Identification and characterization of antigen retaining cells in the putative germinal centres of goldfish (*Carassius auratus*)

by

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ABSTRACT

Follicular dendritic cells (FDCs) present within the germinal centres (GCs) capture the antigen in the form of an immune complex on their cell surface and facilitates the selection of high-affinity antibody-producing B-cells. Recruitment of B-cell to GC and the survival of B-cells within GC is regulated by FDCs. Production of cytokines by FDCs facilitates the clearance of apoptotic cells within GC, thereby preventing the production of auto-reactive B-cells.

Fish were initially thought to lack antibody affinity maturation (AAM) partially because they lack a histologically distinct germinal centre. Later studies in channel catfish showed the presence of fully functional AID (activation-induced cytidine deaminase – mutator enzyme required for AAM) co-localized among aggregates of immune cells referred to as melanomacrophage clusters (MMCs). Studies have shown that experimentally injected antigen gets trapped in and around the MMCs. Based on the similarities between MMs and GCs we hypothesized that MMCs in fish could be analogues to mammalian GCs.

The goal of this work was to identify FDC-like cell type in fish lymphoid organs. Goldfish immunization experiments and mRNA based transcriptome analysis revealed pigmented melanomacrophages (MMs) in fish have functional similarities to mammalian FDCs.

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A	Alpha/anti
Ab	Antibody
Ag	Antigen
APS	Ammonium persulfate
AAM	Antibody affinity maturation
AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cell
BSA	Bovine serum albumin
B	Beta
BAFF	B-cell activating factor
BLIMP1	B-lymphocyte-induced maturation protein 1
BLAST	Basic local alignment search tool
BCR	B-cell receptor
BS^3	Bissulfosuccinimidyl suberate
CSF-1R	Colony-stimulating factor 1 receptor
CSR	Class switch recombination
CDR	Complementarity determining regions
CD38	Cluster of differentiation 38
CD40	Cluster of differentiation 40

CD40L	Cluster of differentiation 40 ligand
CXCL13	C-X-C Motif Chemokine Ligand 13
CXCR5	CXC-Chemokine receptor 5
Cy5	Cyanine-5 channel
CRs	Complement receptors
Δ	Delta
D	Diversity
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
E	Epsilon
FR	Framework region
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDCs	Follicular dendritic cells
FITC	Fluorescein isothiocyanate channel
FcRs	Fragment crystallizable receptors
Γ	Gamma
GC	Germinal centre(s)
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IC	Immune complex

IRF4	Interferon Regulatory Factor 4
IP	Immunoprecipitation
J	Joining
K	Kappa
KLH	Keyhole limpet hemocyanin
Λ	Lambda
M	Mu
MFGE8	Milk fat globule-epidermal growth factor-factor 8
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MMR	Mismatch repair machinery
MM	Melano-macrophage
MMC	Melano-macrophage cluster
MWCO	Molecular weight cutoff
NK	Natural killer cells
PKMs	Primary kidney macrophages
PAMP	Pathogen-associated molecular pattern(s)
PE	Phycoerythrin
PBLs	Peripheral blood leukocytes
PBS	Phosphate buffer saline
PIgRs	Polymeric immunoglobulin receptors
PCR	Polymerase chain reaction

RNA	Ribonucleic acid
RAG	Recombination-activating genes
RSS	Recombination signal sequences
RANKL	Receptor activator of nuclear factor-kappa-B ligand
SHM	Somatic hypermutation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T_{FH}	Follicular helper T-cell
T_H	Helper T-cell
TBM	Tingible body macrophage
TMS	Tricane methanosulfonate
TEMED	Tetramethylethylenediamine
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate channel
UNG	DNA glycosylase
V	Variable
VDJ	Variable, diversity and joining segment
V_L	Variable region in immunoglobulin light
V_H	Variable region in immunoglobulin heavy
WCL	Whole-cell lysate
XBP1	X-box binding protein 1

Chapter 1. Introduction

1.1 Overview

The adaptive arm of immune response is initiated within days following the initial encounter with the pathogen. B-cells are key players in adaptive immunity and function by producing immunoglobulins (Igs) or antibodies (Abs), which binds to and neutralize the target pathogen. Igs adapt to recognize the pathogen by acquiring random point mutations in the antigen recognition site of the antibody, which is mediated by a mutator enzyme activation-induced cytidine deaminase (AID), also referred to as somatic hypermutation (SHM) (Kuraoka et al., 2010). In mammals and birds, these processes occur within specialized sites in the secondary lymphoid organs referred to as germinal centres (GCs). These are sites where B-cells proliferate, acquire Ig specific mutations, and differentiate to become antibody-producing plasma cells or long-lived memory cells. Some of these mutations result in the improved affinity of Igs to an antigen and are hence selected for (Reviewed in Kurosaki et al., 2015).

In higher vertebrates, follicular dendritic cells (FDCs) are present in the light zone of GC. FDCs facilitate the B-cell selection process within GC by capturing the unprocessed antigen as an immune complex (IC) on the cell surface and by presenting it to B-cells through FcRs (Fragment crystallizable receptors) or CRs (complement receptors) (Reviewed in Kranich & Krautler, 2016).

B-cells with acquired mutations compete for the antigen captured on the surface of FDCs. Successful B-cells internalize the antigen process and present it to T helper cells (T_H cells) to receive survival signals. In contrast, unsuccessful B-cells undergo apoptosis. Dysregulation or overexpression of FDCs outside lymphoid tissues, such as in the synovial membrane, salivary glands, or skin in response to the microenvironmental inflammatory signals can lead to autoimmune diseases (Berek & Kim, 1997).

Fish were initially thought to lack antibody affinity maturation process partially because they lack histologically distinct GC (Good and Finstad 1967; Manning and Horton 1982; Pitchappan 1980) and due to the absence of high-affinity antibody in circulation following immunization in early studies (Lobb, 1985). However, later work done in sharks showed that they have a novel way of class switch recombination (Zhu et al., 2012; Zhang et al., 2013) and can produce highly antigen-specific IgNAR response (Dooley & Flajnik, 2005). Work done in our lab showed that cells expressing fully functional AID were colocalized among aggregates of immune cells referred to as melanomacrophage clusters (MMCs) in the spleen and kidney of channel catfish (Saunders et al., 2010). These MMCs consisted of IgM expressing B-cells, T_H cells, and reticular cell stroma, a combination of cells seen in conventional GCs (Saunders et al., 2010).

Putting these together, we hypothesized that MMCs in fish could be analogous to GCs in mammals. To test this hypothesis, ongoing work in our is looking at different characteristics of GCs in MMCs, such as the clonal expansion of B-cells, SHM, and Selection. My work focuses on the selection part by looking for a specific cell type with functional similarities to FDCs in fish.

Past studies have shown that MMCs are capable of phagocyting and retaining antigens such as bacteria (Johnson et al., 1999). Melanomacrophages (MMs) found within the MMCs are phagocytic cells known to accumulate auto-fluorescent pigment in them over time (Reviewed in Agius and Roberts 2003). And reticular cells have a close lineage relation to mammalian FDCs (Bofill et al., 2000). Based on these, we hypothesized either MMs or reticular cells in fish could have functional similarities to mammalian FDCs.

To identify the antigen retaining cell, we injected fish with various proteins (Bovine serum albumin and Keyhole limpet hemocyanin) conjugated to a fluorescent tag Alexa fluor 647. We used confocal microscopy to identify the specific cell type retaining the injected protein. Preliminary results suggested that MMs were involved in antigen uptake, and further experiments were done to confirm the presence of intact antigen on the surface of MMs isolated from immunized fish. Protein profiling through mass spectrometry and mRNA-based transcriptome analysis was done on MMs to look for putative FDC genes and to understand their role in MMCs.

1.2 Adaptive immunity

Our immune system can recognize and respond to a foreign body on the encounter. They recognize the molecular pattern which characterizes a group of common pathogens leading to effector response, each uniquely suited for a single pathogen. At the same time, they differentiate self from a foreign particle. The effector response gets stored as a memory resulting in more rapid and heightened response in the subsequent encounter (Kindt et al., 2006).

A substance capable of initiating an adaptive immune response is referred to as an antigen. Many laboratory experiments involve tricking our immune system into believing there is an invasion by a pathogen. It is done by injecting the animal model with a harmless molecule along with immunostimulants, usually referred to as adjuvants, which activate the innate immune system. This process is called immunization (Gahan, 2005).

Antigen receptor-bearing lymphocytes constitute 20-40 % of the body's white blood cells and are central players of adaptive immunity. Lymphocytes continuously circulate in blood and lymph and can migrate into tissue spaces and lymphoid organs, acting as a bridge between the different parts of the immune system (Kindt et al., 2006). Lymphocytes are further subdivided into B-cells, T-cells, and natural killer cells (NK cells) based on their primary functions and cell membrane components. B and T-cells each have their own distinctive family of antigen receptors, whereas NK-cells, which are a part of the innate immune system does not express the set of surface markers that characterize B or T-cells (Kindt et al., 2006).

Bone marrow is the primary site where B-cell maturation occurs in several mammalian species occurs, including humans and mice. B-cells migrate from bone marrow to secondary lymphoid organs such as the spleen, lymph nodes, and mucosa-associated lymphoid tissue (MALT), where they encounter antigen and differentiate into antibody-producing plasma cells or memory B-cells (Reviewed in Kurosaki et al., 2015). T-cells mature in the thymus and acquire unique antigen-binding molecule called the T-cell receptor on their membrane. T-cell receptors only recognize antigens that are bound to cell membrane proteins called major histocompatibility complex (MHC). On recognition of antigen under appropriate circumstances, the T-cells proliferate and differentiate into various effector T-cells and memory T-cells (Kindt et al., 2006).

1.3 B-cell repertoire

Immune cells do not interact with, or recognize, an entire immunogen molecule; instead, lymphocytes recognize discrete sites on the macromolecule called epitopes, or antigen determinants. Previous studies with small antigens have revealed that B and T-cells recognize different epitopes on the same antigenic molecules (Sanchez-Trincado et al., 2017). B-cells recognize soluble antigens when it binds to antibody molecules in the B-cell membrane. Because B-cells bind antigen that is free in solution, the epitopes they recognize tend to be highly accessible sites on the immunogen's exposed surface (Kindt et al., 2006).

1.3.1 Structure of immunoglobulins

Igs or antibodies that recognize the antigen are made up of four peptide chains, two identical light (L) chains around 22,000 Da, and two identical heavy (H) chains around 55,000 Da or more. Each light chain is bound to a heavy chain through a disulfide bond and noncovalent interaction such as hydrogen bonds, hydrophobic interactions to form a heterodimer (H-L), and salt bridge (Cook, 2000). Similar interactions link the two identical heavy and light (H-L) chain combinations to each other to form the basic four-chain $(H\text{-}L)_2$ antibody structure, a dimer of dimers (Kindt et al., 2006) (Figure 1.1).

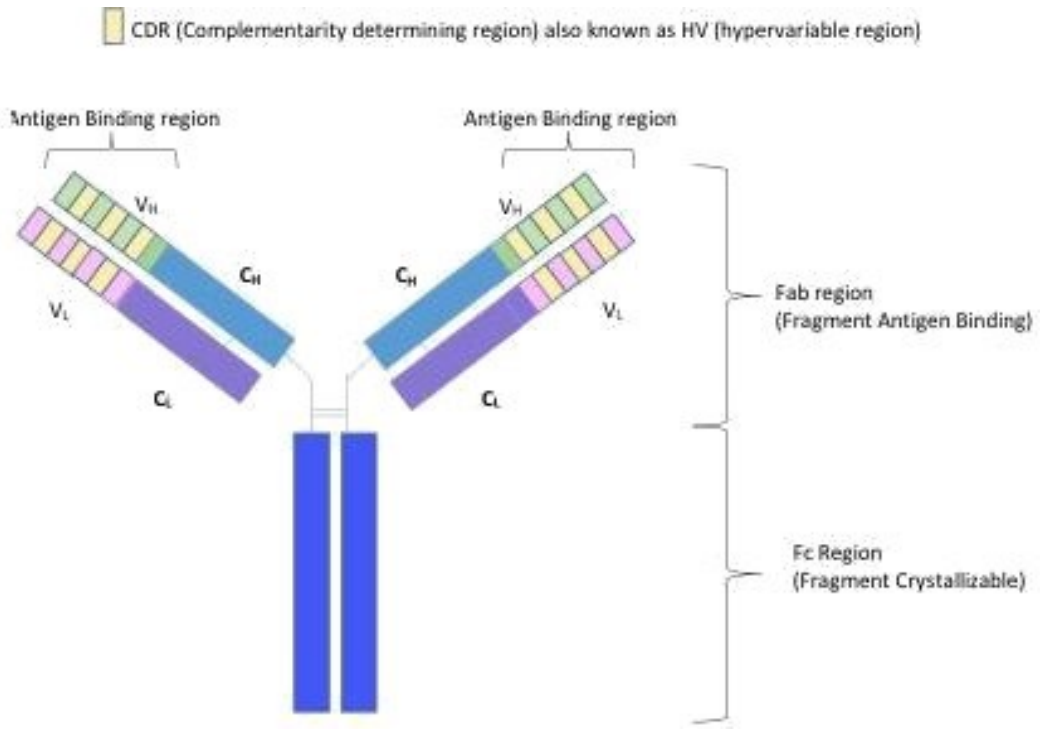

Figure 1.1 Structure of immunoglobulin. The Immunoglobulin molecule has two heavy chains, and two light chains joined by disulphide bonds. The antigen-binding site of the antibody is made up of highly variable amino acid sequences and are referred to as a variable domain (V). And the less variable domain of immunoglobulin is referred to as constant domain (C). The heavy and light chain V regions are represented as V_H and V_L in the figure. C_H and C_L represent the constant domain of heavy and light chain. The V region is further divided into three hypervariable intervals, referred to as complementarity determining regions (CDRs), and are present in between four regions of stable sequence termed frameworks (FRs).

The first 110 or so amino acids of the amino-terminal region of a light or heavy chain varies greatly among antibodies of different antigen specificity. Differences in specificity displayed by different antibodies can be traced to the differences in these regions of amino acid sequences. These highly variable sequences are referred to as variable (V) regions: V_L in light chains and V_H in the heavy chains (Kindt et al., 2006). In contrast, within each class of antibody, far fewer differences are seen in comparison, and these sequences are referred to as constant (C) regions: C_L in light chains and C_H in the heavy chains (Kindt et al., 2006) (Figure 1.1).

Based on the amino acid sequence differences, the constant domains of the heavy chain is sub-classified into alpha (α), delta (δ), epsilon (ε), gamma (γ) or mu (μ) respectively and the light chain can be further sub-classified into kappa (κ) and lambda (λ) chains. The five heavy chain isotypes correspond to the five antibody isotype α – IgA, δ – IgD, γ – IgG, ε – IgE, μ – IgM respectively.

Heavy and light chains of Igs are each encoded by a separate multigene family (Leder, 1982; Tonegawa, 1983), and the individual V and C regions are each encoded by independent elements: V(D)J gene segments for the V region and individual exons for the C region. The V region primary sequences are functionally divided into three hypervariable intervals, referred to as complementarity determining regions (CDRs), and are present in between four regions of stable sequence termed frameworks (FRs) (Schroeder & Cavacini, 2010). The creation of a V region is directed by the recombination signal sequences (RSS) that flank the rearranging gene segments. V(D)J

recombination reaction requires recombination-activating genes (RAGs) 1 and 2, which are almost exclusively expressed in developing lymphocytes (Schroeder & Cavacini, 2010).

1.3.2 B-cell maturation

Production of antibodies or immunoglobulins by B-cells plays a crucial role in producing a pathogen-specific immune response. B-cells develop from hematopoietic stem cells in the fetal liver through hematopoiesis, and the development continues in the bone marrow after birth (Reviewed in De Silva & Klein, 2015). In the bone marrow, transmembrane tyrosine phosphatase CD45R (B220 in mice) is expressed by the progenitor B-cells (pro-B cells). The microenvironment provided by the bone marrow stromal cells allows the pro-B cells to proliferate and differentiate into pre-B cells. Rearrangement of immunoglobulin DNA occurs in the lymphoid stem cells and is essential for B-cell maturation. The heavy chain of the pro-B cell undergoes D_H –to-J_H gene rearrangement, followed by V_H –to- D_HJ_H rearrangement. V_H-D_H-J_H rearrangement continues to the next chromosome when the first rearrangement was not productive. A cell is referred to as a pre-B cell when the heavy-chain rearrangement is complete (Kindt et al., 2006).

Pre-B cell development involves light chain rearrangement, and this commits the immature B-cell to a specific antigenicity. Only one light chain isotype gets expressed on the cell surface due to allelic expression. B-cell development in the bone marrow results in the production of IgM bearing immature B-cells. Engagement of the immature B-cells to antigens results in death or anergy and not proliferation or differentiation since the immature B-cells are not fully functional (Reviewed in Winkler & Mårtensson, 2018). Mature B-cells express IgD and IgM on their cell surface and migrate to secondary lymphoid organs where they become activated and undergo differentiation (Reviewed in LeBien & Tedder, 2008).

1.4 Germinal centre reaction in mammals

Within the secondary lymphoid organs, mature B-cells encounter pathogens and become activated with the help of T_H cells. These activated B-cells start producing antibodies that might have a low affinity toward the specific antigen. As an attempt to produce high-affinity antibodies, some of these B-cells enter specialized sites within the secondary lymphoid organs, which are later referred to as GCs. Three main events occur in the GC, clonal expansion and somatic hypermutation, B-cell selection, and class switch recombination. GC reaction requires crosstalk between T_H cells, FDCs, and B-cells to result in the successful production of plasma and memory cells. The GC can be further divided into dark and light zone reaction (Reviewed in Gatto & Brink, 2010).

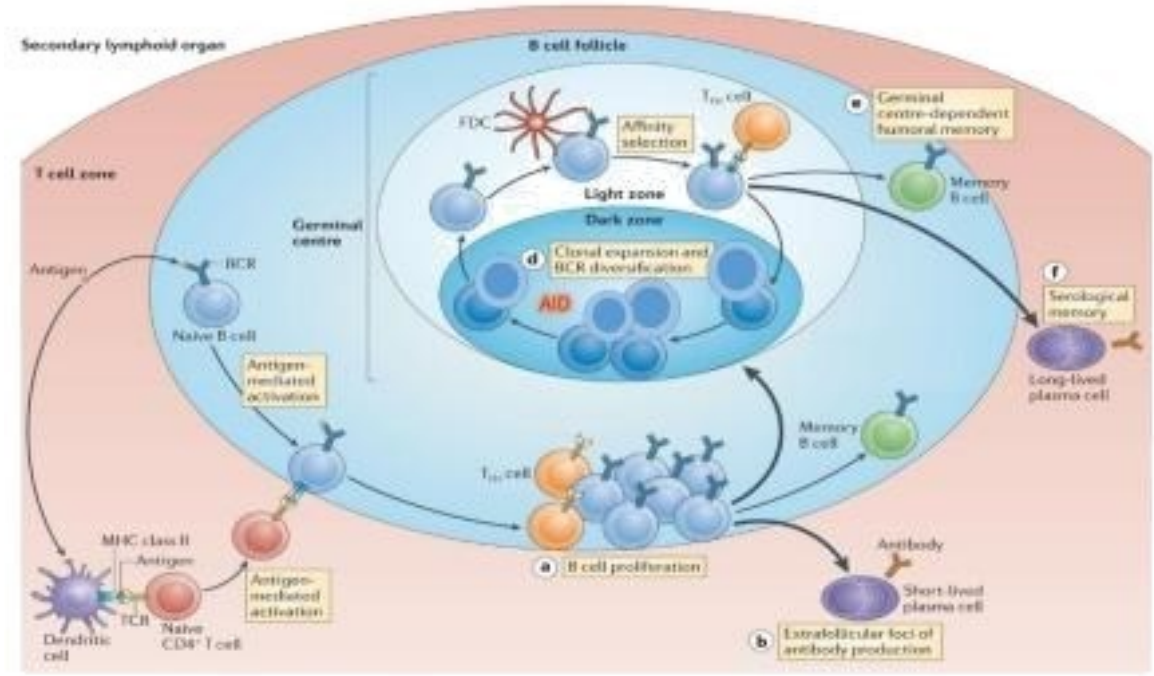

Figure 1.2 Germinal centre reaction in mammals and birds. Germinal centres are specialized sites in the secondary lymphoid organs where antibody affinity maturation occurs. Three major events occur within the germinal centre, which includes clonal expansion and somatic hypermutation that occurs in the dark zone. B-cell selection and class switch recombination occur in the light zone. Long-lived memory B-cells and antibody-producing plasma cells are generated at the end of the germinal centre reaction (Reviewed in Kurosaki et al., 2015).

1.4.1 Dark zone

On entering specialized sites within secondary lymphoid organs, activated B-cells rapidly proliferate and acquire mutations. These proliferating B-cells are referred to as centroblasts. Centroblasts have expanded cytoplasm, diffuse chromatin, and are known to have reduced membrane expression of Igs (Kindt et al., 2006).

1.4.1.1 Somatic hypermutation

In the dark zone, AID results in random point mutations in the exon encoding the antigen recognition site of the antibody. AID deaminates cytosine base to uracil, which leads to U:G mismatch. The generated U:G mismatch can be handled in three different ways. If this error is left uncorrected then during DNA replication, uracil gets recognized as thymine, causing T:A conversion. In the second case, uracil-DNA glycosylase (UNG) removes the uracil leading to the formation of an abasic site (empty site due to the loss of a purine or pyrimidine). Replication over this site can result in both transition and transversion mutations. In the third case, U:G mismatch recruits a mismatch repair (MMR) machinery which can result in mutation at A:T near the initiating U:G lesion (Reviewed in Odegard & Schatz, 2006). These mutations in the variable region of the Ig genes results in the altered affinity of the Igs to specific antigens. B-cells with acquired mutation stop proliferating and enters the light zone of the GC.

1.4.2 Light zone

B-cells entering the light zone are referred to as centrocytes; they are small, non-proliferating, and express Igs on the cell membrane. Within the light zone, centrocytes interact with FDCs and T_h cells to achieve two significant events, selection of high-affinity antibody-producing B-cells and generation of plasma and memory B-cells. (Reviewed in Gatto & Brink, 2010).

1.4.2.1 Follicular dendritic cells (FDCs)

FDCs are non-hematopoietic and are generally found in the stromal network of lymphoid organs. They are large cells with slender, dendritic protrusions, and heterochromatic oval nuclei with prominent nucleoli (Chen et al., 1978). The primary function of FDC is to capture intact ICs (Immune complexes) and present it to B-cell through FcRs or CRs, thereby facilitating the selection of high-affinity antibody-producing B-cells in the light zone of GC (Reynes et al., 1985 ; Szakal et al., 1988). Additionally, they attract activated B-cells to GC by secreting chemokine CXCL13 (C-X-C Motif Chemokine Ligand 13) which is recognized by CXCR5 (C-X-C chemokine receptor type 5) on the B-cells (Cyster et al., 2000)). FDCs enhances the survival of B-cells within the GC by producing B-cell activating factor (BAFF) (Lesley et al., 2004) and facilitate the removal of apoptotic cells by TBMs (tingible body macrophages)

through the production of Mfge8 (Milk fat globule-EGF factor 8 protein) (Kranich et al., 2008).

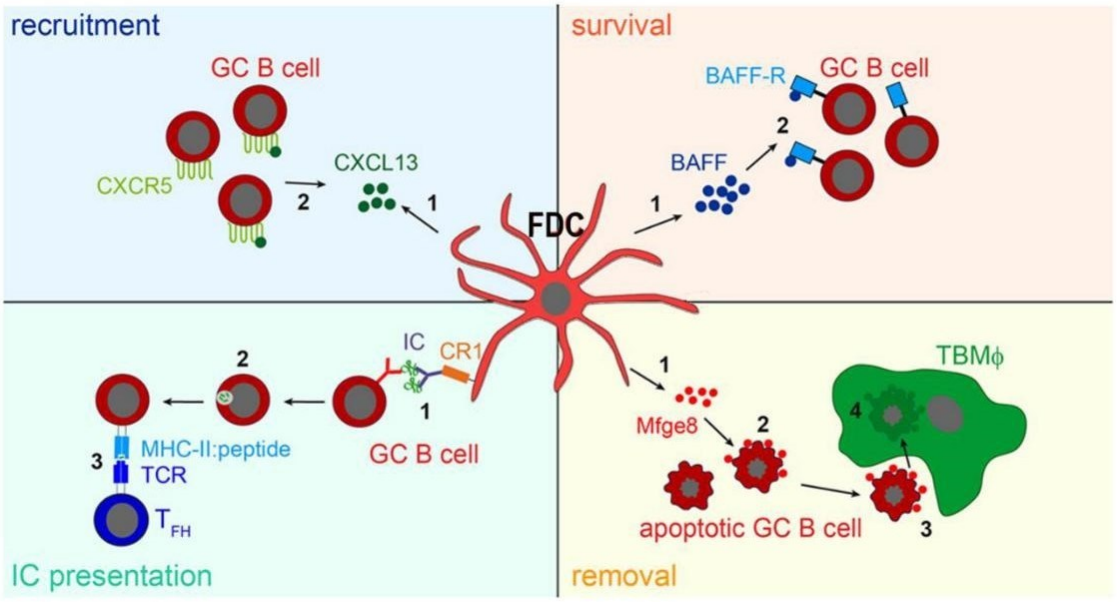

Figure 1.3. Functions of FDCs within mammalian germinal centers. FDCs recruit B-cell to the GC by producing CXCL13 which is recognized by CXCR5 on B-cells (top left panel). The production of BAFF by FDCs ensures the survival of B-cells within the GC (top right panel). Mfge8 secreted by FDCs facilitates the clearance of apoptotic cells within GC by TBMs. The immune complex is captured on the surface of FDCs through FcRs or CRs to select for high-affinity antibody-producing B-cell (Reviewed in Kranich & Krautler, 2016).

