research expand upon the knowledge of the B cell populations present in trout immune tissues through the use of multiple B cell markers measured in individual cells. This method allows us, for the first time, to obtain information on the frequencies of the various B cell populations present in trout immune tissues.

As the demand for fish as a food source makes aquaculture an increasing necessity, the need to understand the teleost immune system becomes an important focus for immunologists. Grown in close proximity to one another, farm-raised fish, especially at the juvenile stage, are highly susceptible to disease and infection. There is an increasing need for vaccines for farm-raised fish; however, the immune system of teleosts is poorly understood, making vaccine development difficult. Identifying sites of B cell activation and antibody secretion can provide target sites for vaccines.

The method and markers used in this project provide preliminary information for the future use of flow cytometry as a diagnostic tool for evaluating immune system activation. As technology continues to evolve, flow cytometry will become more economical and accessible, providing a practical machine to use when analyzing not only fish, but vertebrate health. B cells and their transcription factors have been extensively studied in the mammalian immune system, but little is known about the teleost immune system. The data presented here provide insight into the activation state of B cells in the trout by analyzing B cell marker combinations before and after LPS-activation. The
changes in the expression patterns of the B cell populations can provide markers as to the activation state of the trout immune system and provide information on trout health. These markers and methods, however, have the potential to be used in monitoring immune responses in all vertebrates, as the same cell types and markers are found in mammals and teleosts.

Further investigation of the B cells within trout immune tissues is necessary to better understand the processes of B cell development, activation, and differentiation. Studies with apoptosis markers are necessary to determine the cell death rates in the blood and spleen and experiments with early B cell markers are necessary to identify the B cell populations present in the anterior kidney. As additional B cell markers are developed and tested, future studies can provide further information about the complex B cell populations present in the immune tissues of rainbow trout and other teleosts, both before and after antigenic stimulation. Eventually, flow cytometry may be used to determine vaccine efficiency in immunized fish. Or perhaps, as mentioned previously, specific B cell marker combinations could be analyzed using flow cytometry to determine the immune state of a fish. This method has the potential to not only monitor fish health, but could possibly identify an infection or immune reaction prior to a visible sign. This thesis work provided a starting point for further investigation of the B cell populations present in the trout immune system. Future studies will continue to provide information on the functioning of the teleost immune system and lead to the development of vaccines and treatments to protect these important aquaculture species.
REFERENCES


150


cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. *Developmental and Comparative Immunology* 32:1482-1496.

Vita

Maggie Barr was born in Vienna, Virginia but spent most of her life living in south Florida. She attended the University of Florida where she received a Bachelor of Science in Animal Biology. During her time at the University of Florida, Maggie worked as a veterinary technician at the Micanopy Animal Hospital for two years. Here she gained valuable experience in animal care as well as routine handling of blood and specimens for laboratory testing. Her time as a veterinary technician afforded Maggie the realization that she was interested in immunology and pathology, particularly from a research standpoint.

After graduating from the University of Florida in August of 2007, Maggie was accepted into the Masters program in the Biology Department at the College of William and Mary. The thesis work presented here was conducted over a two-year period from September 2007 to July 2009. Maggie defended her thesis work on November 23, 2009. At the completion of her Masters education, Maggie hopes to pursue a career as a research scientist in the field of immunology.