As shown in Figure 1.3 IC presentation is one of the main functions of FDCs. Fc or CRs trap the IC on FDC surface in an intact form. However these ICs are not directly obtained by FDCs. Antibodies produced by non-specific B-cells in the extrafollicular foci of secondary lymphoid organ (Figure 1.2) are trapped and transported to the surface of FDCs through marginal zone B-cells or subcapsulary sinus macrophages (Ferguson et al., 2004 ; Phan et al., 2009). FDCs are also known to recycle the immune complexes through recycling endosomes. The IC were captured intact within non-degradative cycling compartment and periodically brought to the surface. This allows the FDCs to protect the IC from damage while keeping it accessible to B cells (Heesters et al., 2013).

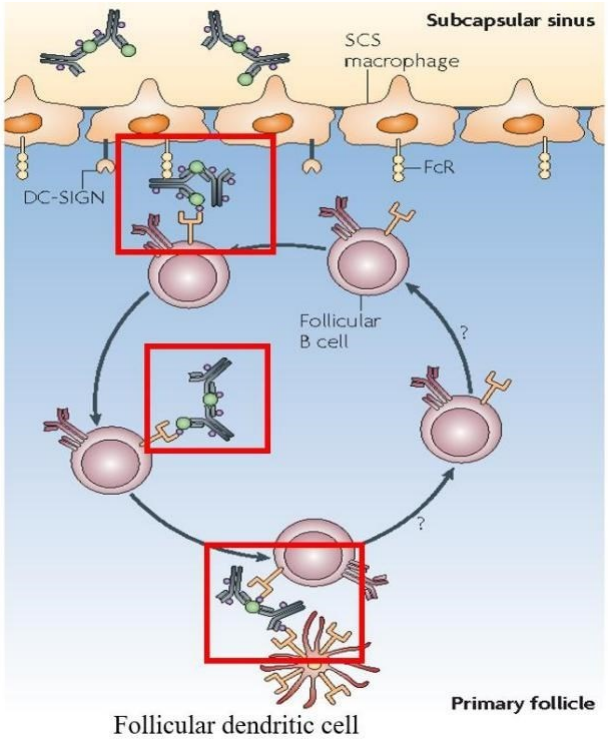

Figure 1.4. Transport of intact immune complex to FDCs. IC captured from the circulation by SCS macrophages gets transferred to follicular B-cells in the lymph node. These follicular B-cells transport ICs to the surface of FDCs through Fc or CRs in an intact form (Reviewed in Batista et al., 2009).

1.4.2.2 Selection of high-affinity antibody-producing B-cells

Once B-cell enters the light zone, they are in a state of activated apoptosis (Hennino et al., 2001). Here, B-cells compete for the antigen that is captured on the surface of FDCs. Successful B-cells internalize the antigen, processes it and presents it on MHC Class II to T_H cells to receive survival signals (Lanzavecchia, 1985). Antigen-dependent FDC–B-cell interaction with appropriate crosslinking of the newly generated BCR (B-cell receptor) by follicular dendritic cell (FDC)-associated immune complexes results in the inactivation of pre-programed cell death (van Eijk & de Groot, 1999; Hennino et al., 2001).

Studies done by Hennino's group showed that activation of caspase-8 and formation of death-inducing signaling complex (DISC) is required for GC B-cell apoptosis. They found that ex vivo GC B-cells contains a preformed inactive DISC containing Fas-associated death domain–containing protein (FADD), procaspase-8, and a long isoform of cellular FADD-like IL-1β–converting enzyme-inhibitory protein (c-FLIPL) but lacks the CD95L. Appropriate crosslinking between IC present on the FDC surface and antibody expressed on the B-cell results in binding of c-FLIPL to FADD domain which inhibits the auto-cleavage of procaspase-8 and prevents apoptosis. Absence of survival signal or lack of crosslinking between FDC and GC B-cell was seen to result in loss of c-FLIPL which lead to cleavage of procaspase-8 into active caspase leading to GC-B cells apoptosis (Hennino et al., 2001).

1.4.2.3 Class switch recombination

Class-switch recombination (CSR) is an intrachromosomal DNA rearrangement event which allows the association of any given V_H domain to the constant region of any isotype. This enables the antibody to alter the biological effector activity while maintaining the specificity. Cytokines produced by T_H cells play a crucial role in the proliferation and differentiation of B-cells. Depending on the type of cytokine produced, the class-switching occurs to result in IgA-, IgE-, IgG- expressing B-cells. Cytokine signalling results in the preferential targeting of AID to one of the Ig heavy chain constant domain located at the 5' end of γ, ε, and α chains. The target switch recombination sequence and the proximal μ chain switch recombination sequence undergoes AID mediated demethylation of deoxycytidine residues. DNA glycosylase (UNG) then removes the resulting deoxyuracil base leading to recombination between the switch recombination sequences. IgG, IgE, or IgA are expressed when the downstream heavy chain gene assumes the original location of the μ heavy chain gene immediately 3′ of the rearranged immunoglobulin heavy chain variable region gene. CSR can occur in the GC, but it is not restricted to GC (Reviewed in Gatto & Brink, 2010).

1.4.2.4 Generation of plasma and memory B-cells

Selected B-cells within the GC receive signals to differentiate into plasma cells. Plasma cells are known to lack membrane-bound Igs and secrete high levels of antibodies. Plasma cells also have increased expression of BLIMP1 (B-lymphocyte-induced maturation protein 1), IRF4 (Interferon Regulatory Factor 4) and XBP1 (X-box binding protein 1). B-cells exiting the GC are found to produce antibodies with up to 1000 fold higher affinity in comparison to B-cells entering the GC (Reviewed in Nutt et al., 2015). Long-lived memory B-cells are also produced as a result of the GC reaction, but this is not the only way by which memory B-cells are generated. Differentiation of an activated precursor cell expressing CD38 and GL7 can result in the formation of memory B-cells. Studies have shown that the CD40 signal provided by the TH cell alone can direct the activated B-cell to differentiate into memory B-cell pathway but not into germinal centre cells. Along with the CD40 signal, cytokine signalling is required for germinal centre B cell differentiation. (Reviewed in Kurosaki et al., 2015). Some of the B-cells in the light zone is seen to re-enter the dark zone and undergo an additional set of SHM events to improve the affinity of antibody produced though the mechanism behind this trigger is not fully understood (Reviewed in Kepler & Perelson, 1993).

1.5 Immunity in teleosts

Fish lack lymph node and bone marrow despite the presence of spleen and thymus. The anterior kidney (head kidney) is one of the main lymphoid organs in fish and considered to be a functional ortholog to mammalian bone marrow. Mucosa-associated lymphoid tissue of teleost constitutes the gut, skin and gills; this plays a vital role in the maintenance of mucosal homeostasis (Reviewed in Sunyer, 2013). The innate arm of the immune system in fish is well developed and widely studies. The characteristic features of the adaptive immune response, that is, immunoglobulins, T-cell receptors, major histocompatibility (MHC) products and recombination activator genes (RAG 1 and RAG 2) are present in the most primitive extant jawed vertebrates and is known to be complete in the teleost (Reviewed in Watts, 2001).

1.5.1 Immunoglobulins in teleost

Fish were initially thought to contain just IgM, but later studies have shown the presence of IgD, IgZ and IgT in the teleost (Reviewed in Zhu et al., 2013). IgZ was identified in zebrafish, and the heavy chain locus of this Ig gene was found to contain independent clusters of V, D, J, and C gene segments (Danilova et al., 2005). IgT was initially identified in rainbow trout, and an IgZ like isoform referred to as IgZ-2 was later identified in zebrafish (Reviewed in Zhu et al., 2013). In zebrafish, an alternate splicing

pathway of Ig heavy chain resulted in a novel IgM membrane isoform (mIgM-2) which lacked the two constant regions domain (Cμ2 and Cμ3) (Hu et al., 2011).

1.5.2 Antibody affinity maturation (AAM) in fish

Fish were initially thought to lack AAM partially due to the absence of histologically distinct GC in their lymphoid tissues (Good and Finstad 1967; Manning and Horton 1982; Pitchappan 1980). Additionally, Early work done in channel catfish looked at the affinities of serum antibodies isolated from vaccinated fish at 1-2 months and 1-2 years following injections. The result of this study showed low levels of antibodies with higher affinity towards the injected antigen and the obtained numbers were not significant when compared to mammals which ideally result in 100 to 1000 fold increase in affinity (Lobb, 1985). It was later argued that the absence of high-affinity antibodies shown by Lobb could be due to the availability of more antigen affecting the selection process of high-affinity antibody-producing B-cells rather than the absence of AAM all together (Reviewed in Magor, 2015). Parameters required for the occurrence of AAM are AID expression and presence of specialized sites (GC like) to allow clonal expansion and selection of B-cells. Later studies in carp showed the accumulation of injected antigen (heat-inactivated bacterin) among aggregates of immune cells (MMCs) in the lymphoid organs. This observation suggested the presence of GC like site in fish lymphoid tissues.

In 2005, a study done in nurse sharks showed that they are capable of producing high-affinity IgNAR and under appropriate circumstances produce memory response (Dooley & Flajnik, 2005). Fully functional AID was seen to be colocalized in catfish spleen MMCs which further supports the hypothesis that MMCs in fish could be analogous to GCs in mammals (Saunders et al., 2010).

1.5.3 Melanomacrophage clusters in fish lymphoid organs

MMCs are aggregates of immune cells seen in the lymphoid organs of most vertebrae except mammals, birds, lamprey and tunicates (Agius, 1980). The MMCs are observed to be diffused in case of sharks, dogfish, rays, and hagfish (Reviewed in Fänge & Nilsson, 1985; Agius, 1980). MMs present within these clusters is pigmented, which makes the MMCs appear histologically distinct under a bright-field microscope (Reviewed in Steinel & Bolnick, 2017). As discussed in the previous sections, MMCs in fish was seen to accumulate injected antigens in and around them for extended periods (Bunton et al., 1987; Pulsford et al., 1992) and AID expressing cells were observed in these MMCs (Saunders et al., 2010). MMCs were also seen to constitute cells expressing transcripts for IgM chain and CD4 which could be B and T-cells (Saunders et al., 2010). Studies done on goldfish MMCs showed that they could be isolated from the surrounding tissues and cultured *in-vitro*. It was noted that reticular cells were vital for the survival of MMCs in culture (Diaz-Satizabal & Magor, 2015).

1.5.4 Characteristics of melanomacrophages

MMs are shown to accumulate auto-fluorescent pigments such as lipofuscin, hemosiderin, and melanin over time. Accumulation of non-degradable products of unsaturated fatty acid in non-dividing cells occurs when vitamin E levels are low, resulting in lipofuscin (Terman and Brunk, 2004). MMs are phagocytic cells, and uptake of dying erythrocytes by MMs results in the accumulation of hemosiderin, suggesting MM involvement in iron recycling (Agius, 1979; Kranz, 1989). Melanin accumulation in MMs is not fully understood; it can be obtained by the phagocytosis of melanin-containing cells or can be produced within MMs during the degradation of the phagocytosed cell membranes (Agius & Agbede, 1984).

Expression of CSF-1R (Colony-stimulating factor 1 receptor) in melanomacrophages suggests that they are tissue macrophages that have accumulated visible amounts of lipofuscin along with hemosiderin and melanin. Tissue resident macrophages are differentiated monocytes, which seed the tissues but are not a homogeneous population, and can be grouped based on the complex signaling events they are exposed to which results in similar functions and phenotypes (Davies & Taylor, 2015). MMs can be FACS (fluorescence-activated cell sorting) sorted based on their auto-fluorescent properties, and the sorted MMs were seen to survive in culture for up to six days (Diaz-Satizabal & Magor, 2015).

Previous studies in our lab looked at the cytokine profile of MMs versus macrophages under healthy and infected conditions. Results showed that MMs did not result in a classical pro-inflammatory response to A. salmonicida like Primary kidney macrophages (PKMs). Differences between the spleen and kidney MMs were also noted which could be explained based on the different functions of the anterior, mid-and posterior kidney, and the anatomical location of MMs within the kidney (Diaz Satizabal, 2013).

1.6 Hypothesis

This study aims to identify a specific cell type that has functional similarities to mammalian FDCs in fish lymphoid organs. Studies have shown similar characteristics between fish MMCs and mammalian GCs, and GCs are the primary site where FDCs facilitate B-cell selection. MMCs contains phagocytic MMs and are surrounded by reticular cell stroma. Reticular cells are also known to have a close lineage to mammalian FDCs. Based on these, we hypothesized either MMs or reticular cells in fish could have functional similarities to mammalian FDCs.

1.7 Aims and Objectives

Aim of this study is to identify and characterize a specific cell type with functional similarities to FDCs in fish.

Objectives of the studies were

- Identify antigen retaining cells in fish lymphoid organs by injecting antigen conjugated to a fluorescent label.
- Show the antigen is retained in an intact form on the cell surface of antigen retaining cells.
- Identification of putative FDC genes in antigen retaining cells through PCR.
- Showing antigen is bound by antibodies or complement proteins (immune complex) using immunoprecipitation and mass spectrometry in goldfish.
- Generating a gene profile for the antigen retaining cell through RNA-sequencing.
- Identification of antigen associated receptor in the antigen retaining cell through Co-IP and mass spectrometry.

Chapter 2: Materials and methods

2.1 Animal maintenance

Goldfish (Carassius auratus L.) were obtained from Aquatic Imports, Calgary, Canada and maintained at the aquatic facility in the Department of Biological Sciences, University of Alberta. All maintenance conditions and animal procedures were approved by the Universities Animal Use and Care Committee, under the guidelines of the Canadian Council for Animal Care. Fish were maintained with static flow water system between 15-20° C and were acclimatized to this environment for at least three weeks before use. Older fish weighing 30-60 gms and 20-30 cms in length along with smaller fish weighing 10-20 gm and 5-10 cm in length were used for experimental purpose.

2.2 TMS for immunizations and euthanization

Tricaine methonsulfonate (TMS) (buffered to ~pH 7 with NaOH) at a concentration of 100 mg/L was used for immunization, and 200 mg/L was used to euthanize goldfish. For zebrafish, 20 mg/L was used for immunizations, and 40 mg/L was used for euthanizations. For immunizations, fish are immersed in a tank with TMS for 5-10 mins. When there is reduced motion, and no mouth opercular moment, the fish are transferred to a workbench followed and the antigen is injected intraperitoneally. Injected fish are allowed to recover in a tank with reduced water flow. For euthanization, fish are

immersed in TMS for 15-20 mins, and the spine is severed above the opercula flap or below the medulla oblongata first before the dissection of organs.

2.3 Vaccine preparation

Fish were injected with one of the following protein such as Bovine serum albumin (BSA) (ThermoFisher), commercially available BSA-Alexa fluor 647 (Invitrogen), BSA-Alexa fluor 647 (Labelled using Alexa fluor 647 labeling kit (ThermoFisher)), Keyhole limpet hemocyanin (Sigma-Aldrich) and Keyhole limpet hemocyanin-Alexa fluor 647 (Labelled using Alexa fluor 647 labeling kit (ThermoFisher)). Proteins or antigen are diluted with 0.9X PBS (pH 7), and the adjuvant constitutes 1/3 rd of the vaccine. Hundred and fifty microliters of vaccine containing 50 µg of protein along with adjuvant is injected per goldfish. In the case of zebrafish, 10 µls of vaccine containing 2 µg of protein and adjuvant is injected into each fish. Initial immunizations were done using complete Freund's adjuvant (Sigma-Aldrich), and successive boost was done using incomplete Freund's adjuvant. During organ removal, it was noted that in some goldfish there remained pockets of an unabsorbed vaccine. The vaccine was not evenly released into the system and instead trapped in vaccine pockets even after a month following immunization. Antigen emulsified in mineral oil constitutes Freund’s adjuvant which could be one of the reasons why we noted unabsorbed vaccine pockets. To resolve these issue alum adjuvants were used in goldfish instead of Freund’s adjuvant. Aluminum-based adjuvants include aggregate formation enabling continuous

release of antigens. Alum adjuvant was prepared following the protocol described in Jiang et al. (2015). Five percent of Al2(SO4)3 and five percent NaOH were filtered through 0.22µm Millex-GP Syringe Filter Unit (Millipore Sigma). The solutions were incubated at 60 °C for 30 min. Five volumes of 5% Al2(SO4)3 and two volumes of 5% NaOH were mixed with stirring, followed by centrifugation at 10,000× g for 5 min. After washing twice with sterile PBS, the mixtures were re-suspended in PBS to 0.2 mg/mL. Equal volumes of alum adjuvant and protein vaccine were mixed before injecting fish.

2.4 Fish immunizations

Fish are injected with vaccines containing protein or antigen along with adjuvant diluted in 0.9X PBS. TMS (Syndel Laboratories) was used to anaesthetize fish, and protein or antigen was injected intraperitoneally with the needle angled forward and toward midline, entering the fish on the body wall just up from the ventral surface of the fish, between the pelvic fin and the anus. In goldfish BD Luer-Lok™ 1 mL syringe and 23G x 1 (0.6 mm x 25 mm) BD PrecisionGlide™ Needle was used, and in zebrafish, BD Insulin Syringes with BD Ultra-Fine™ needle 6 mm x 31 G 3/10 mL/cc was used to inject the antigen intraperitoneally. Concentrations and volume of vaccines injected are discussed in section 2.3. Fish were allowed to recover in separate tanks and used for future experiments. Protein injected intraperitoneally drains into the portal vein and pass through the liver before reaching the systemic circulation (Turner et al., 2011).

2.5 Tissue collection from goldfish

TMS (Syndel Laboratories) at lethal concentrations were used to euthanize the fish, followed by serving of spine above the opercula flap or below the medulla oblongata. Sterilized dissection tools were used to remove the spleen and kidney of the fish. Obtained tissues were placed in cold MGFL-16 media (Neumann et al., 2000) containing 10% FBS and heparin (50,000 U/mL, Sigma).

2.6 Isolation of spleen and kidney leukocytes

Tissues were mashed through 500 µm cell strainers (pluriStrainer) using 1 ml syringe plunger and passed followed by 40 µm cell strainers (BD Falcon) to obtain individual cells. Cells were suspended in MGFL-16 complete media and layered on top of 25.5% Precoll (GE Healthcare) in 0.9X PBS at equal volume. Centrifugation was done at 230 x g for 30mins. Leukocytes are separated from Percoll-MGFL-16 media interface and washed thrice with 0.9X PBS. Purified leukocytes were suspended in 0.9X PBS and stored at 4°C with 5% FBS and 0.0013% sodium azide. Cytospin was used to prepare microscopic slides with 10^5 cells per slide at 800 rpm for 8 minutes as described in Koh (2013).

2.7 Isolation of peripheral blood leukocytes.

TMS (Syndel Laboratories) was used to anaesthetize goldfish and blood was drawn from the caudal vein using BD Luer-Lok™ 1 mL syringe and 23 G x 1 (0.6 mm x 25 mm) BD PrecisionGlide™ Needle. The obtained blood was diluted 1:5 with MFGL-15 containing heparin and antibiotics. Percoll (25.5%) (GE Healthcare) centrifugation was done for 30 minutes at 400 x g to remove erythrocytes. Leukocytes are removed from the percoll-MFGL-15 interface and washed twice with 0.9 X PBS by centrifugation for 10 minutes at 230 x g and 4 ºC.

2.8 Isolation of MMs through FACS

MMs can be sorted based on their auto-fluorescent property, size and complexity. Peripheral blood leukocytes (PBLs) lack auto-fluorescent MMs; hence PBLs can be used to establish gates to sort MMs. Leukocytes were isolated from goldfish according to protocols described in section 2.6. Isolated leukocytes were washed with PBS containing serum to avoid clumping of cells. PBLs isolated from unvaccinated fish (described in section 2.7) is used to set the gates for FACS. Based on the cell size and complexity sorting gates were set and cells with fluorescence intensity higher than the background in both the green and the red channels were isolated as MMs (Wang 1995; Barreda, Hanington et al. 2005). Cells were suspended in 0.9 X PBS – 0.05 mM EDTA (High pressure during sorting compromises buffer capacity hence EDTA is used to help prevent cell adhesion) at a concentration of 5-7 x 10^6 cells/mL (Basu et al., 2010) and sorted

using Aria lll flow cytometer (BD Biosciences) at the flow cytometry unit at the Heritage Medical Research Centre, University of Alberta. Sorted melanomacrophages were collected into complete MFGE8 media, and further experiments (Isolation of RNA or protein) were performed within 4-6 hours after sorting.

2.9 RNA isolation from goldfish leukocytes or sorted MMOs

Total RNA isolation was done using the Qiagen RNeasy micro kit for cell number $<5 \times 10^5$ and Qiagen RNeasy mini kit was used for 10^7 cells by following the manufacture's protocol. After isolation, the concentration and purity of the RNA were determined by reading the absorbance of the samples at 230, 260 and 280 nm using a NanoDrop (ND-1000 spectrophotometer, NanoDrop) and Qubit (RNA HS Assay Kit, Thermo Fisher Scientific). Obtained RNA was stored at -20°C until further use.

2.10 cDNA preparation

Isolated total RNA was reverse transcribed into cDNA using anchored Oligo (dT)16 primer (Nam et al., 2002) and the SuperScript III™ First-Strand cDNA Synthesis System (Invitrogen™). cDNA synthesis from isolated leukocytes was performed using 200-500 ng of RNA and 100 U of the enzyme. Twenty microliter reactions were incubated 25°C for 5 minutes, followed by 50°C for 2 hours, heat-inactivated at 70°C for

15 minutes and 2 U of RNase H (Invitrogen™) was added to the samples and incubated for 20 minutes at 37°C to remove residual RNA. The first-strand cDNA synthesized was stored at -20°C for PCR.

2.11 Polymerase chain reaction

Polymerase chain reactions were carried out at a reaction volume of 20 µl. The reaction mix constitutes 2 µl of 10X PCR buffer (Invitrogen™), 2 µl of 2.5 mM dNTP's (Invitrogen™), 0.6 µl of 50 mM MgCl2 (Invitrogen™), 13.6 µl of MilliQ H2O along with 0.5 µl of forward and reverses primers (0.5µM) and cDNA (1 µg) and 0.3 µl of Taq polymerase. The initial denaturation was carried out at 95°C for 1 minute followed 35-40 cycles of 94°C for 15 seconds, primer specific annealing temperature for 15 seconds, 72°C for 30 seconds. Final cycle is set at 72°C for 7 minutes, and the obtained product is run on 0.8% agarose gel or stored at 4°C.

2.12 Reticular cell culture and RNA isolation

Leukocytes from goldfish were isolated using the protocol described in section 2.6. Following percoll centrifugation, leukocytes were washed thrice using 0.9X PBS and re-suspended in complete MGFL-15 media. Cells were allowed to grow in MGFL-15 media containing penicillin (100 units/mL), streptomycin (100 µg/mL), fungizone (0.5 µg/mL) in T-25 flasks at 23°C with no CO2. Reticular cells adhere to the flask, whereas

melanomacrophages tends to stay in the media. Media was changed regularly to remove the non-adherent cells and cells were cultured until there was no evidence of remaining auto-fluorescent MMs. Adherent cells were treated with trypsin, and RNA was collected from these cells using the RNeasy Micro kit (Qiagen). Absence of melanomacrophages was confirmed based on the absence of CSF1-R expression by PCR.

2.13 Laser scanning confocal microscopy.

Spleen and kidney leukocytes from goldfish were isolated, and microscopic slides were prepared (previously described in section 2.6). Zebrafish whole spleen and kidney tissues were formalin-fixed, and paraffin-embedded cross-sections were prepared following standard protocols (Canene-Adams, 2013) at the Microscopy Facility in the Biological Sciences department, University of Alberta. Imaging was done using the laser scanning confocal microscope (Zeiss LSM 510 META) at Katz microscope facility, University of Alberta. Fluorescence emission spectra were established for unvaccinated zebra fish spleen and kidney cross sections and unvaccinated goldfish spleen and kidney leukocytes based on scans with 10 nm windows over a range of 410–680 nm wavelengths (emission).

2.14 Identification of specific cell type retaining intact antigen on the cell surface

Leukocytes were isolated from BSA immunized fish at day 14 and 21 following protocols described in section 2.3, 2.4 and 2.5. Cells were fixed using 4% paraformaldehyde (PFA) in 0.9X PBS (Harlow & Lane, 2006) for 30 mins on ice in the dark. Cells were then washed with cold 0.9X PBS. Magnetic protein G beads (2.8µm, Invitrogen) were conjugated to rabbit BSA polyclonal antibody (Thermo Fisher Scientific) according to manufacturer's protocol and exposed to the fixed cells for 15 mins with rotation (30-40 RPM) at room temperature. Beads were placed on magnetic stand and washed thrice with 0.9X Phosphate-Buffered Saline, 0.1% Tween (PBS-T) (Sigma-Aldrich) and eluted using 0.1M glycine (pH 2) (Sigma-Aldrich) and neutralized using 0.1M tris (Thermo Fisher Scientific). Eluted cells were re-suspended in cold 0.9X PBS, and cytospin was used to prepare microscopic slides. Leukocytes isolated from the spleen and kidney of unvaccinated fish were exposed to just Protein G beads and to Protein G beads conjugated to anti-BSA antibodies to serve as negative controls.

2.15 Hybridoma culture and isolation of monoclonal antibodies

WCI 12 hybridoma cell line were used to generate monoclonal IgG1 antibodies that binds to heavy chain of carp Igs (Rombout et al., 1993; Secombes et al., 1983). Cell line was cultured at 37°C with CO_2 in RPMI 1640 media containing 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) the cells were passaged regularly, and with each passage, the concentration of FBS in media was gradually reduced to 0%. The cells were grown in RPMI 1640 media without FBS for 3-5 days in T-175 flasks, before the collection of supernatant.

The supernatant was isolated and filtered using 0.22µm Millex-GP Syringe Filter Unit (Millipore Sigma) from WCI12 cells cultured in 0% FBS containing RPMI-16 media. Saturated ammonium sulphate (SAS) at a concentration ranging from 37%-45% was gradually added to the supernatant at 4°C under continues mixing for at least an hour (B.T. Tey, S.H.S. Mariam et al. 2015). The solution was then centrifuged at 12,000g for 20 mins, and the obtained pellet was washed with chilled phosphate buffer saline (PBS) containing 33% SAS to the original volume of serum. The solution was subjected to centrifugation again, and the obtained pellet containing IgG was re-suspended in PBS [10% (v/v) of the original volume of serum]. The IgG solution was dialyzed (Slide-A-Lyzer dialysis cassette, 10K MWCO, Thermo fisher scientific) in PBS for 24 h at 4°C to remove (NH4)2SO4. The obtained IgG was then conjugated to Alexa fluor 350 using Alexa Fluor™ 350 Antibody Labeling Kit (Thermo Fisher Scientific).

2.16 Antibody isolation from serum

Fish were immunized with BSA Freund's adjuvant (Sigma-Aldrich) or BSA Alum adjuvant once a month over some time, and after at least three months peripheral blood from these fish was isolated from caudal vein using 3 ml (BD syringes) and 23Gx1 needles (BD precision glide needle). Blood was allowed to clot at room temperature for 1 hr, and the sample was centrifuged at 1,500 x g for 10 mins at 4°C. Obtained serum was allowed to interact with CNBr activated Sepharose beads (Sigma-Aldrich) conjugated with BSA. Bound beads were washed and later eluted with 0.1 M glycine (pH 11) followed by neutralization with 0.1 M tris buffer (pH 7.2). The sample was then dialyzed (Slide-A-Lyzer dialysis cassette, 10K MWCO, Thermo fisher scientific) against 0.9X PBS for one hour at room temperature on the magnetic spinner followed by overnight dialysis at 4°C. Dialyzed sample was then concentrated using Amicon centrifugal filter devices, 10K MWCO and stored at 4 °C.

2.17 Protein isolation from cells

Melanomacrophages were sorted according to the protocol described in section 2.8, and cells were kept on ice at all times. Cells were washed thrice with cold 0.9X PBS to remove any FBS from the cells and pelleted through centrifugation at 230 x g for 10 mins. BS^3 (Bissulfosuccinimidyl suberate) (Thermo Fisher Scientific) cross-linking of cell surface proteins was done following manufacturers instructions. BS^3 (5mM) was prepared by dissolving in 20 mM sodium phosphate containing 0.15 M NaCl (pH 7-9).

The cell pellet was added with 250 µl of 5 mM BS^3 and incubated for 30 mins on ice. Following incubation, cells were added with quenching buffer (to stop cross-linking) (1 M Tris HCl pH 7.5) to a final concentration of 20-50 mM and incubated for 15 mins at RT. Cells were washed twice with cold 0.9X PBS, and the cell pellet was added with lysis buffer (pH 7.5) (50 mM Tris HCl, 150 mM NaCl, 1% Triton X 100) containing protease inhibitor cocktail (Catalogue number-11836153001, Sigma-Aldrich) and incubated overnight at 4°C. On the following day, cells were sonicated twice at a frequency of 20 kHz for 10 secs with a 10 min interval on ice. Samples were then centrifuged in 4°C for 30 mins at 12,000 RPM, and the supernatant containing the protein was stored at 4°C and used for later experiments. In initial experiments involving protein isolation and immunoprecipitations non-denaturing lysis buffer containing 1% Triton X (Sigma-Aldrich) was used to isolate protein. After optimization, 1% SDS (sodium dodecyl sulfate) denaturing detergent was added to the lysis buffer to ensure complete lysis of cell membrane proteins.

2.18 Immunoprecipitation

Co-immunoprecipitations were carried out to isolate antigen retaining, or antigen associated receptors from MMs and also to isolate immune complexes from goldfish immune serum. Protein G magnetic beads (2.8 µm, Invitrogen) were conjugated to rabbit BSA polyclonal antibody (Thermo Fisher Scientific) according to manufacturer protocol. Whole-cell lysate obtained from sorted MMs were crosslinked (specific for cell surface

proteins) with BS^3 (Bissulfosuccinimidyl suberate) and exposed to antibody-conjugated beads for 15 mins with rotation (80 RPM) at room temperature. Beads were then washed thrice with PBS-T, and the bound protein was eluted using 0.1 M glycine (pH 2) (Sigma-Aldrich) and neutralized using an equal volume of 0.1 M tris (Thermo Fisher Scientific). Eluted protein was concentrated using Pierce™ Protein Concentrators, 10 K MWCO (Thermo Fisher Scientific) and samples were run on SDS-PAGE gel.

2.19 SDS PAGE electrophoresis and Coomassie staining

The separating gel was prepared by mixing 1.88 ml of 40% Acrylamide/Bis solution 37:5:1 (Bio-Rad), 2.5 ml of filter-sterilized (0.2 µm) Tris-HCl (1.5M, pH 8.8), 100 µL of 10 % SDS and 5.47 ml of MilliQ water. The stacking gel was prepared by mixing 1 ml of 40 % Acrylamide/Bis solution 37:5:1 (Bio-Rad), 2.5 ml of filter-sterilized Tris-HCl (0.5 M, pH 6.8), 100 µL of 10 % SDS and 6.36 ml of MilliQ water. Finally, 10 µl of TEMED and 50 µl of 10 % APS was added to both the mixtures just before pouring the gel. Separating gel was poured first followed by 1 ml of 80 % ethanol, and the gel was allowed to polymerize for 45 minutes. After polymerization ethanol was removed and the stacking gel was poured on top and allowed to polymerize for another 30 minutes. The electrophoresis buffer was prepared by dissolving Tris (15.14 g), glycine (72.05 g), and SDS (5 g) in 500 ml distilled water and the pH was adjusted to 8.30 using 10 N NaOH. A vertical polyacrylamide gel system was used, consisting of separating (7.5 %, m/v) and stacking (4.0 %, m/v) gels. Laemmli buffer was added to samples at a final concentration of 1X and samples were boiled for 5 minutes at 95 °C before loading on

gel. Sample loading volume was 20 µL, and the samples were run at a constant voltage of 100 V for 40-50 minutes. The gels were stained with Coomassie Brilliant Blue R-250 or silver stain. Coomassie stain was prepared by dissolving 0.25 gms of Coomassie Brilliant Blue R-250 in 90 ml of methanol: H_2O (1:1, v/v) and 10 ml of glacial acetic acid and filtered through Whatman filter No. 1 and stored at room temperature. After electrophoresis, the gels were washed twice in distilled water for 5 minutes and stained in Coomassie for 30 minutes with rotation and followed by two de-stainings (60% H2O, 30% methanol and 10% acetic acid).

2.20 Silver staining

Silver stain is more sensitive in comparison to Coomassie stain and gives better results for low protein concentration samples. SDS-PAGE gels containing protein samples were washed twice using MilliQ water for 5 minutes, and silver staining was done by using Pierce™ Silver Stain Kit (Thermo Scientific). Staining was done following manufactures instructions. The gels were incubated in the staining solution for 5 minutes, followed by developed for 30-60 seconds, and stopping solution (5% acetic acid) was added to avoid overdeveloping the gel. Bands were visualized using Porta-Trace illuminator, and specific bands were cut using a razor blade for mass spectrometry analysis.

2.21 Isolation of immune complexes from goldfish serum.

Peripheral blood was isolated from unimmunized, and BSA immunized goldfish. Collected blood was allowed to coagulate for 30 minutes at room temperature and centrifuged at 2,000 x g for 10 minutes at 4°C to isolate serum. The obtained immune and non-immune serum was divided and exposed to three different treatments. 1) Inactivation of complement proteins by incubation at 56°C incubation for 1hr (Giard, 1987), 2) Removal of antibodies (IgM) (Rombout et al., 1993) by using protein G beads (2.8µm, Invitrogen) conjugated to WCI 12 antibody (followed manufactures protocol), 3) Untreated serum-containing both complement proteins and antibodies. The three treated serum samples were then exposed to protein G beads conjugated to rabbit BSA polyclonal antibody (Thermo Fisher Scientific). Beads were later eluted, and samples were run on SDS-PAGE electrophoresis gel followed by silver staining (discussed in section 2.19 & 2.20) and mass spectrometry analysis of specific bands.

2.22 Sample preparation for RNA-Sequencing.

RNA was obtained from 5 different unvaccinated goldfish MMs (FACS sorted) from spleen and kidney leukocytes following protocols described in sections 2.8 and 2.9. RNA isolated from the five fish were combined and divided into spleen MM RNA, and kidney MM RNA. Concentration and quality of the obtained RNA were verified using Agilent 2100 Bioanalyzer (MBSU) and sent to LC Sciences for library preparation, RNA-sequencing and analysis.

2.23 RNA-Sequencing and analysis.

Poly(A) RNA sequencing library was prepared following TruSeq-stranded-mRNA sample preparation protocol (Illumina). Illumina's NovaSeq 6000 sequencing system was used, and 2 X 150 bp PE, 6 Gb data per sample (~40 M reads) was obtained. Generated reads were analyzed using bioinformatics tools.

Table 2.1 Number of goldfish used in different experiments.

Type of experiment	Number of goldfish used
Identification of antigen retaining cells	16
Retention of intact antigen on MMs	15
RNA isolation from MMs	22
Reticular cell culture	12
Identification of immune complex components	5
Co-IP to identify antigen retaining receptor	44
LSCM of goldfish	2

Table 2.2 Number of zebrafish used in different experiments.

Type of experiment	Number of goldfish used
Identification of antigen retaining cells	4
LSCM of zebrafish	2

Table 2.3 Composition of MGFL-15 medium (pH 7.4).

Components	Amount per 1 L
HEPES	3.5 g
KH2PO4	0.344 g
K2HPO4	0.285 g
NaOH	0.375 g
NaHCO3	0.17 g
10X Hank's balanced salt solution	40 mL
L-Glutamine (GLUTAMAX)	10 mL
Insulin	0.005 g
MEM amino acid solution	12.5 mL
MEM non-essential amino acid solution	12.5 mL
MEM sodium pyruvate solution	12.5 mL
MEM vitamin solution	10 mL
Nucleic acid precursor solution	10 mL
2-Mercaptoethanol solution	3.5 µL
GFL-15 medium*	500 mL
Milli-Q Water	Fill to 1 L

Filter sterilized with a 0.2 µm filter—store at 40C. *GFL-15 medium is made by mixing one package of dry powder of Leibovitz's L-15 medium and 1 of Dulbecco's modified eagle medium in 2 L of Milli-Q water. GFL-15 medium is filter sterilized with a 0.2 µm filter and store at 40C

Table 2.4 List of PCR primer sequences.

Primer	Sequence (5'-3')
BAFF FWD	ATG ATG CCG TAA CAG ACC ATA
BAFF REV	C CCT CCA GTG TAG CAT GTG TTG
CXCL13 FWD	ATG CAT TGC CAA TGG AAG
CXCL13 REV	TTT GCG ACA ATG TGC TCC CCA
B-actin FWD	TCG AGC ACG GTA TTG TC CCA
B-actin REV	TCT CCT GCT CGA AGT C GCT AAT
RANKL FWD	GAT TAT CGC GTG TAC C GCA
RANKL REV	GCC ATG CAT TGG ATA C GAA
CSF1-R FWD	AAT GCT CAA AGC CAG CG CAG
CSF1-R REV	TGA TGA CTA ACA CAG GAC GGC
EF-α FWD	AAG CCC ATG TGT GTA GAG TCG
EF-α REV	CCC TTT GAC ACC AAC AG

Chapter 3: Results

3.1 Identification of antigen retaining cells in goldfish and zebrafish lymphoid organs

In this section, we are trying to identify which cell type, within the spleen and kidney leukocytes population, is retaining the antigen. We hypothesize that the antigen retaining cell will be either MMs or RCs. (We used the term 'capture' to describe antigen trapping or holding by FDCs, and the term 'retain' is used to describe similar processes in fish).

3.1.1 Characterization of melanomacrophage autofluorescence in goldfish and zebrafish lymphoid organs using LSCM

MMs can be distinguished from RCs and other cell types based on their autofluorescent property. However, autofluorescence may interfere with the detection of experimentally injected fluorescently labelled antigens. To select a label with appropriate emission spectrum, we first generated a lambda profile for autofluorescence in the goldfish and zebrafish leukocytes using laser scanning confocal microscopy (LSCM). Figure 3.1 shows an image of leukocytes isolated from unvaccinated goldfish kidney in bright field, FITC and Cy5 channels. Figure 3.1.a shows an image of leukocytes with Hoechst (nuclear staining) isolated from unvaccinated goldfish kidney in TRITC, FITC

and DAPI channels. DAPI channel in Figure 3.1.a shows the nuclei along with autofluorescence emission. Presence of autofluorescent pigments can interfere with underlying fluorescent dyes, and though not done here, these signals can be separated through Z-stacks as done in Saunders 2010.

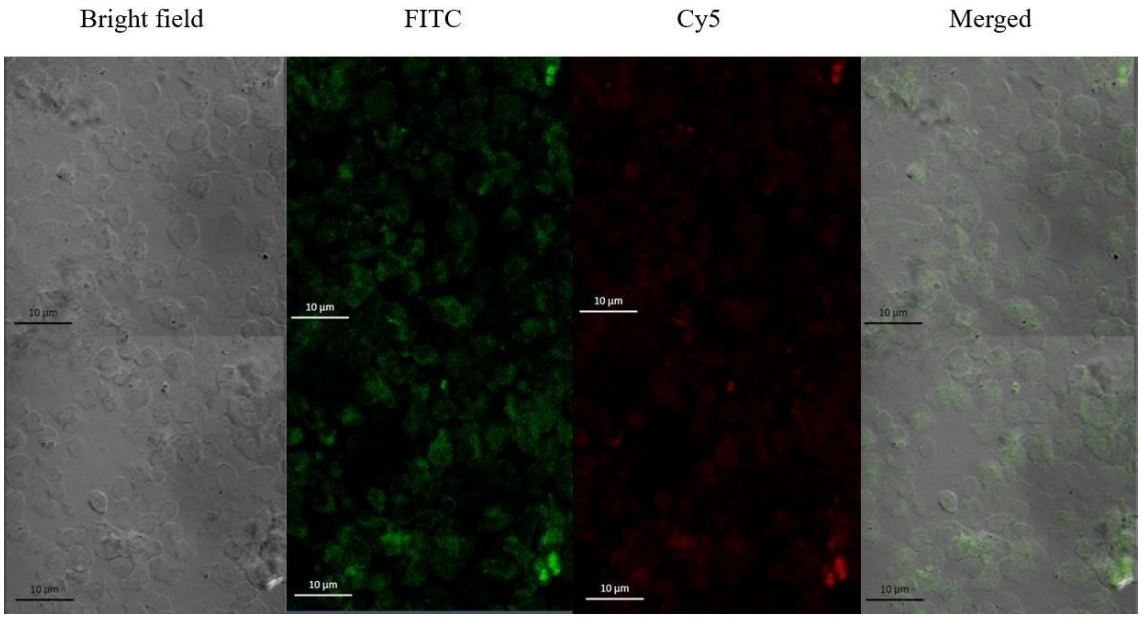

Figure 3.1. Mixed leukocytes isolated from unvaccinated goldfish kidney. FITC and Cy5 channel shows MMs containing autofluroscent pigments with different emission spectrum in green and red respectively. Cells observed in green in the merged image channel represents autofluorescent MMs. Colocalization of autofluroscent pigments can be noted in the merged channel (Scale bar – 10 µm).

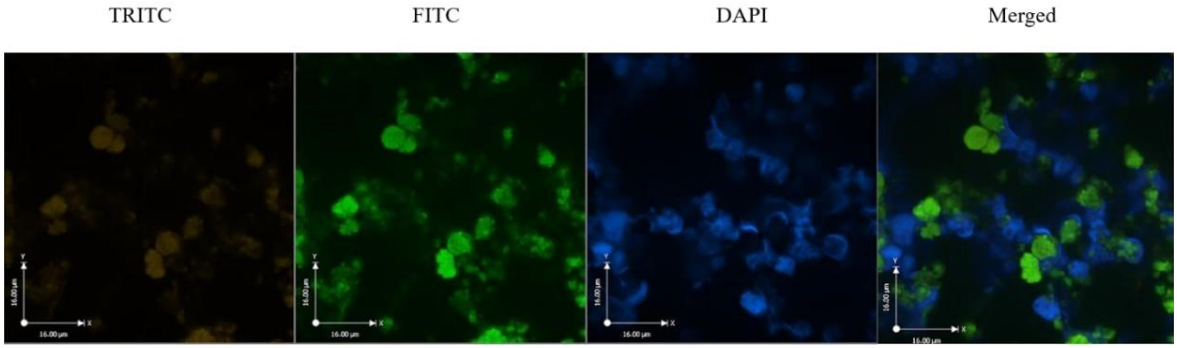

Figure 3.1.a. Mixed leukocytes isolated from unvaccinated goldfish kidney with nuclear staining. TRITC and Cy5 channel shows MMs containing autofluroscent pigments with different emission spectrum in orange and green respectively. Cells observed in DAPI channel showed the nuclei along with autofluorescence emission in DAPI channel. Colocalization of autofluroscent pigments can be noted in the merged channel (Scale bar – 16 µm).

Figures 3.1.1. a, 3.1.1. b, 3.1.1. c, & 3.1.1. d represents the emission spectrum of auto-fluorescent pigments in the goldfish kidney MMs when excited with DAPI (405 nm), FITC (488 nm), TRITC (543 nm) and Cy5 (633 nm) lasers respectively. Similar observations were seen in goldfish spleen samples.

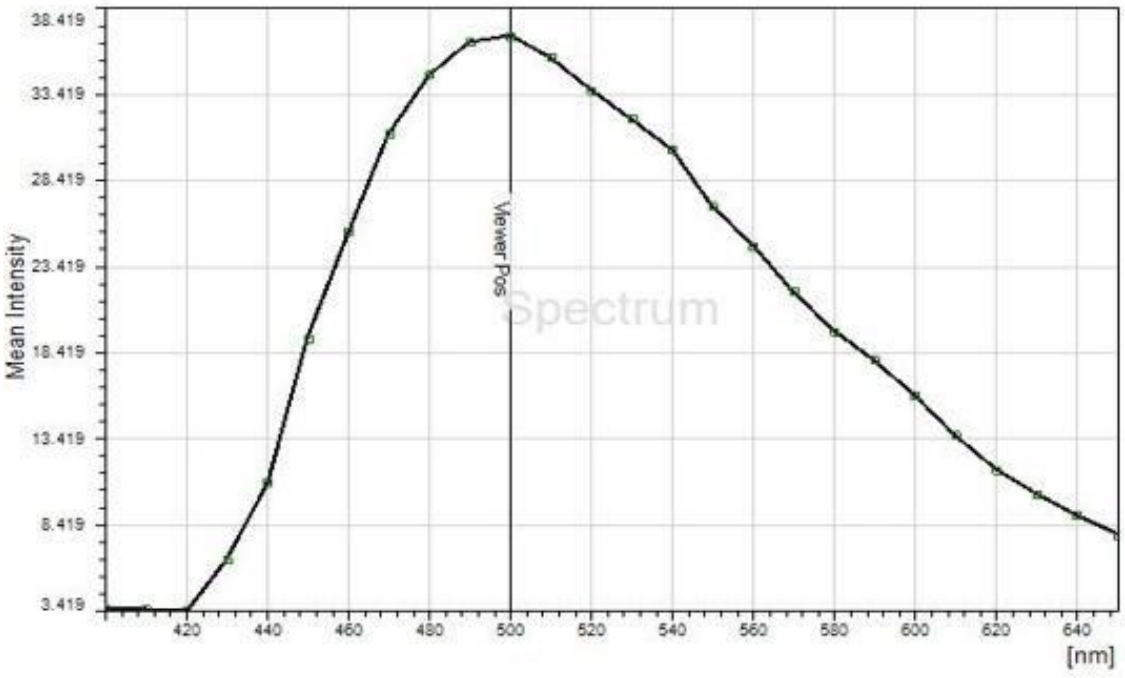

Figure 3.1.1 a) Lambda profile of unvaccinated goldfish MMs on excitation with DAPI laser (405 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Leukocytes were isolated from the kidney of unvaccinated goldfish. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

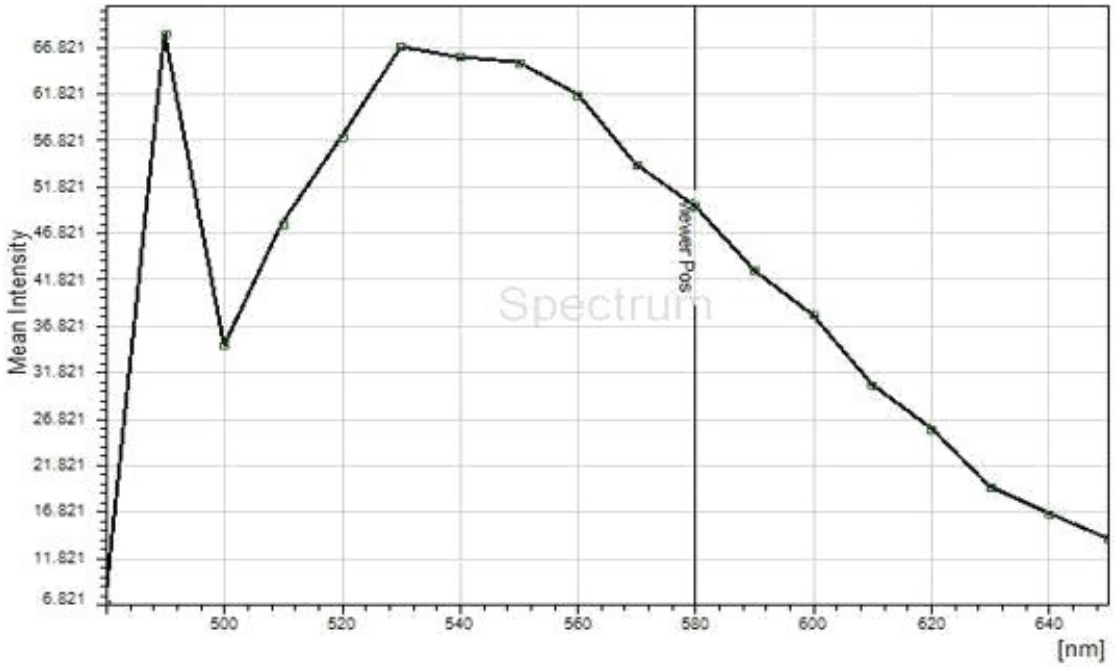

Figure 3.1.1 b) Lambda profile of unvaccinated goldfish MMs on excitation with FITC laser (488 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Leukocytes were isolated from the kidney of unvaccinated goldfish. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

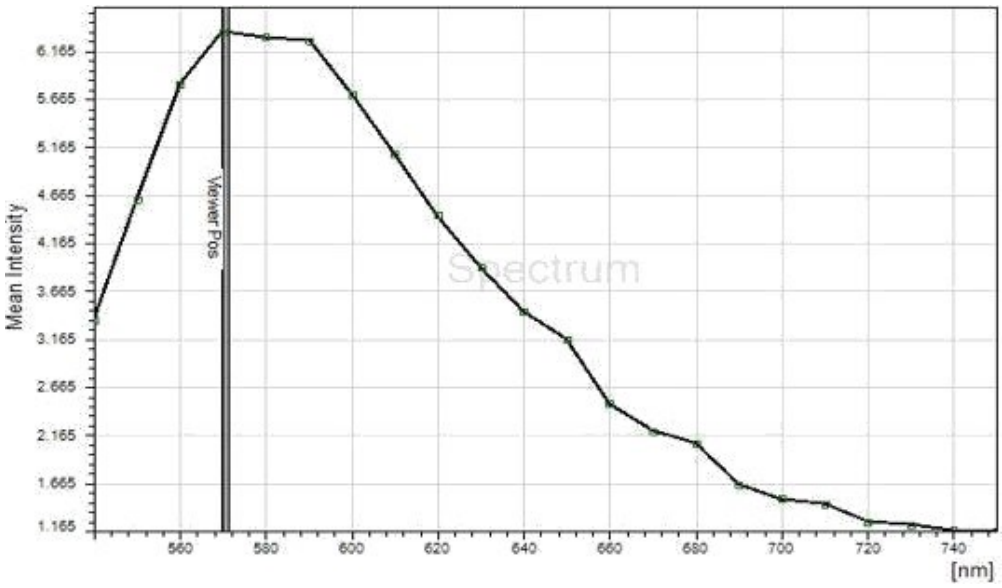

Figure 3.1.1 c) Lambda profile of unvaccinated goldfish MMs on excitation with TRITC laser (543 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Leukocytes were isolated from the kidney of unvaccinated goldfish. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

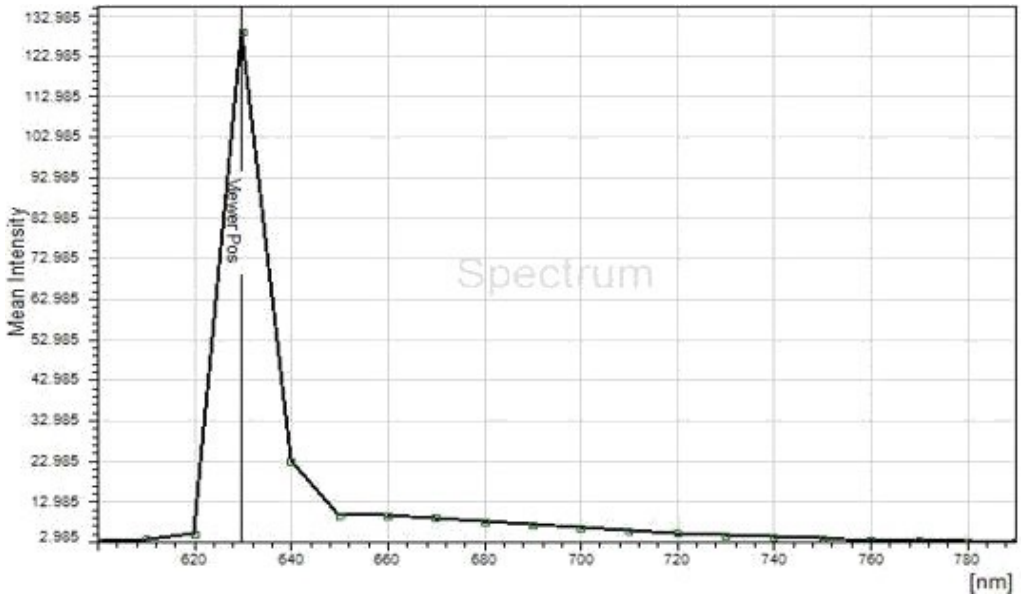

Figure 3.1.1 d) Lambda profile of unvaccinated goldfish MMs on excitation with Cy5 laser (633 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Leukocytes were isolated from the kidney of unvaccinated goldfish. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

Figures 3.1.2. a, 3.1.2. b, 3.1.2. c, & 3.1.2. d represents the emission spectrum of auto-fluorescent pigments in the zebrafish kidney MMs when excited with DAPI (405 nm), FITC (488 nm), TRITC (543 nm) and Cy5 (633 nm) lasers respectively. Similar observations were seen in zebrafish spleen samples.

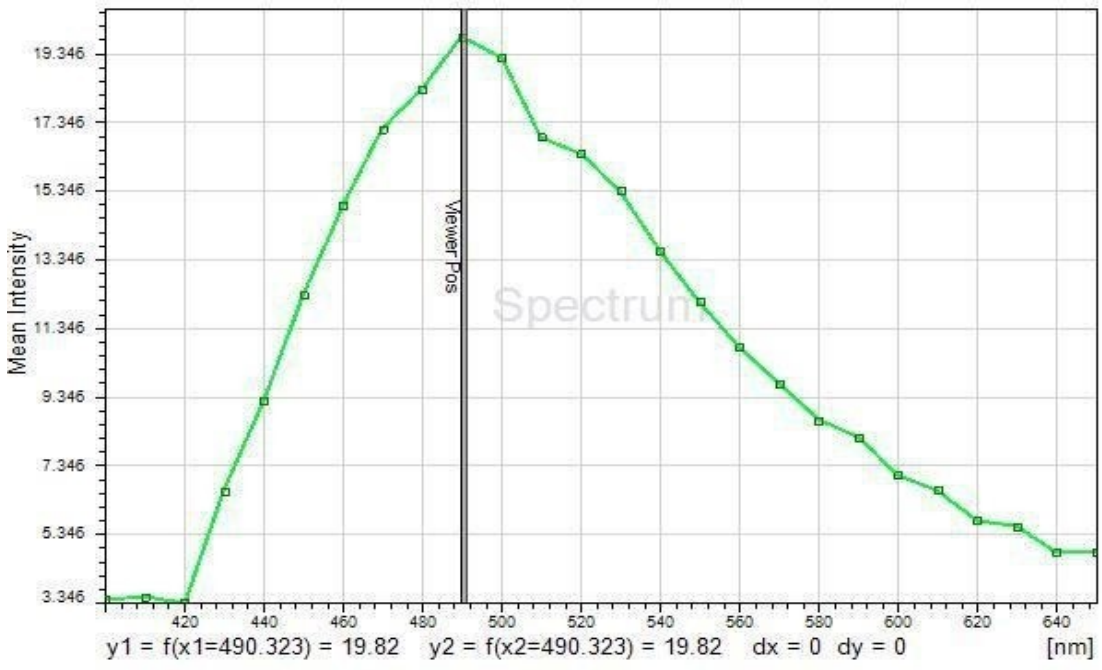

Figure 3.1.2. a) Lambda profile of unvaccinated zebrafish MMs on excitation with DAPI laser (405 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Whole kidney tissue isolated from unvaccinated zebrafish was used to perform LSCM. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

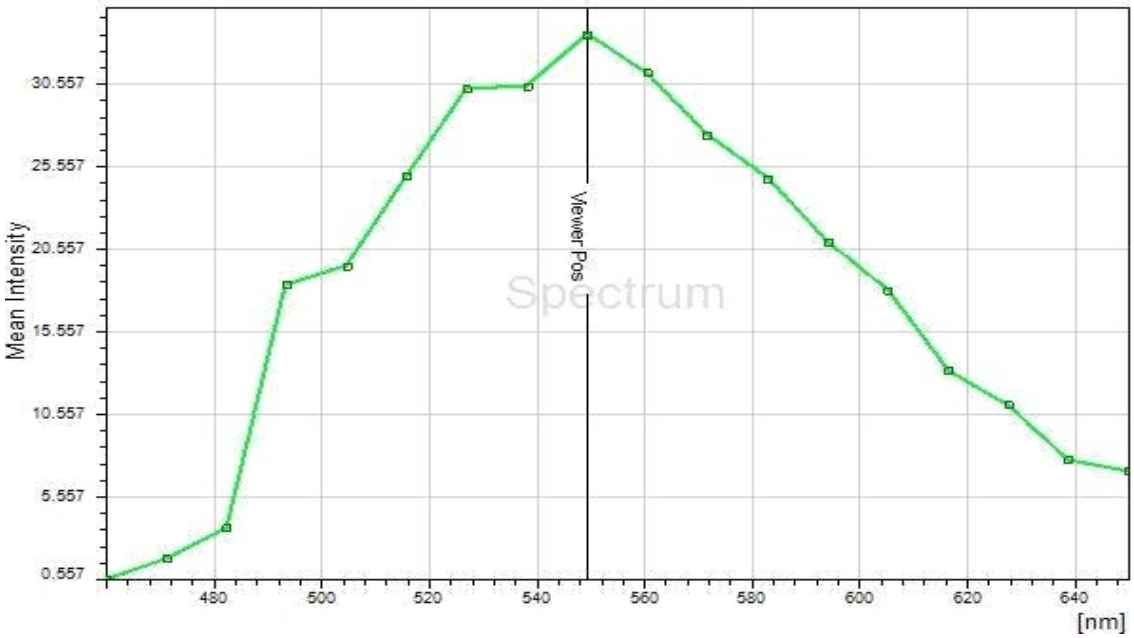

Figure 3.1.2. b) Lambda profile of unvaccinated zebrafish MMs on excitation with FITC laser (488 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Whole kidney tissue isolated from unvaccinated zebrafish was used to perform LSCM. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

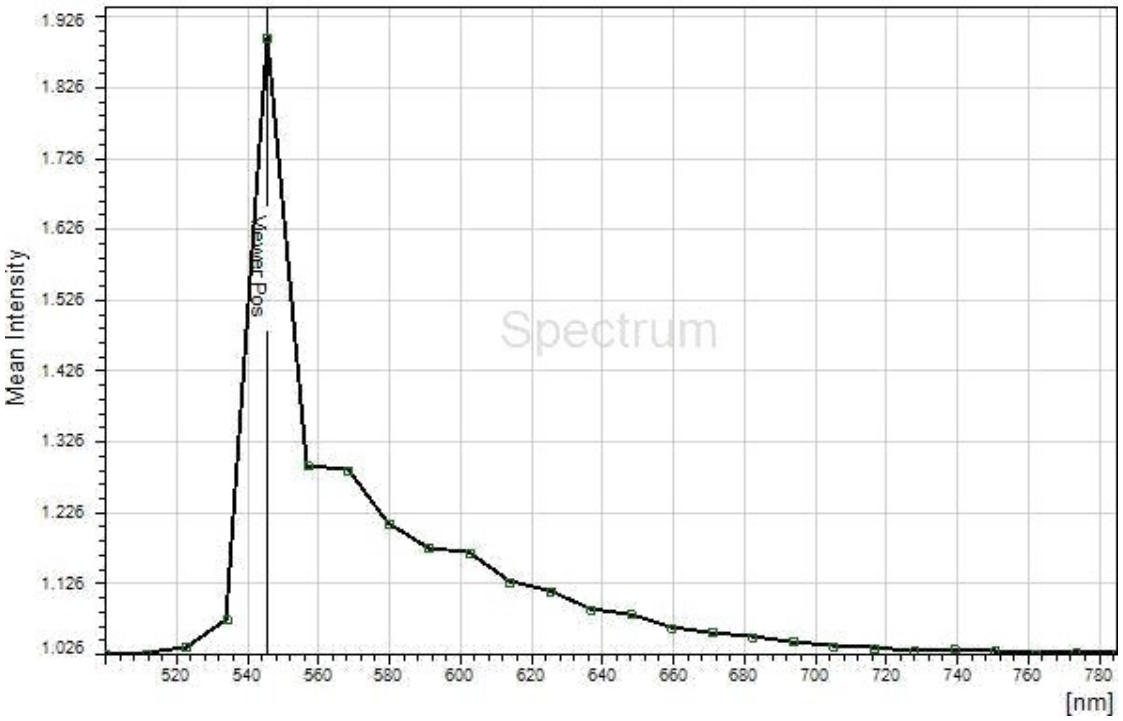

Figure 3.1.2. c) Lambda profile of unvaccinated zebrafish MMs on excitation with TRITC laser (543 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Whole kidney tissue isolated from unvaccinated zebrafish was used to perform LSCM. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

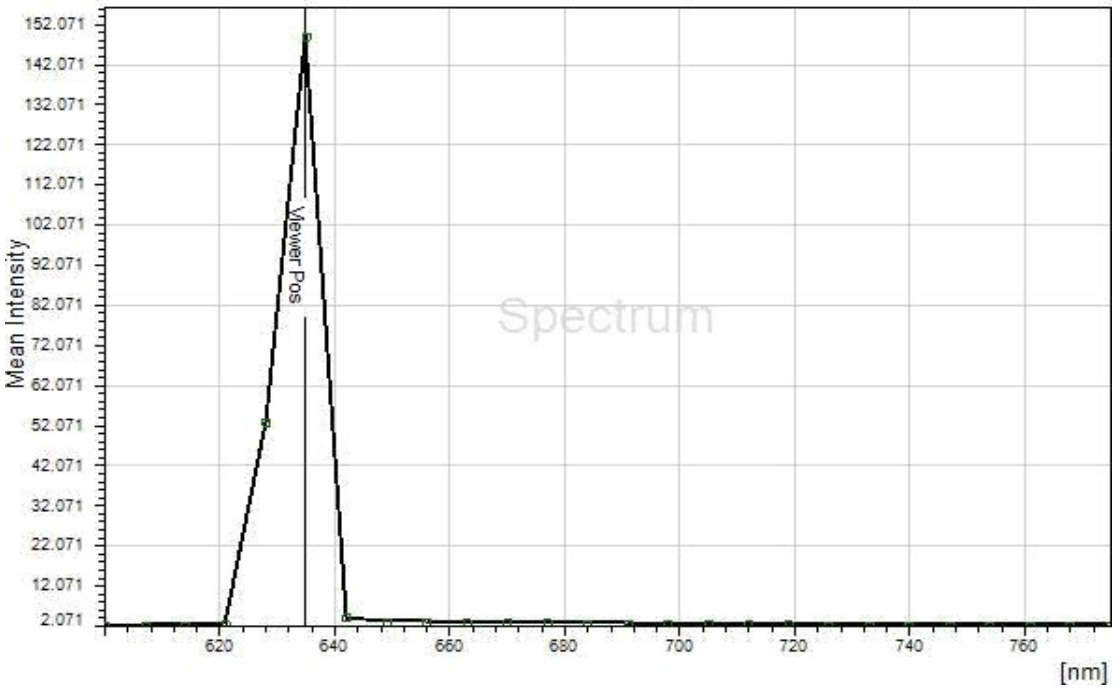

Figure 3.1.2. d) Lambda profile of unvaccinated zebrafish MMs on excitation with Cy5 laser (633 nm). The graph shows the emission spectrum of the auto-fluorescent pigments present in the MMs. Whole kidney tissue isolated from unvaccinated zebrafish was used to perform LSCM. The X-axis shows the wavelength, and Y-axis shows the mean intensity.

As seen from laser scanning confocal microscopy data (Figure 3.1.1 & 3.1.2), there is emission from the autofluorescent pigments in all four channels DAPI (405 nm), FITC (488 nm), TRITC (543 nm), and Cy5 (633 nm) in both goldfish and zebrafish. Nevertheless, Cy5 appears to be the only channel with a narrow emission peak (620-650 nm range). Lasers with excitation above 633 nm or below 405 nm are not available at the University of Alberta microscope facility at the moment. Due to this, we decided to use Alexa fluor 647 a fluorescent protein label (emission in the Cy5 channel) for our experiments.

3.1.2 Identification of antigen retaining cells in goldfish and zebrafish lymphoid organs using confocal microscopy

Commercially available antigen BSA-conjugated to Alexa fluor 647 was used to immunize multiple goldfish and zebrafish. Lymphoid tissues were isolated, and cells were collected from these fish at day 14 and day 21 following the initial immunization with antigen. Confocal microscopy images of these cells showed that autofluorescent MMs vastly took up the injected BSA-Alexa fluor 647. Based on the confocal images, we cannot identify the exact position of antigen in the MMs, but on looking at the Z-stacks and Imaris 3D surface constructions it appears that some of the antigen was present closer to the cell membrane or near the cell surface and some of the antigens are present within the cells possibly in vacuoles. The signals from the different autofluorescent pigments appeared to be colocalized and can be differentiated from Alexa fluor 647 signal in the merged image. The Alexa fluor 647 was seen as distinct signals in the Cy5 channel within the goldfish melanomacrophages (Figure. 3.1.3). Similar observations were noted in zebrafish as well (Figure 3.1.4).

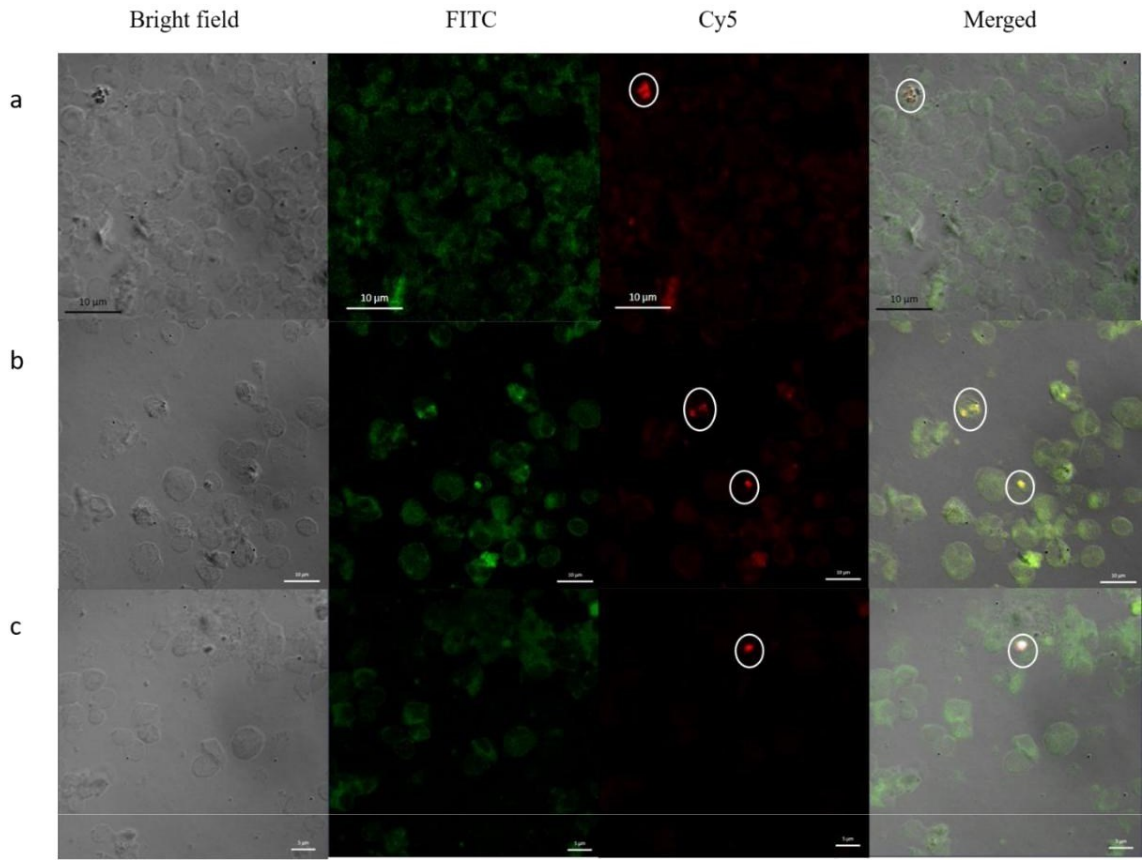

Figure 3.1.3) Retention of experimentally injected labelled antigen by MMs in the goldfish spleen and kidney. Leukocytes isolated from goldfish immunized with BSA-Alexa fluor 647 at day 14. **Bright reddish yellow spots** in the merged image represent the signal from Alexa-fluor 647. a, b) Kidney leukocytes isolated from 2 different immunized goldfish (scale bar - 10 µm), c) Spleen leukocytes isolated from goldfish immunized with Alexa-fluor 647 (scale bar - 5 µm).

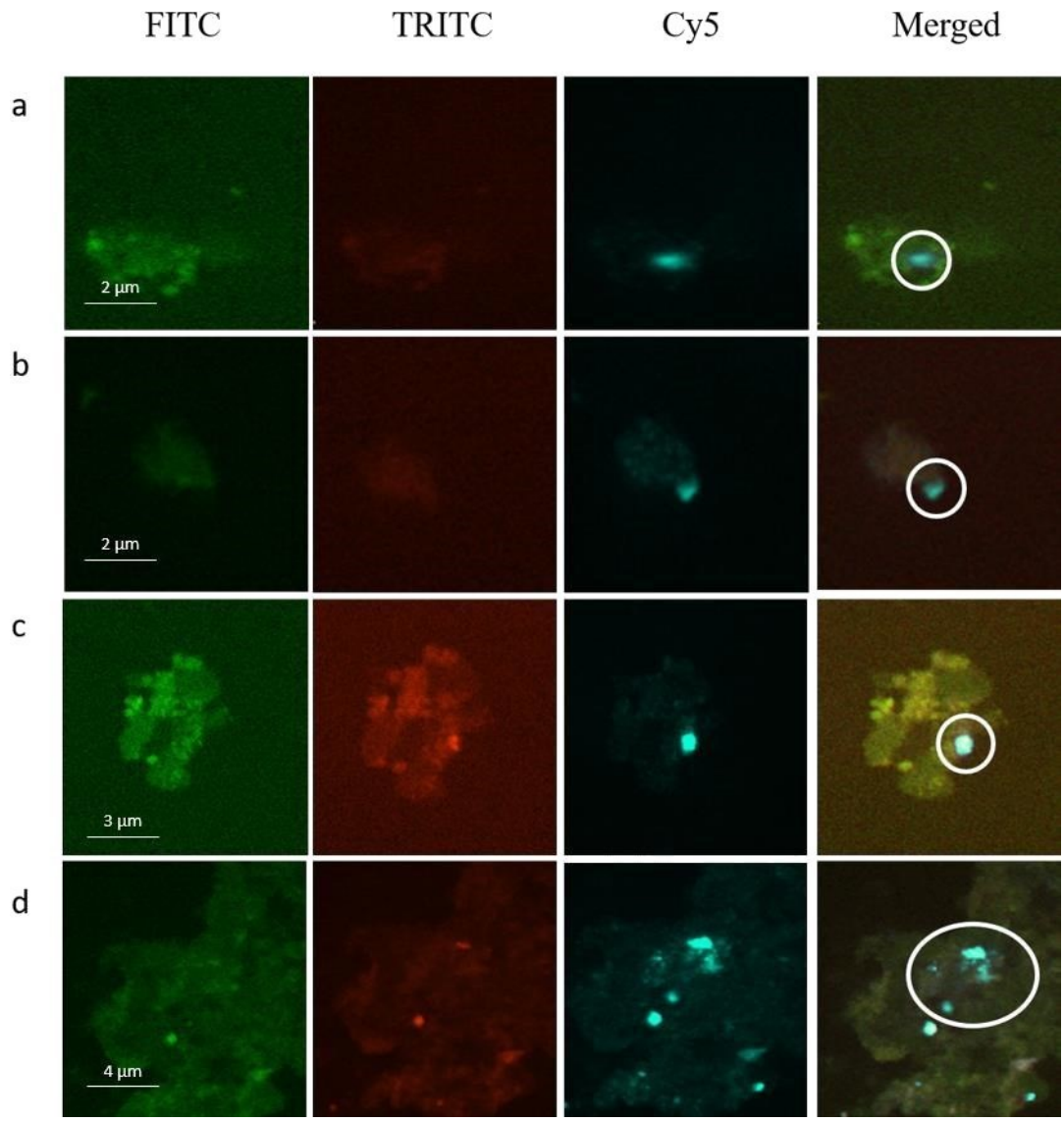

Figure 3.1.4) Retention of experimentally injected labelled antigen by MMs in the zebrafish spleen and kidney. Leukocytes isolated from zebrafish immunized with BSA-Alexa fluor 647 at day 14. Distinct blue spots in the merged image indicate the signal from Alexa-fluor 647. a, b) Spleen leukocytes isolated from immunized zebrafish c & d) Are kidney leukocytes isolated from 2 different zebrafish immunized with Alexa-fluor 647.

Figure 3.1.5 shows the emission spectrum of melanomacrophages in BSA-Alexa fluor 647 immunized goldfish when excited with Cy5 laser (633 nm). On comparing Figure 3.1.5 and 3.1.1. d, we can see that the excitation peak in unvaccinated fish is in the range of 620-650 nm, whereas, in the case of vaccinated fish, there are two peaks. The first peak is in between 620-650 nm with a maximum mean intensity of 42; the second peak is in the range of 640-750 nm with a maximum mean intensity of 243. From these two figures, it is clear that there is a fivefold difference in mean intensity values of the two peaks seen within the vaccinated fish. These observations also suggest that the emission spectrum seen in the 620-650 nm range is due to auto-fluorescent pigments, and the emission seen in 640-750 nm range is due Alexa-fluor 647.

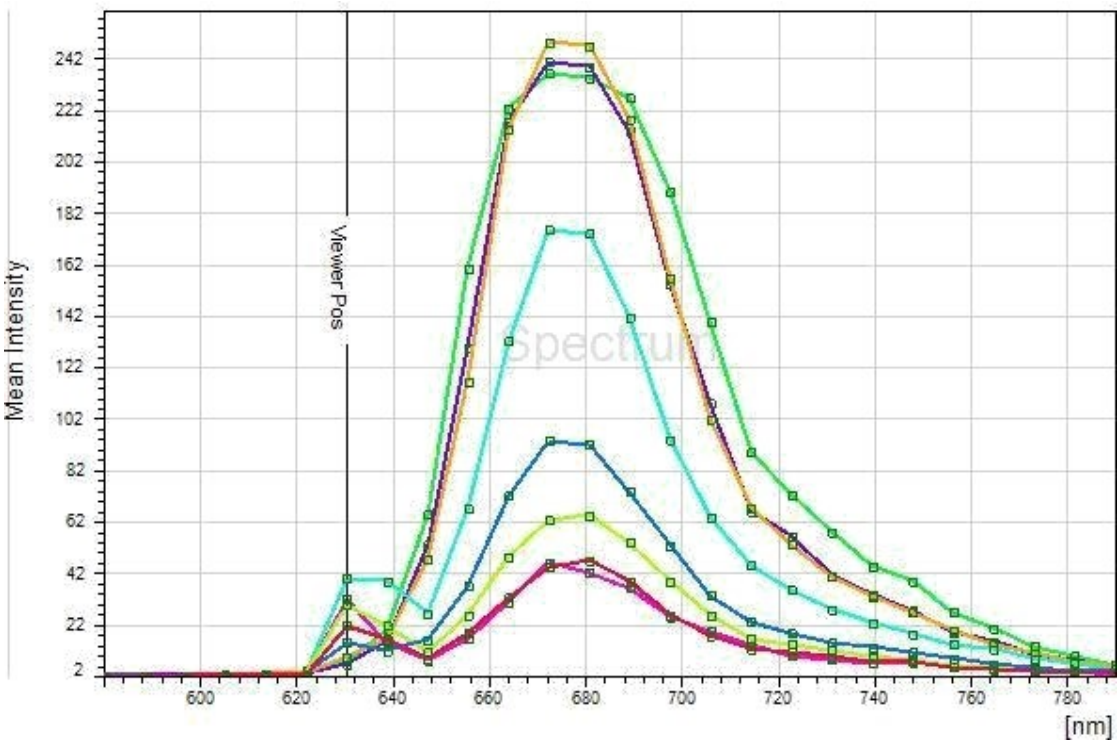

Figure 3.1.5 Lambda profile of MMs isolated from BSA-Alexa fluor 647 injected goldfish on excitation with Cy5 laser (633 nm). Leukocytes were isolated from the kidney of immunized goldfish at day 14. The X-axis shows the wavelength, and Y-axis shows the mean intensity. Each coloured line graph represents the emission spectrum of individual cells present in Figure 3.1.6. 620-650 nm peak represents the emission from auto-fluorescent pigments and 640-740 nm peak represents the emission from Alexa fluor 647.

Based on Figure 3.1.3 & 3.1.4, we can say that among our two candidates MMs and reticular cells, MMs appears to be retaining the antigen. MMs are also phagocytes, so just based on the confocal images it is not possible to conclude MMs are capturing the antigen in a way similar to that of FDCs.

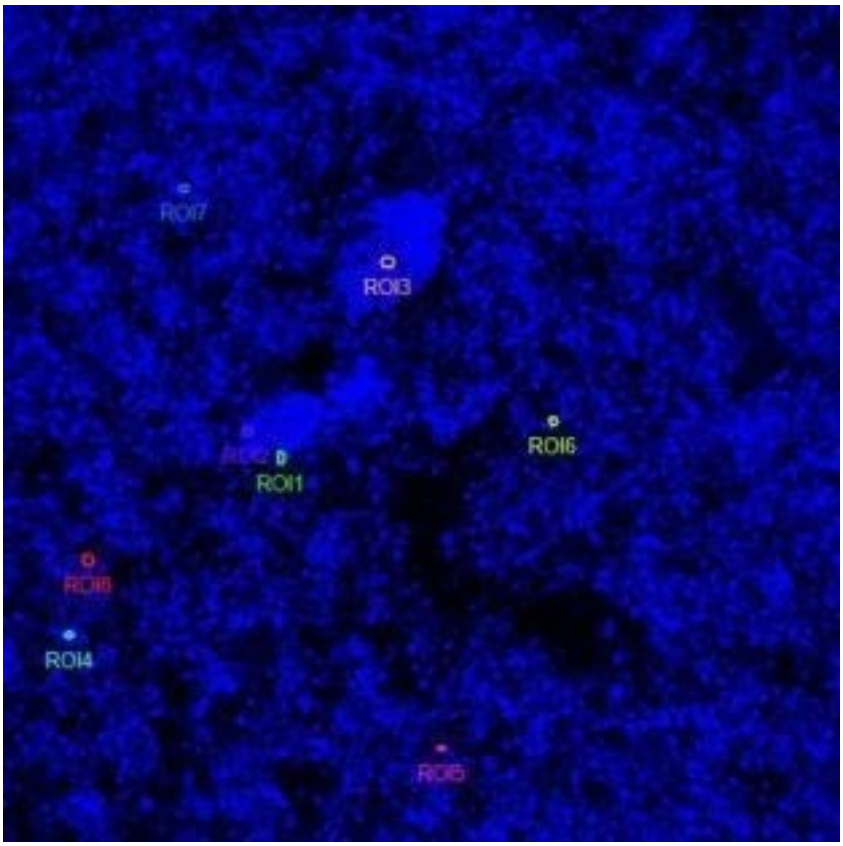

Figure 3.1.6 Cells corresponding to lambda profile of MMs isolated from BSA-Alexa fluor 647 injected goldfish on excitation with Cy5 laser (633 nm). Leukocytes were isolated from the kidney of immunized goldfish at day 14. Blue color in the figure represents cells emitting auto–fluorescence when hit with 633 nm and emission from BSA-Alexa fluor 647. Circled region of interests (ROI) corresponds to individual cells and emission intensities of each ROI is represented in figure 3.1.5. Variation in the emission intensity between ROIs cannot be visualized by this single image since analysis was performed using Z-stack and emission intensities at different wavelengths within the same cell is represented in the Figure 3.1.5.

3.2 Identification of the specific cell type in goldfish lymphoid organs that are involved in the retention of experimentally injected antigen on the cell surface in an intact form

To differentiate phagocytosis from antigen retention (intact form) on the cell surface, we isolated leukocytes from BSA immunized goldfish and exposed these cells to protein G beads conjugated to an anti-BSA antibody. Cells with the intact BSA on the cell surface is expected to bind to the beads (Figure 3.2.1). To look for non-specific interactions leukocytes isolated from the spleen and kidney of unvaccinated goldfish were exposed to Protein G beads – anti-BSA antibody complex and a second negative control group looked at leukocytes from unvaccinated fish exposed to protein G beads (no conjugated antibody).

The beads were later eluted, and eluted samples were analyzed using a confocal microscope. Results of this assay (BSA-immunized goldfish leukocytes exposed to protein G beads-anti-BSA antibody) showed that among the cells that bound the beads more than 80% of the eluted cell population constituted the autofluorescent MMs. At the same time, the identity of the remaining 20% of cells that bound the beads is unknown. Negative controls showed low levels of non-specific binding of cells to the beads, but on comparing the number of bounds cells from the negative control to the treatment group, there is a six-fold difference (Table 3.2.1). These data suggest that MMs obtained from the BSA-immunized fish contain some amount of intact antigen on their cell surface (Figure 3.2.2). Based on the numbers from this assay, it appears that 1-6% of the total MM population is involved in retaining the antigen on the cell surface (Table 3.2.2).

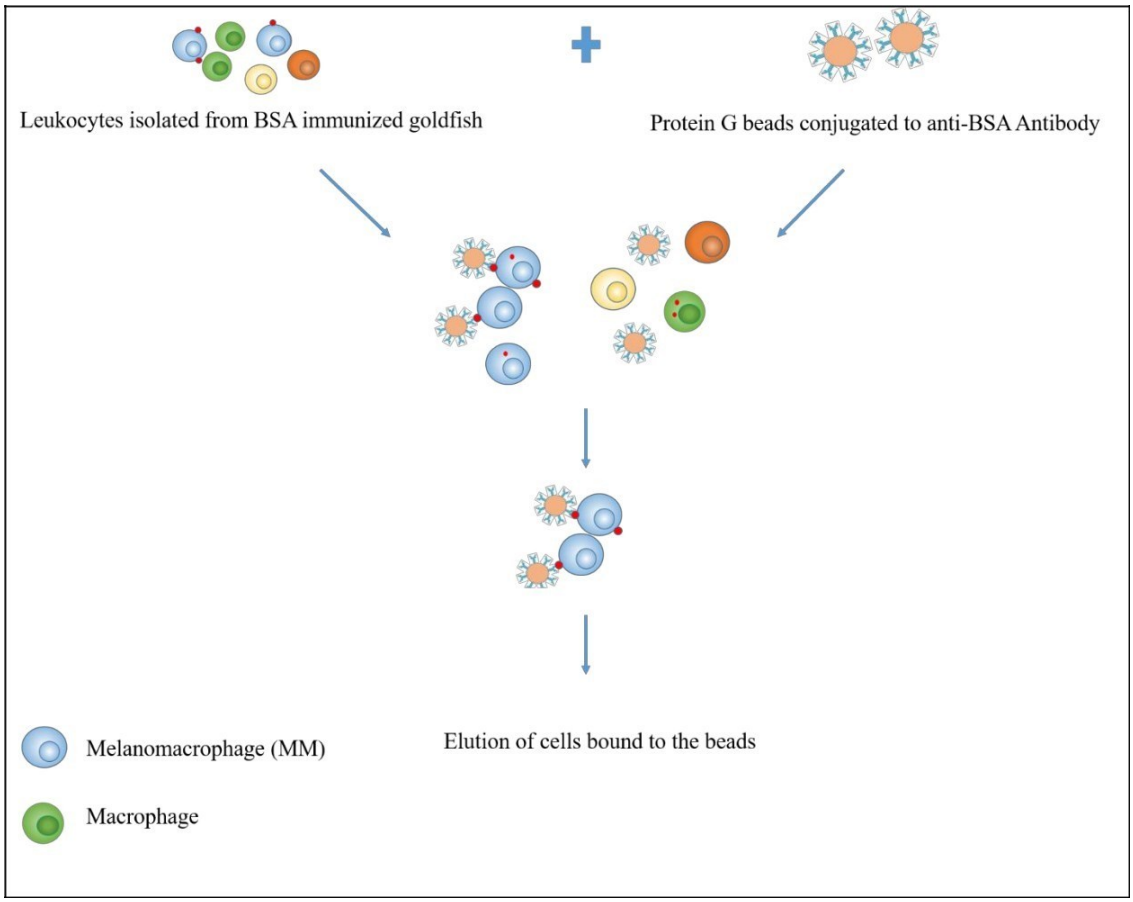

Figure 3.2.1. Schematic showing the binding of cells retaining intact antigen on the cell surface to protein G beads - anti-BSA antibody. As shown in the schematic MMs (blue cells) and macrophages (green cells) both are expected to phagocyte the antigen. Macrophages degrade the internalized antigen (except for SCS macrophages-discussed in Chapter 4), and FDCs captures the intact antigen on the cell surface. If MMs are retaining the antigen on the cell surface in an intact form, then they will be able to bind to the protein G beads conjugated to the anti-BSA antibody.

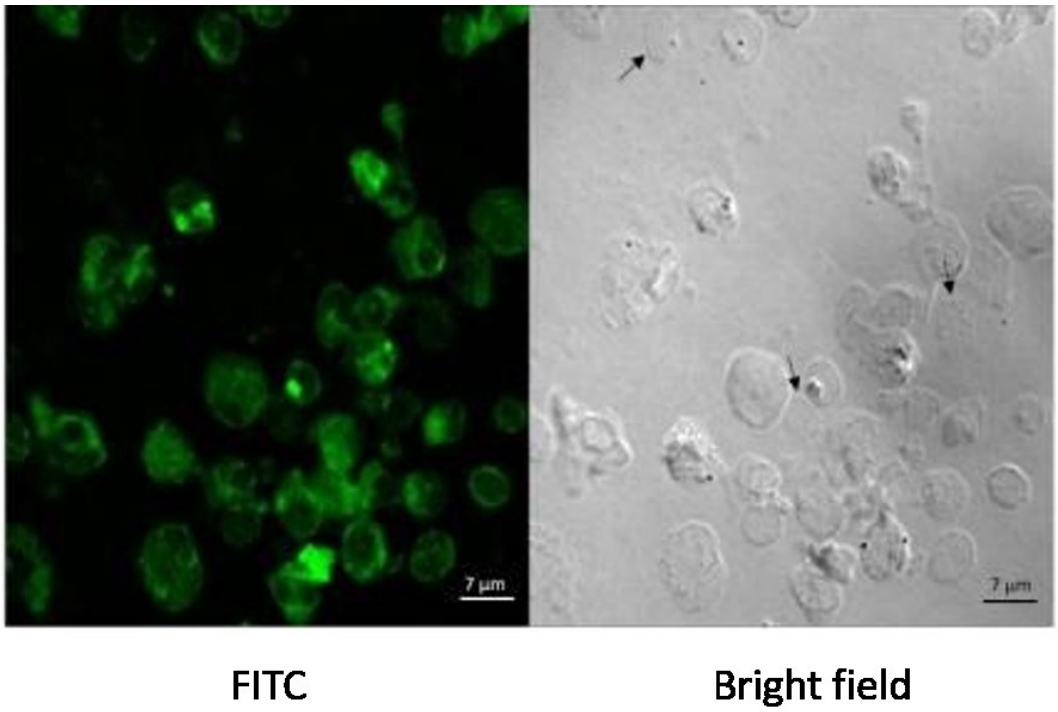

Figure 3.2.2. Intact antigen retained on the surface of MMs. Elution of anti-BSA - Protein G beads exposed to spleen and kidney leukocytes isolated from BSA immunized goldfish showed the presence of MMs indicating retention of intact antigen on the surface of MMs. Green cells are the autofluorescent MMs observed in the FITC channel, and the bright field image shows the all the cells present in the eluted sample. Arrowheads in the bright field image point three different non-autofluorescent cells (not MMs) in the slide (Scale bar – 7 µm).

	Leukocytes from BSA vaccinated goldfish + (Protein G – anti-BSA Ab)		Leukocytes from unvaccinated goldfish + (Protein G – anti-BSA Ab)		Leukocytes from unvaccinated goldfish + Protein G beads	
	Autofluroscent Cells (MMs)	Non-autofluroscent cells	Autofluroscent Cells (MMs)	Non-autofluroscent cells	Autofluroscent Cells (MMs)	Non-autofluroscent cells
Goldfish 1	1134	224	137	109	221	123
Goldfish 2	3216	518	296	131	108	336
Goldfish 3	1381	349	56	204	74	129

Table 3.2.1. Counts for the different cells eluted from the three different bead treatments for 9 different goldfish.

	Average of the total number of cells bound to the beads	Average of the total number of MMs bound to the beads	% of MMs bound to the beads
Leukocytes from BSA vaccinated goldfish + (Protein G – anti-BSA Ab)	2274	1910	84
Leukocytes from unvaccinated goldfish + (Protein G – anti-BSA Ab)	311	163	52
Leukocytes from unvaccinated goldfish + Protein G beads	330	134	40

Table 3.2.2. Average number of cells bound to beads for the three different treatments from 9 different goldfish.

3.3 Identification of FDC markers in antigen retaining cells through PCR in goldfish

Our preliminary results suggested that MMs are involved in antigen retention, and to investigate this further, we isolated MMs from the total leukocyte population (spleen and kidney) of goldfish through FACS. Peripheral blood leukocytes obtained from goldfish are known to lack the autofluorescent MMs; hence they are used to set the negative gates based on previously established parameters (Wang 1995; Barreda, Hanington et al. 2005) (appendix Figure A.2, A.3 &A.4).

Total RNA was isolated from the sorted MMs, and PCR was performed using primers for FDC markers like BAFF and CXCL13. Results showed that the MMs do express some of the FDC markers like BAFF, CXCL13 (Figure 3.3.1 & 3.3.2). We also found that MMs are positive for RANKL (discussed in Chapter 4). The identity of all the obtained bands was confirmed through Sangers sequencing. Presence of Mfge8, which is another FDC marker, was confirmed in MMs (Work by undergrad - unpublished data).

PCR performed on reticular cells (discussed in section 2.12) showed that they do not express BAFF or CXCL13, thereby lowering the chances of reticular cells being the antigen retaining cell.

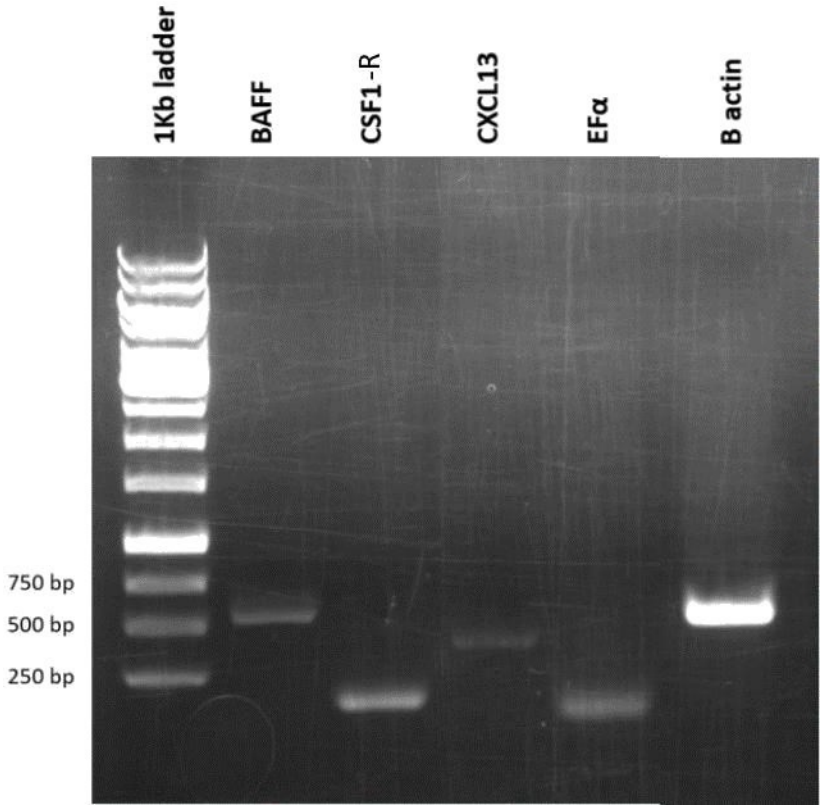

Figure 3.3.1 Polymerase chain reaction showing the genes present in MMs (BAFF, CSF1-R, CXCL13). MM shows the presence of expected bands for BAFF, CSF1-R, CXCL13. EF1αand B actin was used as a positive control (Annealing temperature 64º C and number of cycles 35).

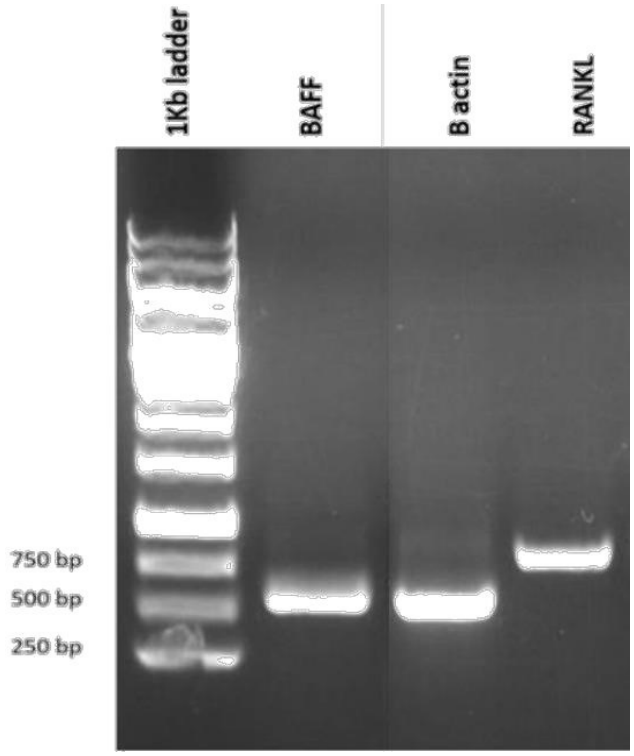

Figure 3.3.2 Polymerase chain reaction showing the genes present in MMs (BAFF and RANKL). MM shows the presence of expected bands for BAFF and RANKL. B actin used as a positive control (Annealing temperature 64º C and number of cycles 35).

3.4 Isolation of immune complexes from goldfish immune serum and identification of isolated components through mass spectrometry

In mammals and birds, immune complexes (antigen bound by antibodies and/or complement proteins) can bind to FcRs or CRs. FDCs are known to capture these opsonized antigens (immune complexes) on their cell surface. To see if the injected antigen in goldfish binds to an antibody or complement protein or both we obtained serum from BSA, KLH or PE immunized goldfish and performed immunoprecipitation using protein G beads bound to antibodies against the injected antigen (anti-BSA, anti-KLH or anti-PE).

Samples for the immunoprecipitation included three immune serum treatments, one stripped of antibodies (antibody removal using protein G beads conjugated to WCI12) (Rombout et al., 1993), second heat denaturation of complement proteins (56 ºC for 1hr) (Giard, 1987), and third an untreated immune serum which includes both antibodies and complement proteins. These three treatments were then exposed to Protein G beads conjugated to anti-BSA, anti-KLH or anti-PE antibodies. Silver staining of the SDS-PAGE gel showed the presence of specific bands around 35KDa in the first (absence of antibody, presence of complement proteins) and third (has both antibodies and complement proteins) treatment samples (Figure.3.4).

Based on the size, the obtained bands were predicted to be inactivated C3d (~35 kDa) or other cleaved complement proteins. However, the identity of the obtained band was not revealed through in-gel digestion and mass spectrometry analysis (further discussed in chapter 4).

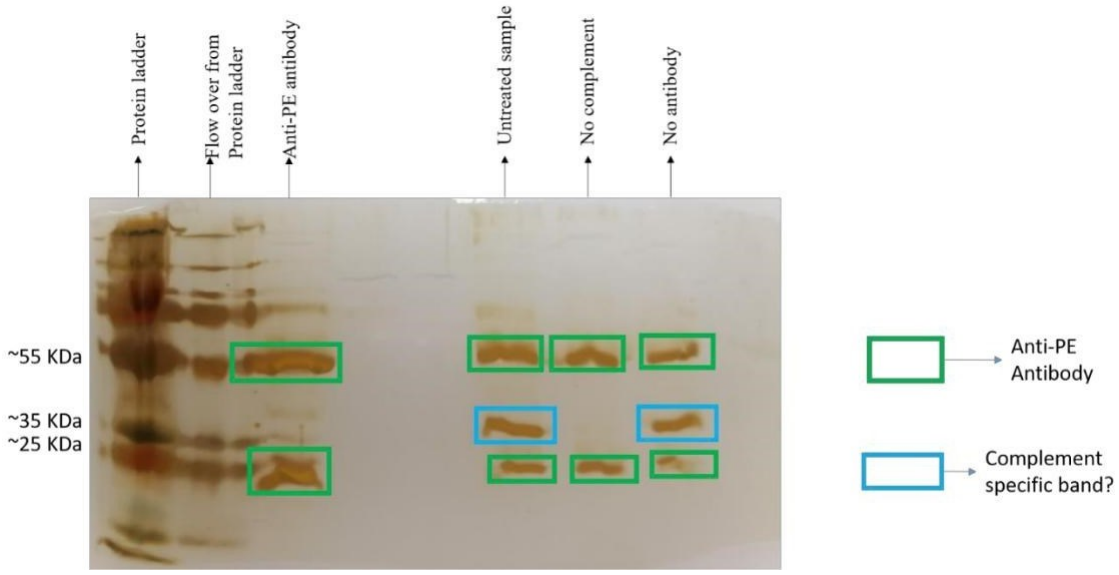

Figure 3.4. IP of goldfish immune serum (PE injected fish) followed by silver staining. Bands highlighted with green indicates the heavy (~55KDa) and light chains (~25KDa) of the anti-PE antibody, whereas the bands highlighted with blue indicates the specific bands (~ 35KDa) obtained.

3.5 In-gel digestion and whole lane analysis of goldfish melanomacrophage whole cell lysate

Experimental attempts made to isolate the receptor-associated to antigen retention in goldfish MMs through Co-IPs were unsuccessful after multiple trials (discussed in chapter 4). Hence, as an alternative, a non-specific approach was used where the protein profile of MMs was obtained by performing mass spectrometry analysis on MM whole cell lysate. Mass spectrometry analysis showed the presence of different housekeeping proteins, some of which are represented in Table 3.5. The score column shown in Table 3.5 is the sum of all peptides that were identified. Coverage represents the percentage of the protein sequence that is covered by the identified peptides. It is the total number of identified amino acids in the peptides divided by the number of amino acids in the protein.

Along with housekeeping proteins, we observed the presence of transferrins and C3 complement protein (Carassius gibelio) in goldfish MMs (further discussed in chapter 4). We did not observe the presence of potential antigen retaining receptors such as FcR-like or Poly IgR or complement receptors, which we hoped to see in MMs.

Table 3.5. Results from mass spectrometry whole lane analysis of goldfish MMs (isolated from BSA immunized fish) whole cell lysate.

Accession	Description	Score	Coverage	# Unique Peptides
Q8UVE7	Serotransferrin OS=Cyprinus carpio	320.26	44.84	14
Q8JHD1	Serotransferrin OS=Carassius auratus	189.16	16.34	1
U5XYT4	Vitellogenin B variant 1 OS=Carassius auratus ssp.	157.29	21.73	1
Q7T1G9	Serotransferrin OS=Carassius cuvieri	153.99	26.08	6
U5XWG2	Vitellogenin B variant 2 (Fragment) OS=Carassius auratus ssp.	153.31	22.84	2
Q156A2	Vitellogenin (Fragment) OS=Carassius auratus	147.74	18.84	1
Q7T1G8	Serotransferrin OS=Carassius cuvieri	106.12	19.55	3
T1WGW0	Complement C3 OS=Carassius gibelio	103.38	13.52	22
I6QQA0	Transferrin b (Fragment) OS=Cyprinus carpio	86.97	58.87	1
I6QXU6	Transferrin a (Fragment) OS=Cyprinus carpio	75.09	62.41	1
A0A2Z6E9K4	Beta-actin OS=Carassius auratus	70.40	33.60	4
I3WTR0	Vitellogenin (Fragment) OS=Carassius auratus	66.31	30.18	2
H2DQ56	Glutamate dehydrogenase OS=Carassius auratus red var.	59.72	27.96	14
R4NII6	Keratin 8 OS=Carassius auratus	59.46	25.72	14
Q8UVE9	Serotransferrin OS=Carassius gibelio	54.25	12.26	3

3.6 RNA-Sequencing data analysis of spleen and kidney melanomacrophages

PCR analysis of goldfish MMs (Chapter 3.3) showed the presence of few FDC like genes (BAFF, CXCL13 and Mfge8) in them. The goldfish genome has more than 30-40 predicted sequences available for FcR-like, CR-like and PIgR-like isoforms in the database. And It is time-consuming to look into each of these candidates. To narrow down the list of potential antigen retaining receptors in goldfish MMs, and to look at the gene profile of goldfish melanomacrophages we did RNA-seq on MMs isolated from the spleen and kidney of 5 different unvaccinated goldfish. Low Input RNA sequencing with 2 X 150 bp PE was done, and 6 Gb data per sample (~40 M reads) was obtained (Sequencing and analysis were done at LC Sciences, Texas, USA).

FastQC report showed that 51041414 and 57385958 valid reads were obtained from the raw data (53645656 & 69061580) of kidney and spleen MMs. The valid ratio of 95.15 and 83.09 was obtained for the kidney and spleen MM samples, respectively. The Q20 % score (Probability of Incorrect Base Call (1 in 100)) was above 99.9 (Base call accuracy) for both samples, and Q30 % score (Probability of Incorrect Base Call (1 in 1000)) was 98.23 for kidney MMs and 96.72 for spleen MMs suggesting the reads are of good quality.

On mapping the obtained reads to goldfish genome, we found that in the kidney MMs sample, 57.93 % of the reads were mapped among which 39.72 % were unique reads (reads that align to just one region of the reference genome), and 18.22 % were

multi mapped reads (reads that align to more than one region of the reference genome). In the case of the spleen MMs sample, only 10.10% of the obtained reads mapped to the goldfish genome, among which 6.67 % were unique reads, and 3.43 % were multi mapped reads. The low overall alignment rate in spleen MM data suggest that any analysis done on spleen MMs sample would be less reliable and not accurate (further discussed in chapter 4).

3.6.1 FDC like genes identified in melanomacrophages

Follicular dendritic cells are known to express BAFF, CXCL13, and Mfge8 along with FcRs and CRs. These were few genes we expected to see on antigen retaining cells in fish. Polymeric immunoglobulin receptors (PIgRs) were also among the potential candidates for antigen retaining receptors in fish. Read count-based analysis performed using HTseq-counts showed the presence of BAFF-like, FcR-like, CR-like and PIgR-like isoforms in the goldfish kidney MMs (Table 3.6.1). Read counts indicates the number of reads that maps to each gene in the reference genome (Law et al., 2014). As seen in Table 3.6.1, most of the genes have a higher read count in kidney MM sample but has a read count value of zero in the spleen MM sample. This drastic difference in the two samples is most likely due to low alignment rates of spleen sample rather than the absence of gene (discussed in Chapter 4).

Table 3.6.1. List of FDC like genes and their read counts in the spleen and kidney MMs.

Gene_ID	Transcript_ID	Description	Kidney MM counts	Spleen MM counts
113042005	XM_026200605.1	high affinity immunoglobulin gamma Fc receptor I-like [Carassius auratus]	367	1704
113052058	XM_026216338.1	IgGFc-binding protein-like [Carassius auratus]	33	0
113065145	XM_026236359.1	Fc receptor-like protein 5 isoform X5 [Carassius auratus]	232	0
113067641	XM_026240016.1	Fc receptor-like protein 2 isoform X1 [Carassius auratus]	9	0
113108918	XM_026272338.1	tumor necrosis factor ligand superfamily member 13B-like isoform X1 [Carassius auratus] (BAFF)	3415	0
113065508	XM_026236798.1	complement receptor type 1-like isoform X2 [Carassius auratus]	6365	0
113070510	XM_026243819.1	complement receptor type 1-like isoform X1 [Carassius auratus]	333	0
113066877	XM_026238969.1	lactadherin-like isoform X1 [Carassius auratus] (Mfge8)	0	0
113106241	XM_026267950.1	lactadherin-like [Carassius auratus] (Mfge8)	0	0
113086155	XM_026255346.1	polymeric immunoglobulin receptor-like isoform X3 [Carassius auratus]	564	459
113063187	XM_026233413.1	polymeric immunoglobulin receptor-like isoform X2 [Carassius auratus]	2401	0
113086158	XM_026255349.1	polymeric immunoglobulin receptor-like isoform X2 [Carassius auratus]	422	69
113115126	XM_026282462.1	polymeric immunoglobulin receptor-like [Carassius auratus]	1916	0
113119817	XM_026289485.1	polymeric immunoglobulin receptor-like [Carassius auratus]	222	0
113119814	XM_026289482.1	polymeric immunoglobulin receptor-like [Carassius auratus]	6021	0
113096944	XM_026262157.1	polymeric immunoglobulin receptor-like [Carassius auratus]	959	0
113086588	XM_026255458.1	polymeric immunoglobulin receptor-like [Carassius auratus]	79	0
113086587	XM_026255457.1	polymeric immunoglobulin receptor-like [Carassius auratus]	592	531
113082659	XM_026254202.1	polymeric immunoglobulin receptor-like [Carassius auratus]	246	0
113082657	XM_026254201.1	polymeric immunoglobulin receptor-like [Carassius auratus]	1337	73
113082655	XM_026254198.1	polymeric immunoglobulin receptor-like isoform X1 [Carassius auratus]	154	108

3.6.2 Gene expression profile of melanomacrophages

Table 3.6.2. shows the list of genes present in the spleen and kidney MM along with the read counts. Most of the genes shown in Table 3.6.2 are involved in regulating cell cycle, protein degradation, cytoskeleton arrangement and other housekeeping functions. We did find a few unexpected genes like cilia- and flagella-associated protein 206 and T-cell activation Rho GTPase-activating protein in MMs (not shown in the table) (discussed in Chapter 4).

Table 3.6.2. Gene profile of MMs isolated from spleen and kidney of the goldfish

Gene ID	Description	Kidney MMs read counts	Spleen MMs read counts
113037696	activin receptor type-1-like [Carassius auratus]	2379	0
113037699	cilia- and flagella-associated protein 206 isoform X2 [Carassius auratus]	473	0
113037705	uncharacterized protein LOC113037705 isoform X2 [Carassius auratus]	597	0
113037711	ribosome production factor 2 homolog [Carassius auratus]	3282	65
113037714	protein yippee-like 5 [Carassius auratus]	620	168
113037726	connector enhancer of kinase suppressor of ras 3-like [Carassius auratus]	3187	414
113037739	midasin [Carassius auratus]	493	0
113037742	serine/threonine-protein kinase Nek1-like [Carassius auratus]	218	0
113037743	palladin-like isoform X1 [Carassius auratus]	437	0
113037749	transcription regulator protein BACH2-like isoform X1 [Carassius auratus]	4839	56
113037757	tyrosine-protein kinase Blk-like [Carassius auratus]	330	0
113037760	eukaryotic translation initiation factor 5-like [Carassius auratus]	2912	0
113037763	PIN2/TERF1-interacting telomerase inhibitor 1-like [Carassius auratus]	1326	645
113037766	L-threonine 3-dehydrogenase, mitochondrial-like [Carassius auratus]	6039	0
113037767	probable G-protein coupled receptor 132 [Carassius auratus]	1824	8

Chapter 4. Discussion

4.1 Overview

Antigen captured by FDCs within the GC facilitates the selection of high-affinity antibody-producing B-cells and is a characteristic feature of GC. Fish were initially thought to lack antibody affinity maturation process partially because they lack histologically distinct GCs (Good and Finstad 1967; Manning and Horton 1982; Pitchappan 1980). Later work showed that fish retain experimentally injected antigen in and around the MMCs (Lamers & De Haas, 1985) raising the hypothesis that MMCs in fish could be analogous to GCs in mammals. Previous work done in our lab showed the presence of fully functional AID (mutator enzyme required for SHM) in channel catfish MMCs (Saunders et al., 2010) and ongoing work in our lab is showing evidence of clonal expansion and mutations within the VDJ exons of Ig genes of B-cell present with MMCs of zebrafish by tracing AID mediated mutations. These observations support the hypothesis that MMCs in fish could be analogous to GCs in mammals. However, further studies need to be done to look at the affinity of B-cells within MMCs and to fully understand the intercellular interactions occurring between different cell types in MMCs.

The goal of this work was to identify a specific cell type that has functional similarities to mammalian FDCs. FDCs are known to capture antigen for extended periods, hold the antigen in an intact form on the cell surface through FcRs or CRs, recruit B-cell (CXCL13), enhance the survival of B-cells within GC (BAFF) and

facilitate apoptotic cell clearance by TBM (Mfge8). These were some of the characteristic features we expected to see in our target cell.

Previous work has shown that MMs are known to retain pigments (lipofuscin, melanin, and hemosiderin) in them for extended periods (Fulop et al., 1984). Reticular cells are known to have a close lineage relation with mammalian FDCs (Bofill et al., 2000). Based on these observations, we hypothesized either MMs or reticular cells in fish could have functional similarities to mammalian FDCs.

Results of this study showed that MMs in goldfish are involved in the retention of antigen in an intact form, and the presence of FcR-like, CR-like and PIgR-like isoforms were seen in MMs through RNA-sequencing. MMs were also seen to express other molecular markers of FDCs like BAFF suggesting functional relation to mammalian FDCs.

4.2 Melanomacrophages in the lymphoid organs of goldfish and zebrafish are involved in the retention of experimentally injected antigen

Confocal microscopy images of leukocytes isolated from the spleen and kidney of immunized goldfish and zebrafish revealed that the autofluorescent MMs are involved in antigen retention. Around 70-80% of the cells that retained the antigen were identified to be MMs. The remaining 20-30% of the antigen retaining cells were non-autofluorescent, and identification of these cell types was not possible based on just the confocal images (Appendix Figure A.1). These non-fluorescent cells taking up the antigen are most likely tissue-resident macrophages. Macrophages are antigen-presenting cells and are known to process the antigen and present it through MHC II (Reviewed in Unanue, 1984). If macrophages are taking up some of the injected antigens, then we do not expect to see the injected antigen held on the cell surface of macrophages in an intact form which is a characteristic feature of FDCs. However, there are few exceptions, in case of subcapsular sinus macrophages, they hold the antigen on the cell surface in an intact form on FcRs or CRs and transport the antigen to the surface of FDCs (Reviewed in Batista et al., 2009) through nonspecific B-cells. Images obtained from confocal microscopy alone are not conclusive. Nevertheless, based on the experimental observations, it is safe to say most of the retained antigen is seen on MMs.

4.3 Goldfish spleen and kidney melanomacrophages retain the injected BSA or KLH (intact form) on the cell surface

Experiments showing antigen uptake by MMs were performed in both goldfish and zebrafish but, later experiments were performed just with goldfish due to the bigger size of the goldfish and availability of more cell. Preliminary experiments utilizing Freund's adjuvant showed the presence of unreleased antigen 30 days following the initial injection in goldfish. Alum adjuvant was used in later experiments to ensure the uniform release of antigen. This prevents the mix up of MMs taking up injected antigen from MMs holding the antigen on the cell surface.

The total number of MMs taking up the antigen can be influenced by multiple factors like the time of dissection, size of the fish, and concentration of the antigen injected. The total number of MMs present in the lymphoid organs is going to vary with each fish. Other factors like the age of the fish, size of the fish, and exposure to environmental antigen influences the number of MMs present in each fish. Based on our work so far, we have observed that MMs constitute about 5-10% of the total leukocyte population in the spleen or kidney. The actual cell number varies for each fish, but 5-10% is approximately around 40,000 cells in small fish (2 inches) and around 1,000,000 cells in bigger fish (>5 inches) (Appendix Figure A.2, A3, & A4; Table A.1).

Within the GC, FDCs do not directly capture the antigen on Fc or CRs. Marginal zone B-cells and subcapsular sinus macrophages hold the antigen as an immune complex through Fc or CRs and transport the intact antigen to non-specific B-cells in the primary follicle and these non-specific B-cells transport the antigen to the surface of FDCs (Reviewed in Batista et al., 2009). Our findings showed the presence of intact antigen on the surface of 1-6% of MMs. Ideally in macrophages, degradation of internalized product begins when the phagosomes are delivered to endolysosomes. This process can begin as early as 4 hr or 24 hrs following internalization based on the signals encountered by the cell (Wong et al., 2017). All the results shown in Chapter 3.1, Chapter 3.2 represents data at day 14 following immunization. These observations could indicate FDC-like antigen capture in MMs or could suggest that MMs are involved in antigen transport to MMCs rather than directly retaining the antigen.

FDCs are also known to internalize and recycle the immune complex back to the cell surface in an intact form through recycling endosomes (Heesters et al., 2013). The captured antigen is not necessarily exposed on the cell surface of FDCs at all time, thereby allowing the antigen to be stored in an intact form for extended periods (up to a year or more). If MMs are functionally similar to FDCs, then the antigen recycling by FDCs suggests the possibility that more than 1-6% of MMs could be involved in antigen capture. Live cell tracking experiments involving antigen-coated microspheres and Z-stack imaging along with endosomal membrane label could be performed in the future to track antigen recycling in MMs.

4.4 Melanomacrophages express FDC markers like BAFF and CXCL13

MMs were seen to be positive for FDC markers like BAFF, CXCL13 and Mfge8. Along with these presences of few FDC markers, expression of RANKL (Receptor activator of nuclear factor-kappa B ligand) was noted in MMs. RANKL is primarily expressed by lymphoid tissue organizers (mesenchymal stromal cells), marginal reticular cells and is thought to be absent in B cells and macrophages (Reviewed in Katakai, 2012). Studies have shown that RANK-RANKL interactions are essential for the formation of lymph nodes. In RANK and RANKL-deficient animals, there was a complete absence of lymph node and defects were noted in Peyer's patches and spleen (Dougall et al., 1999; Kim et al., 2003; Knoop et al., 2011). Currently, the role of RANKL in MMs is not known but the presence of RANKL in MMs could be of interest in the future. Presence of BAFF is not unique to FDCs other cells types such as monocytes, macrophages, dendritic cells, bone marrow stroma cells are also known to express BAFF (Schneider et al., 1999; Nardelli et al., 2001). CXCL13 is expressed by both follicular dendritic cells (Wang et al., 2011) and GC T follicular helper cells (Kroenke et al., 2012) in the B-cell follicles.

RNA-Sequencing on MMs (discussed in Chapter 3.6.1) showed the presence of two CR1-like isoforms in MMs. Even though MMs are phagocytic cells, presence of BAFF, CXCL13, CR1-like isoforms, FcR-like isoforms (Chapter 3.6.1) along with the assay showing retention of intact antigen on MMs surface (Chapter 3.2) suggest that MMs have a complement-mediated function.

4.5 Potential C3 like complement protein isolated from goldfish immune serum

To show antigen is bound by antibodies or complements co-immunoprecipitation was done on goldfish immune serum. Results obtained from the assay showed the presence of 35 KDa protein band in the first (presence of complement, proteins absence of antibodies) and third (presence of both antibodies and complements) treatment groups. Absence of this specific band in the complement inactivated treatment (second treatment group) suggests that the isolated protein could be a complement protein. Despite having an intense band (sufficient concentration of protein available for MS) identity of the obtained protein band was not revealed through MS.

The technique used to identify the protein was a database search approach where the experimentally generated spectra are compared against theoretical spectra that were generated in silico from a given protein sequence database. Therefore, the identification of the protein is only possible if the protein of interest is already known, and only if the database contains the correct sequences (Wang et al., 2013). The current protein database available for goldfish has a partial sequence for complement C3, but it appears that it was not a match with our experimental spectra. Open database search was also performed to identify any match in other species but no candidates were identified which suggest using database search approach was not the only issue.

In the future MS experiments, de novo sequencing approach can be tried to identify the protein as the protein sequence information is directly extracted from peptide

fragment iron derived spectral peaks rather than using any protein database (Wang et al., 2013). This approach is feasible and used to identify novel proteins.

Although the identification of antigen-bound complement proteins from goldfish immune serum was unsuccessful using the current approach, MS was done on MM whole cell lysate for a different purpose (discussed in Chapter 4.6) and the results showed the presence of C3 complement protein that aligned with *Carassius gibelio*. This observation partially supports the possibility that complement C3 bound antigen were retained on the surface of MMs but the absence of CRs-like isoforms in the mass spectrometry of MM whole cell lysate is a caveat.

These observations suggest the possibility that the immune complex retained on the cell surface or internalized immune complex component showed up on mass spectrometry or MMs are producing C3 complement protein (discussed in section 4.6). Although the presence of C3 complement protein in serum was not direct confirmed through this assay identification of C3 complement protein in Section 4.6 supports its presence.

4.6 In-gel digestion and mass spectrometry analysis of melanomacrophage whole cell lysate showed the presence of housekeeping proteins along with C3 complement protein (*Carassius gibelio*) and transferrin

Previous attempts to isolate and identify antigen retaining receptor in MMs through Co-IP were unsuccessful, and one of the crucial factors affecting the experiment was the concentration of protein isolated. The goldfish used for these experiments were <2 inches in size, and as discussed in Chapter 4.1 after FACS sorting MMs, we obtain a total of 70,000-80,000 cells from one fish (spleen and kidney MMs pooled). Our experimental data (Chapter 3.2) suggests that around 1-6% of the MMs are involved in holding the antigen on the cell surface in an intact form. From the obtained MMs (80,000 cells) of 1 fish, 6% would be 4,800 cells. On pooling samples from 10 different goldfish (<2 inches), the total number of cells holding the intact antigen would be 48,000, which is still a deficient number. Ideally, 0.5-2 x 10^7 cells are required to perform a successful IP (Bonifacino et al., 2016).

During Co-IP, there are multiple washing steps which could further result in the loss of target protein. Loss of protein in Co-IP is unavoidable, but considering the low concentration of the target protein in our sample loss of protein in washing steps could have a significant effect on the experimental outcome. In the future attempts to isolate antigen holding receptors in MMs bigger goldfish should be used (>5 inches) and a biotin-conjugated antigen should be used to increase specificity.

A non-specific and straightforward approach was used as an alternative for looking at potential antigen holding receptors in goldfish MMs. MS analysis of MM whole cell lysate showed the presence of multiple housekeeping proteins, but we did not observe the presence of Fc-like receptors or CRs that we hoped to see.

MS analysis also showed the presence of transferrin in MMs. Previous studies in fish showed the expression of transferrin in activated macrophages (Stafford & Belosevic, 2003) and another study in mouse showed the production of transferrins in mouse macrophages in a dose-dependent way to γ-IFN (Djeha et al., 1992). Cellular damage was seen to result in the release of transferrin cleaving enzymes and truncated forms of transferrin are found to be essential in modulating nitrogen oxide production by goldfish macrophages (Stafford & Belosevic, 2003). Isolation of MMs through FACS can be harsh on cells resulting in danger signals and this could be one possible reason for the presence of multiple transferrin molecules.

Presence of complement protein C3 in MMs was also noted in the MS results. In humans, macrophages were seen to express C3 complement at higher levels when stimulated with low-density lipoprotein (Mogilenko, et al., 2012). And increased levels of IgA and IgG immune complexes were seen to increase the biosynthesis of C3 in human macrophages (Laufer et al., 1995; Lubbers et al., 2017). Based on the MS results it's not possible to conclude if MMs are producing the complement protein C3 or if this is the complement component that is bound to the retained antigen on MMs. The RNA-Sequencing analysis showed the presence of three complement C3-like isoforms in MMs

so there's a possibility that MMs are producing C3 (read counts - 30,11 and 446) (shown in appendix Table A.6).

4.7 Data analysis of melanomacrophage RNA-Sequencing

Transcriptome analysis of goldfish MMs showed the presence of few FDC-like genes such as BAFF, FcRs, CRs, and Poly IgRs. Most of these genes were present in kidney MMs but absent in spleen MMs and this is likely due to errors in sequencing or analysis rather than having a biological significance. As discussed in Chapter 3.6 reads obtained from spleen had low alignment rates with the reference genome (~10%).

Alignment rate of 10% means only 10% of the obtained reads from the sequencing align to the reference genome. Which means the remaining 90% of the reads are not included in the downstream analysis pipeline. Suggesting that analysis done on this 10% aligned spleen reads is not going to produce biologically significant results or any results altogether. Low alignment rates are observed when there is adaptor contamination, or when the quality of the reads obtained reads is too low. In our case, we did not see any of these issues and the quality score of the spleen and kidney MMs were comparable (discussed in 3.6). We did find the presence of multiple overrepresented sequences in the spleen MM quality report and this was not seen in kidney MMs (shown in appendix Table A.4 & A.5). Cutadapt was used to remove the overrepresented sequences and alignments were done again using HISAT2 but the alignment rate was still 10% and the reason behind the low alignment rate is not known. We can generate an

individual file of all the unmapped reads through HISAT2 and blast the reads against the goldfish genome individually. However, individually blasting over 5 x 10^6 reads is not efficient.

Presence of different isoforms of Poly IgRs is seen in goldfish MMs (Table 3.6.1). In case of mammals Poly IgRs are involved in transporting Poly Ig across mucosal epithelial cells they are specifically involved in the transcytosis of secreted IgA. Structurally mammalian Poly IgR (type I transmembrane glycoprotein) consists of an extracellular region, a transmembrane region, and a cytoplasmic region (Kaetzel, 2005). In mammals, the extracellular region is composed of five Ig-like domains and a cleavage site (Braathen et al., 2007; Hamuro et al., 2007). Where as in case of fish its shown that the extracellular region of poly IgR consists of two ILDs, where each region of two ILDs indicates a high similarity among the different fishes based on multiple alignments (Wang et al., 2017). And functionally fish Poly IgR in mucosal immunity is involved in the binding of Ig to poly IgR and studies have shown that fish Poly IgRs are capable of binding to IgM although the fish Poly IgR is structurally different from mammalian Poly IgR (Zhang et al., 2010; Xu et al., 2013).

Table 3.6.2 showed the presence of genes like cilia- and flagella-associated protein 206 and T-cell activation Rho GTPase-activating protein which is not associated with macrophages or FDCs. MMs are phagocytic cells its possible that the clearance of bacteria or apoptotic T-cell by MMs resulted in these showing up in transcriptome data. FDCs produce Mfge8 to facilitate the clearance of apoptotic cells within the GC and this plays a major role in preventing the generation of auto-reactive antibodies (Kranich et al.,

2008). RNA-Sequencing should be done on primary kidney derived macrophages to compare their gene expression profile to MMs.

For this work, we were interested in finding out if the FDC like genes are present or absent in MMs and this can be answered based on the read counts generated by HTseq-counts. So conclusions can be drawn for our purpose just by using the kidney MM counts. To perform future analysis like differential gene expression or comparisons among treatment groups its important to have sufficient replicates.

CONCLUSION

The focus of this study was to identify and characterize antigen retaining cells in fish lymphoid organs and thereby better understand the antibody affinity modification process in putative germinal centres (MMCs) of fish. Experimental injection of antigen into goldfish and zebrafish revealed the auto-fluorescent MMs are involved in antigen retention. Additional experiments showed that some of the MMs retain the antigen on the cell surface in an intact form.

FDC markers such as BAFF, FcR-like isoform, CR-like isoform were found to be expressed in MMs through RNA-Sequencing of goldfish kidney MMs. Although further experiments need to be performed to fully understand the role of MMs in MMCs, based on the presence of FDC markers in MMs and characteristics similarities between FDCs and MMs its safe to say that MMs in fish have some functional similarities to mammalian FDCs.

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APPENDICES

1) Uptake of experimentally injected labelled antigen by non-autofluorescent cells

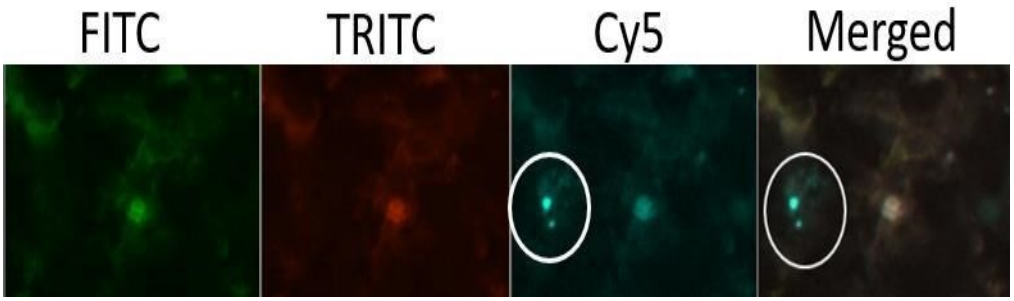


Figure A.1. Antigen retention by non-autofluorescent cells in the kidney of BSA-Alexa fluor 647 injected fish at day 14. FITC and TRITC channel show the absence of auto-fluorescence in the circled cell.

2) Isolating MMs through FACS

Sorting double-positive MMs from BSA-Alexa fluor 647 injected goldfish.

Alexa fluor 647 has emission in Cy5, MMs autofluorescent pigments have emission in PE channel.

So we are trying to sort antigen retaining MMs here.

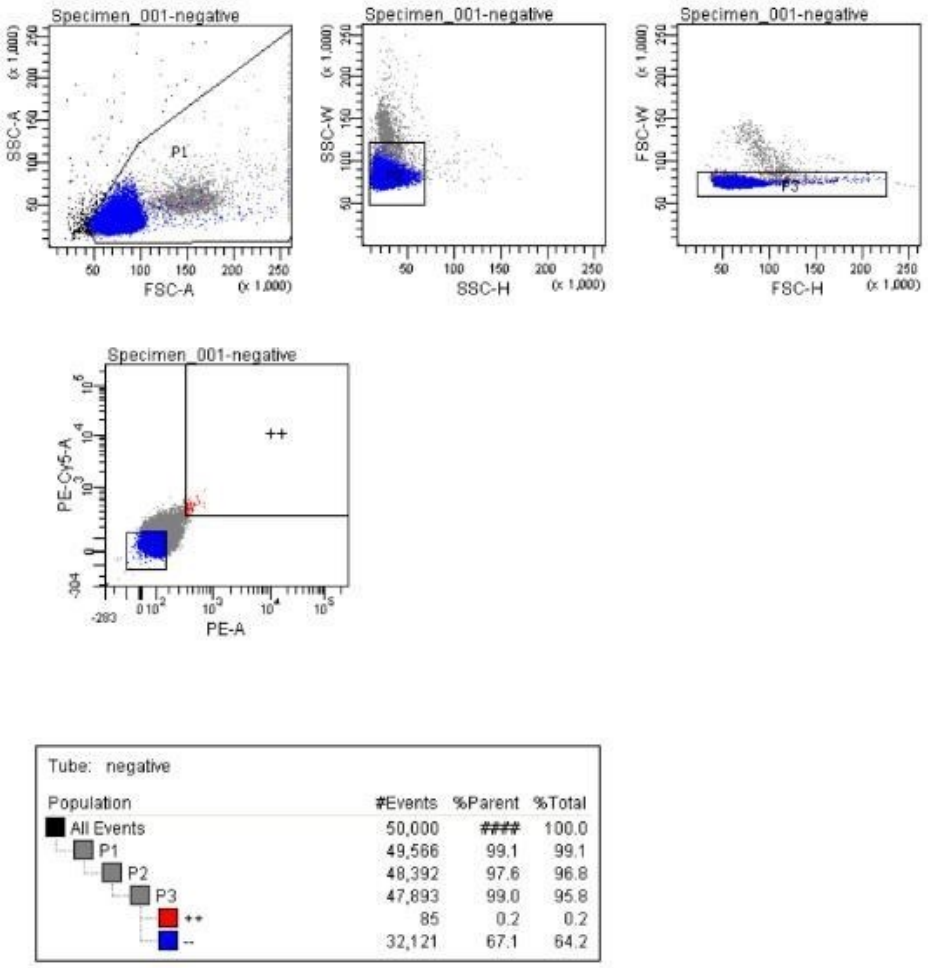
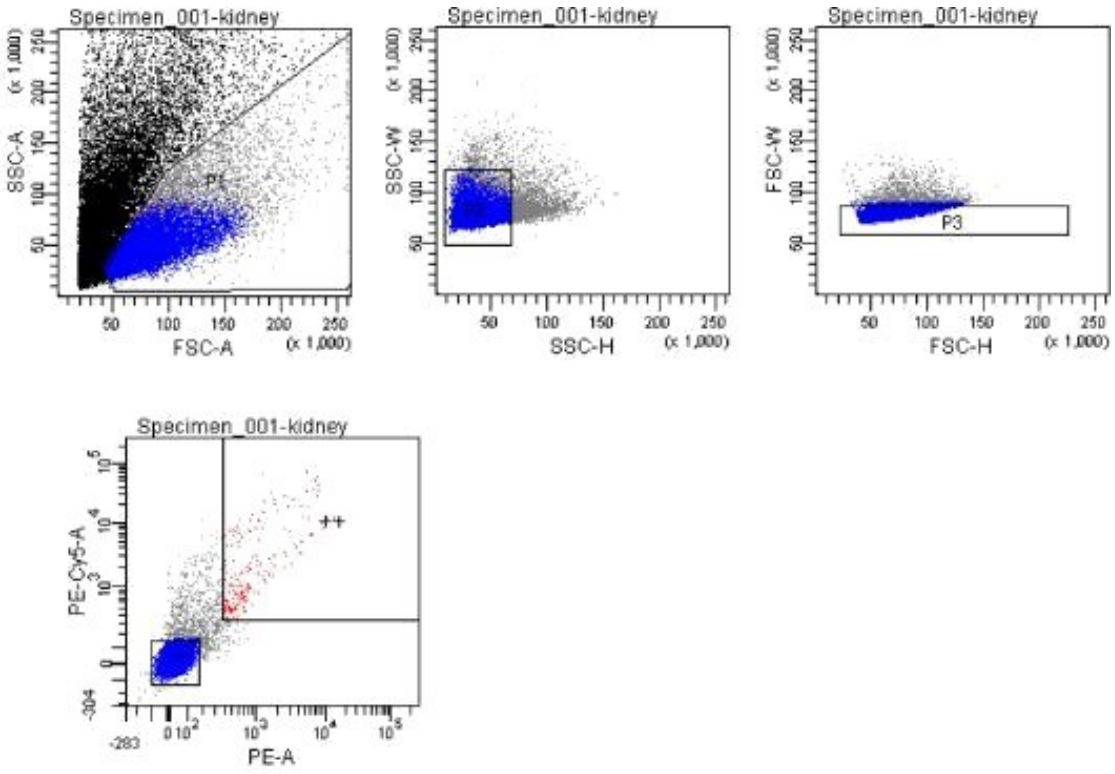


Figure A.2.i Gates showing the percentage of double-positive MMs present in BSA-Alexa fluor 647 injected goldfish. (negative control-PBLs) Leukocytes isolated from 2> inch goldfish.



Tube: kidney

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
P1	21,429	42.9	42.9
P2	19,314	90.1	38.6
P3	18,273	94.6	36.5
++	190	1.0	0.4
--	17,213	94.2	34.4

Figure A.2.ii Gates showing the percentage of double-positive MMs present in BSA-Alexa fluor 647 injected goldfish (kidney leukocytes). Leukocytes isolated from 2> inch goldfish. Pooled MMs from the two sorted samples (kidney an spleen) had a total of ~63,000 cells.

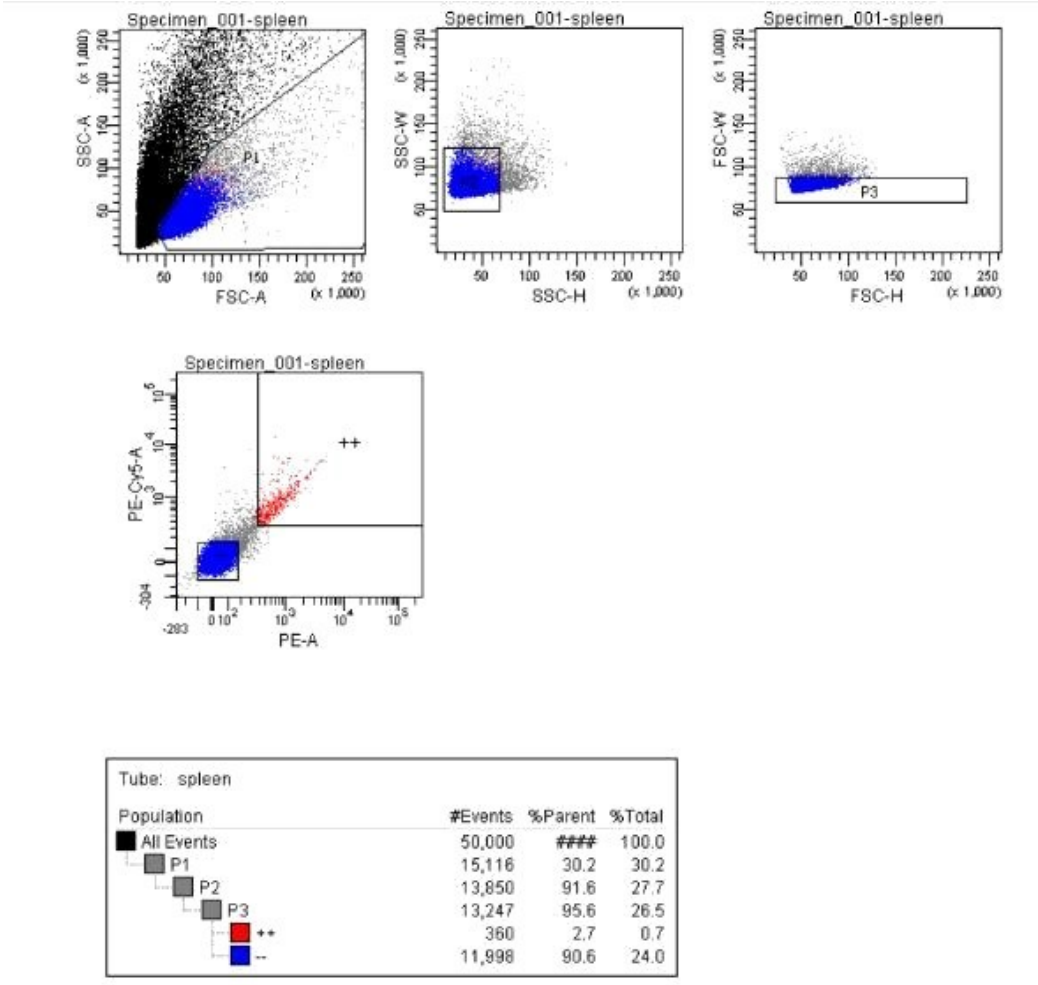
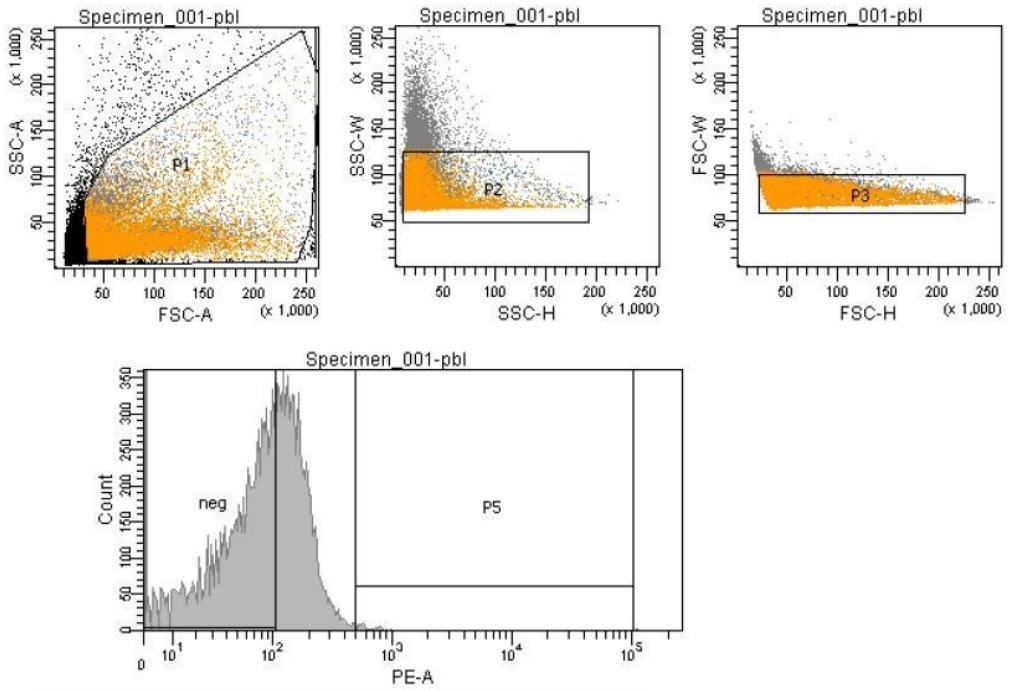


Figure A.2.iii Gates showing the percentage of double-positive MMs present in BSA-Alexa fluor 647 injected goldfish (Spleen leukocytes). Leukocytes isolated from 2> inch goldfish. Pooled MMs from the two sorted samples (kidney an spleen) had a total of ~63,000 cells.

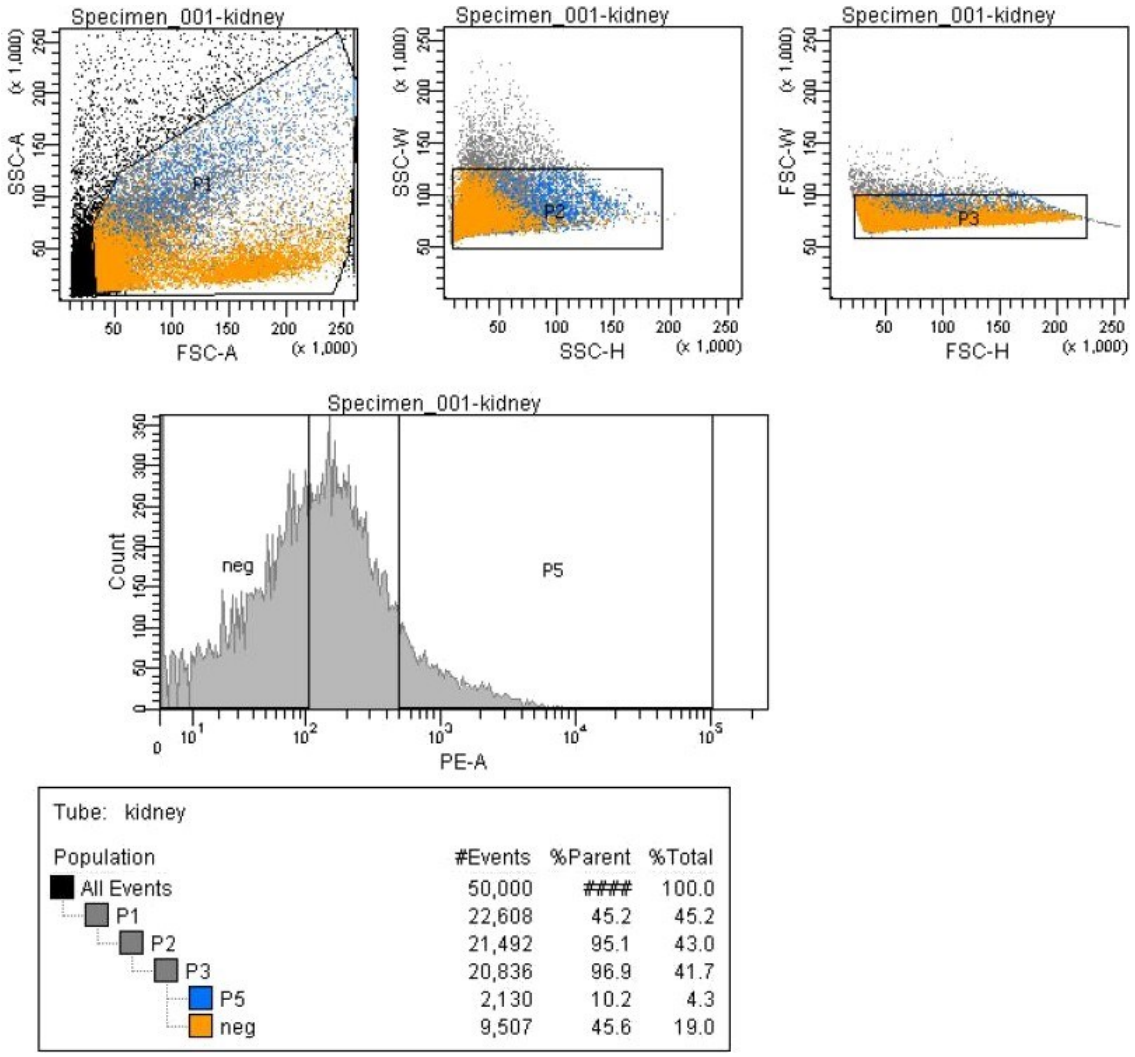
Isolating MMs from BSA (no fluorescent label) vaccinated fish (<2 inches) based on the presence of auto-fluorescent pigments



Tube: pbl

Population	#Events	%Parent	%Total
All Events	43,567	####	100.0
P1	19,327	44.4	44.4
P2	15,682	81.1	36.0
P3	14,379	91.7	33.0
P5	49	0.3	0.1
neg	8,247	57.4	18.9

Figure A.3.i Gates (P5) showing the percentage of MMs present in BSA vaccinated goldfish (negative control-PBLs). Leukocytes isolated from <2 inches goldfish.



Tube: kidney

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
P1	22,608	45.2	45.2
P2	21,492	95.1	43.0
P3	20,836	96.9	41.7
P5	2,130	10.2	4.3
neg	9,507	45.6	19.0

Figure A.3.ii Gates (P5) showing the percentage of MMs present in BSA vaccinated goldfish (kidney leukocytes). Leukocytes isolated from <2 inches goldfish. Sorted MMs from spleen-246,956 cells and kidney-626,194 cells.

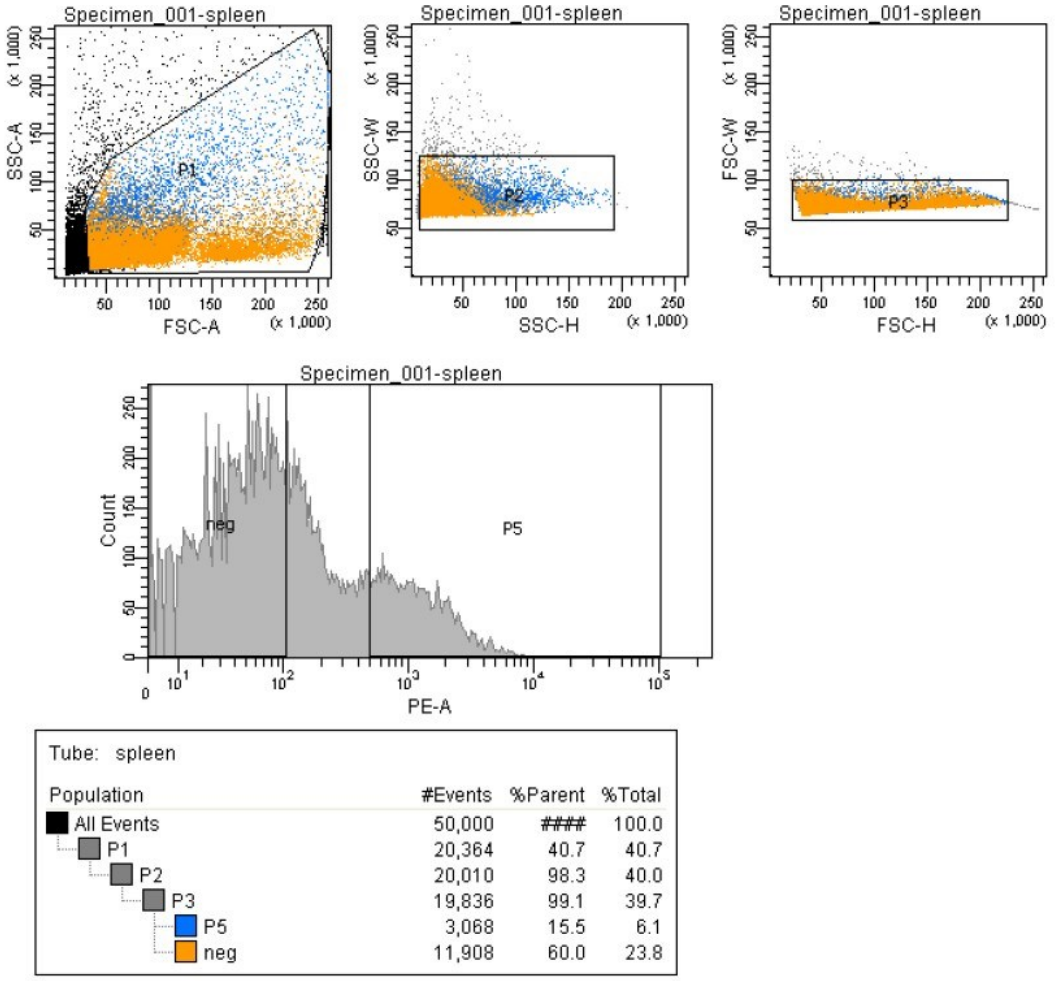
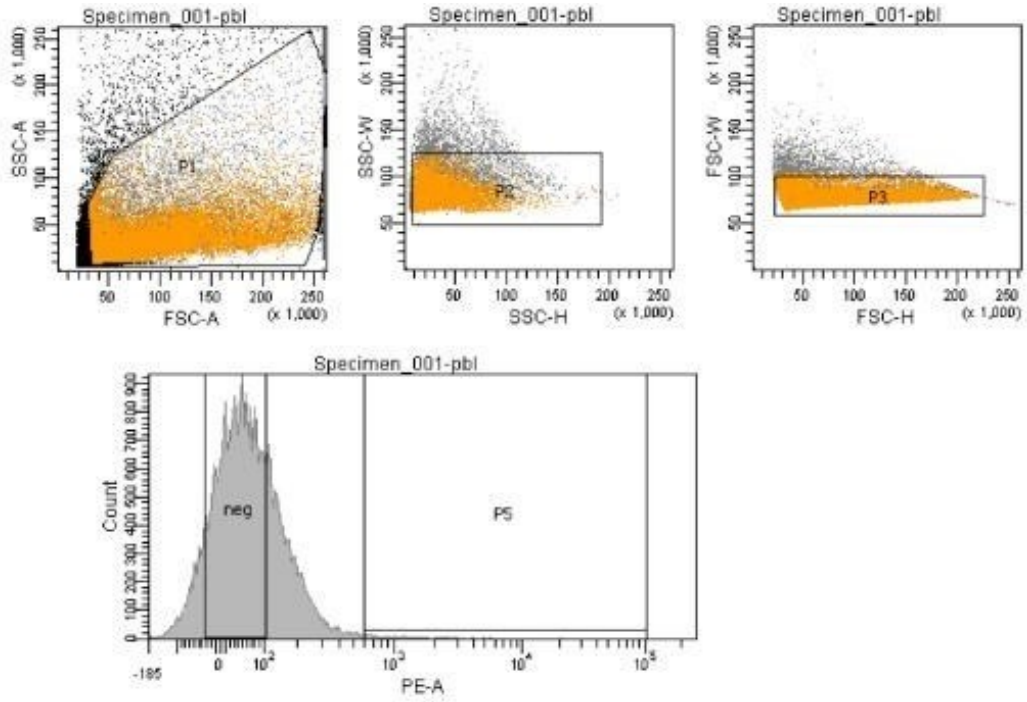


Figure A.3.iii Gates (P5) showing the percentage of MMs present in BSA vaccinated goldfish (spleen leukocytes). Leukocytes isolated from <2 inches goldfish. Sorted MMs from spleen-246,956 cells and kidney-626,194 cells.

Isolating MMs from BSA (no fluorescent label) vaccinated fish (>5 inches) based on the presence of auto-fluorescent pigments



Tube: pbl

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
P1	33,621	67.2	67.2
P2	32,250	95.9	64.5
P3	30,845	95.6	61.7
P5	161	0.5	0.3
neg	19,412	62.9	38.8

Figure A.4.i. Gates (P5) showing the percentage of MMs present in BSA vaccinated goldfish (negative control-PBLs). Leukocytes isolated from ≥5 inches goldfish.

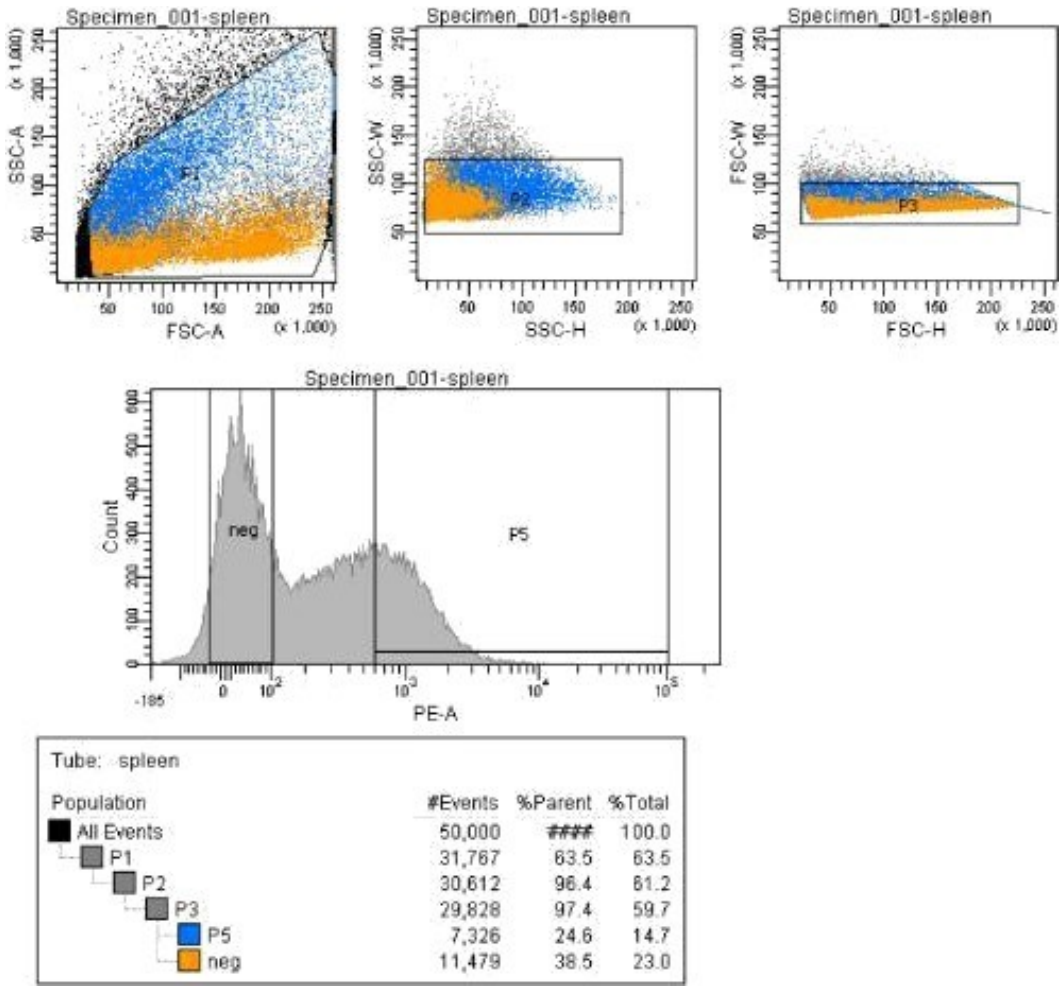
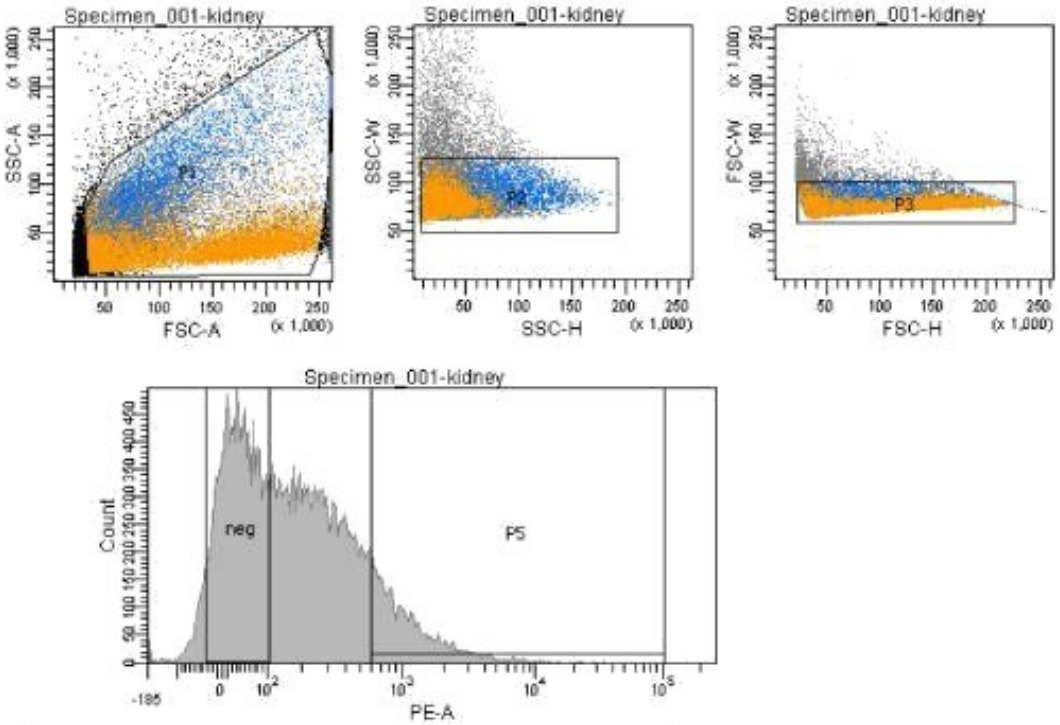


Figure A.4.ii. Gates (P5) showing the percentage of MMs present in BSA vaccinated goldfish (spleen leukocytes). Leukocytes isolated from ≥5 inches goldfish. Sorted MMs from spleen- 2,544,889 cells and kidney-1,280,732 cells.



Tube: kidney

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
P1	31,340	62.7	62.7
P2	29,153	93.0	58.3
P3	27,531	94.4	55.1
P5	3,488	12.7	7.0
neg	10,370	37.7	20.7

Figure A.4.iii. Gates (P5) showing the percentage of MMs present in BSA vaccinated goldfish (kidney leukocytes). Leukocytes isolated from >5 inches goldfish. Sorted MMs from spleen- 2,544,889 cells and kidney-1,280,732 cells.

	FACS sorted MMs from goldfish spleen	FACS sorted MMs from goldfish kidney	FACS sorted MMs from goldfish- spleen and kidney combined
Double positive MMs isolated from BSA-Alexa fluor 647 injected (<2 inches) goldfish	-	-	~63,000 cells
Single positive MMs isolated from BSA-injected goldfish (<2 inches)	246,956 cells	626,194 cells	873,105 cells
Single positive MMs isolated from BSA-injected goldfish (5< inches)	2,544,889 cells	1,280,732 cells	3,825,621 cells

Table A.1. Showing FACS sorted MM cell counts from Figures A.2, A.3 & A.4

3) **Non-uniform colocalization of auto-fluroscence in small gf (<2 inches)**

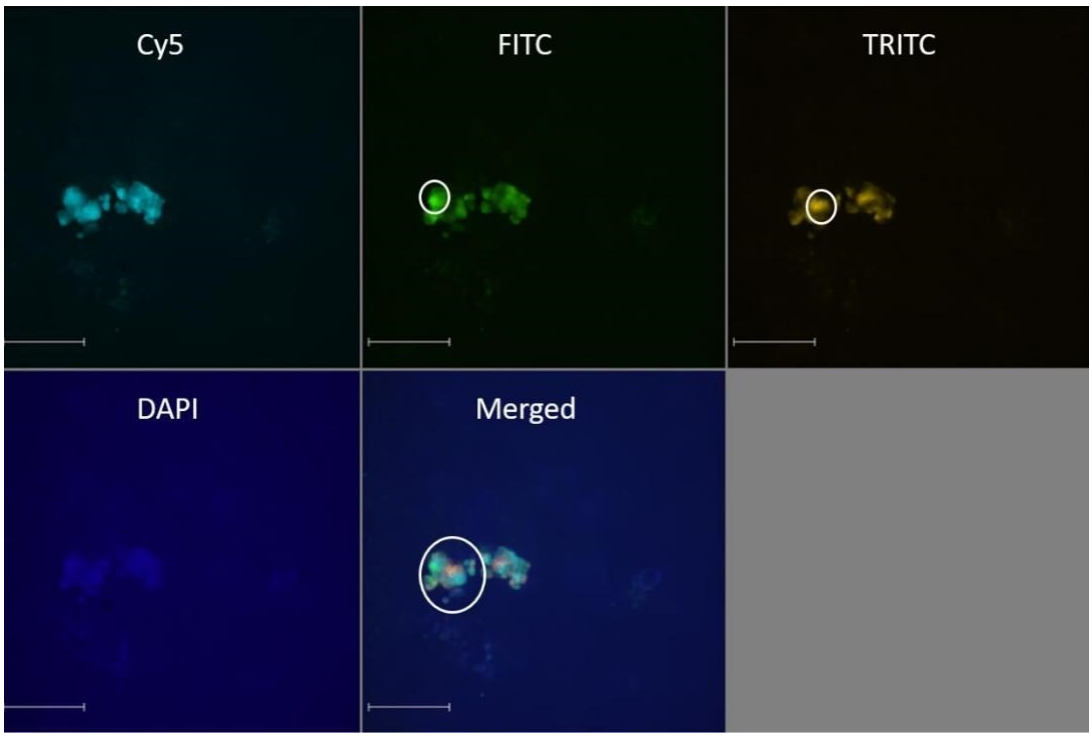


Figure A.5. Confocal image of small goldfish immunized with BSA Alexa fluor 647 and stained with WCI 12 conjugated to BSA Alexa fluor 350.

FITC and TRITC channel showing spots where there is no co-localization of auto fluorescent pigments. This observation was never seen in older goldfish (5< inches).

4) Transcriptome analysis

HISAT2 alignment of our sequencing reads to the reference genome (NCBI_GCF_003368295.1_ASM336829v1).

```
The following have been reloaded with a version change:
  1) samtools/1.9 => samtools/1.10

perl: warning: Setting locale failed.
perl: warning: Please check that your locale settings:
        LANGUAGE = (unset),
        LC_ALL = (unset),
        LANG = "C.UTF-8"
    are supported and installed on your system.
perl: warning: Falling back to the standard locale ("C").
  File "/cvmfs/soft.computecanada.ca/easybuild/software/2017/avx2/Compiler/intel2018.3/hisat2/2.2.0/bin/hisat2_read_statistics
.py", line 182
    length_map = sorted(length_map.iteritems(), key=lambda (k,v):(v,k), reverse=True)
                                                           ^
SyntaxError: invalid syntax
25520707 reads; of these:
  25520707 (100.00%) were paired; of these:
    12030972 (47.14%) aligned concordantly 0 times
    12227821 (47.91%) aligned concordantly exactly 1 time
    1261914 (4.94%) aligned concordantly >1 times
    ----
    12030972 pairs aligned concordantly 0 times; of these:
      609370 (5.07%) aligned discordantly 1 time
    ----
    11421602 pairs aligned 0 times concordantly or discordantly; of these:
      22843204 mates make up the pairs; of these:
        20803435 (91.07%) aligned 0 times
        1742654 (7.63%) aligned exactly 1 time
        297115 (1.30%) aligned >1 times
59.24% overall alignment rate
[bam_sort_core] merging from 26 files and 1 in-memory blocks...
[aradana@cedar1 kidney]$
```

Figure A.6. Screenshot of HISAT2 output showing overall alignment percentage of goldfish kidney MM reads to the reference genome.

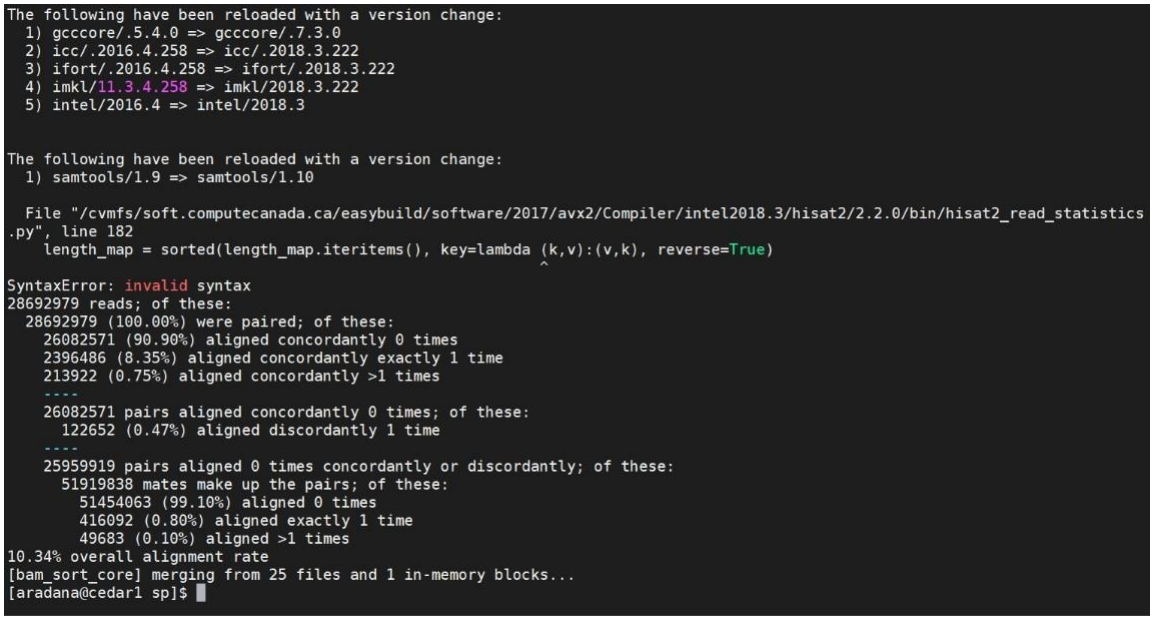


Figure A.7. Screenshot of HISAT2 output showing overall alignment percentage of goldfish spleen MM reads to the reference genome.

The observed alignment rates were 59.24% and 10.34% for the kidney and spleen MM samples, respectively. This is considered very low, and the quality of overall alignment is really bad.

FastQC report did not show any issues with the quality of the reads, but it did show quite a few overrepresented sequences in the spleen sample. The identity of the overrepresented sequences was unknown since it did not match any adaptor sequences (Table A.4 & A.5). This may or may not be of biological relevance, but there is a high possibility that this is resulting in a lower alignment rate.

Sequence	Count	Percentage	Possible Source
CTTATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAG	139468	0.48607012886323164	No Hit
ATTTTAGTCTGTTCGACTACGTAACTTTACATGATTTGAGTTCAGACCGA	129574	0.45158782571861916	No Hit
TATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGAT	85849	0.299198629741443	No Hit
AGGGATAACAGAGCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGAT	72297	0.2519675632146805	No Hit
CTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAA	66832	0.2301329534308724	No Hit
GGATAACAGAGCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGATTG	61179	0.213219488134652	No Hit
GGCTTAATTTTAGTCTGTTCGACTACGTAACTTTACATGATTTGAGTTCA	53011	0.18475251384668007	No Hit
GACTTACGTCGGTCTGAACTCAAATCATGTAAAGTTACGTAGTCGAACAG	52109	0.18160888766551567	No Hit
CCTTTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTT	50643	0.17649962382783607	No Hit
TTATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGA	46153	0.16085119638501114	No Hit
TTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTTACG	38410	0.13386550068572525	No Hit
TCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAAC	32723	0.11404512098253026	No Hit
GTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	32711	0.11400349890473206	No Hit
GCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGATTGCGACCTCGAT	31388	0.10939261482748096	No Hit
CCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTTACGTCGGTCTG	30646	0.10680661635029252	No Hit
GACTAAAATTAAGCCCGAGCTACCGGGCTTTAAACTTTAATTCAACATCG	30599	0.10664281321224958	No Hit
CTCCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTTACGTCGGTC	30459	0.10615488897127064	No Hit
AAACTAACCTGACTTACGTCGGTCTGAACTCAAATCATGTAAAGTTACGT	29611	0.10319946214019814	No Hit
TAACAGAGCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGATTGCGA	29232	0.10187858151640512	No Hit

Table A.4. FastQC report showing overrepresented sequences in goldfish spleen MM reads1.

Sequence	Count	Percentage	Possible Source
CTTATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAG	141511	0.49319033760837455	No Hit
ATTTTAGTCTGTTCGACTACGTAACTTTACATGATTTGAGTTCAGACCGA	134487	0.46871048140383054	No Hit
TATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGAT	87876	0.30626307571618827	No Hit
AGGGATAACAGAGCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGAT	72513	0.252720360615048	No Hit
CTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAA	65968	0.22990990234928202	No Hit
GGATAACAGAGCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGATTG	64559	0.22499929338114386	No Hit
GGCTTAATTTTAGTCTGTTCGACTACGTAACTTTACATGATTTGAGTTCA	54085	0.18849558980961859	No Hit
GACTTACGTCGGTCTGAACTCAAATCATGTAAAGTTACGTAGTCGAACAG	49683	0.17315385760398042	No Hit
CCTTTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTT	48382	0.16861964733602602	No Hit
TTATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGA	46352	0.1615447458418312	No Hit
TTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTTACG	37482	0.13063126000266478	No Hit
GTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	34835	0.12140600667501272	No Hit
TCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAGATAGAAACTAAC	32749	0.11413593548442635	No Hit
GCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGATTGCGACCTCGAT	31985	0.11147326319794122	No Hit
CCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTTACGTCGGTCTG	31224	0.10882104643090564	No Hit
TAACAGAGCTATAACAAGTTAGGTGACCAAAGTTTAATTTGTGATTGCGA	30652	0.10682752738919161	No Hit
CTCCTGGAGGAAAATTAAAAAGATAGAAACTAACCTGACTTACGTCGGTC	30136	0.1050291780438692	No Hit
AAACTAACCTGACTTACGTCGGTCTGAACTCAAATCATGTAAAGTTACGT	29087	0.1013732314096769	No Hit

Table A.5.FastQC report showing overrepresented sequences in goldfish spleen MM reads2.

Cutadapt was used to remove these overrepresented sequences, and the alignments were performed again.

```
d mismatches (0)
Warning: skipping mate #1 of read 'A00133:192:HCVWKDSXY:3:2678:7229:37027 1:N:0:
rs long
28692979 reads; of these:
  28692979 (100.00%) were paired; of these:
    26075731 (90.88%) aligned concordantly 0 times
    2401654 (8.37%) aligned concordantly exactly 1 time
    215594 (0.75%) aligned concordantly >1 times
    ----
    26075731 pairs aligned concordantly 0 times; of these:
      119368 (0.46%) aligned discordantly 1 time
    ----
    25956363 pairs aligned 0 times concordantly or discordantly; of these:
      51912726 mates make up the pairs; of these:
        51430360 (99.07%) aligned 0 times
        431506 (0.83%) aligned exactly 1 time
        50860 (0.10%) aligned >1 times
10.38% overall alignment rate
[bam_sort_core] merging from 24 files and 1 in-memory blocks...
[aradana@cedar5 ali]$
[aradana@cedar5 ali]$
```

Figure A.8. HISAT2 output showing overall alignment percentage after removing the overrepresented sequences in the spleen MM sample.

Alignment with HISAT2

Building indexes command lines

File Edit Format View Help

```
#!/bin/bash
#SBATCH --account=def-bmagor
#SBATCH --nodes=8
#SBATCH --ntasks-per-node=1
#SBATCH --mem-per-cpu=8190mb
#SBATCH --time=4:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=muthupan@ualberta.ca

module load nixpkgs/16.09  intel/2018.3
module load  hisat2/2.2.0
module load samtools

hisat2-build -p 16 GCF_003368295.1_ASM336829v1_genomic.fna ref
```

Alignment with HISAT2

File Edit Format View Help

```
#!/bin/bash
#SBATCH --account=def-bmagor
#SBATCH --nodes=8
#SBATCH --ntasks-per-node=1
#SBATCH --mem-per-cpu=8190mb
#SBATCH --time=4:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=muthupan@ualberta.ca

module load nixpkgs/16.09  intel/2018.3
module load  hisat2/2.2.0
module load samtools

hisat2 -q -x ref -1 Kidney_mmo_Clean_Data1.fq -2 Kidney_mmo_Clean_Data2.fq -S kidney_mmo.sam
samtools view -bS kidney_mmo.sam > kidney_mmo.bam
samtools sort kidney_mmo.bam -o kidney_mmo.sorted.bam
```

Counting with HTseq counts

File Edit Format View Help

```
#!/bin/bash
#SBATCH --account=def-bmagor
#SBATCH --nodes=8
#SBATCH --ntasks-per-node=1
#SBATCH --mem-per-cpu=8190mb
#SBATCH --time=2:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=muthupan@ualberta.ca

module load nixpkgs/16.09  intel/2018.3
module load  hisat2/2.2.0
module load samtools
module load python
virtualenv ENV
source ENV/bin/activate
pip install numpy
pip install HTSeq
pip install matplotlib

htseq-count kidney_mmo.sam anno.gtf -s yes -t exon -i gene_id --nonunique all > k+.txt
```

Cutadapt

File Edit Format View Help

```
#!/bin/bash
#SBATCH --account=def-bmagor
#SBATCH --nodes=8
#SBATCH --ntasks-per-node=1
#SBATCH --mem-per-cpu=8190mb
#SBATCH --time=6:00:00
#SBATCH --mail-type=ALL
#SBATCH --mail-user=muthupan@ualberta.ca

module load nixpkgs/16.09  intel/2018.3
module load python/3.8.2
pip install --user --upgrade cutadapt

cutadapt -a CTTATTAACCCCTCATTCTCCTTTCGTACTCCTGGAGGAAAATTAAAAAG -a ATTTTAGTCTGTTCGACTACG
GTGATTGCGA -o s1.fq Spleen_mmo_Clean_Data1.fq
```

Table A.6 Showing read counts for complement C3 like isoforms in spleen and kidney goldfishMMs

Gene ID	Transcript ID	Description	Kisney MM read counts	Spleen MM read counts
113049880	XM_026212635.1	complement C3-like [Carassius auratus]	0	0
113049778	XM_026212447.1	complement C3-like isoform X1 [Carassius auratus]	0	0
113049778	XM_026212516.1	complement C3-like isoform X2 [Carassius auratus]	0	0
113053025	XM_026217662.1	complement C3-like [Carassius auratus]	0	0
113049957	XM_026212713.1	complement C3-like [Carassius auratus]	0	0
113049641	XM_026212199.1	complement C3-like [Carassius auratus]	0	0
113053189	XM_026218051.1	complement C3-like [Carassius auratus]	0	0
113049555	XM_026212030.1	complement C3-like [Carassius auratus]	0	0
113049706	XM_026212324.1	complement C3-like [Carassius auratus]	0	0
gene-LOC113040803	gene-LOC113040803	PREDICTED: complement C3-like [Sinocyclocheilus grahami]	0	0
113040479	XM_026198806.1	complement C3-like [Carassius auratus]	0	0
113040480	XM_026198808.1	complement C3-like [Carassius auratus]	0	0
113040521	XM_026198842.1	LOW QUALITY PROTEIN: complement C3-like [Carassius auratus]	0	0
113039944	XM_026198119.1	complement C3-like isoform X1 [Carassius auratus]	0	0
113065133	XM_026236340.1	complement C3-like [Carassius auratus]	30	0
gene-LOC113078665	gene-LOC113078665	complement C3-like isoform X1 [Carassius auratus]	0	0
113097118	XM_026262319.1	complement C3-like [Carassius auratus]	0	0
113097116	XM_026262316.1	complement C3-like [Carassius auratus]	0	0
113097117	XM_026262318.1	complement C3-like [Carassius auratus]	0	0
gene-LOC113097114	gene-LOC113097114	complement C3-like [Carassius auratus]	11	0
113097364	XM_026262614.1	complement C3-like [Carassius auratus]	0	0
113097359	XM_026262610.1	complement C3-like, partial [Carassius auratus]	0	0
gene-LOC113097365	gene-LOC113097365	complement C3-like [Carassius auratus]	0	0
113097362	XM_026262612.1	complement C3-like [Carassius auratus]	446	0
gene-LOC113098137	gene-LOC113098137	PREDICTED: complement C3-like [Cyprinus carpio]	0	0
113097363	XM_026262613.1	complement C3-like [Carassius auratus]	0	0