University of Alberta

Melano-macrophage characterization and their possible role in the goldfish (*Carassius auratus*) antibody affinity maturation

by

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ABSTRACT

During the antibody affinity maturation process, changes to the immunoglobulin genes are initiated by the B-cell specific enzyme activation-induced cytidine deaminase (AID). These changes are randomly generated and produce antibodies with different affinities for the antigen. In mammals, this process occurs in highly regulated environments known as germinal centres (GCs). Although conventional GCs are absent in fish, AID expressing B-cells are found in close proximity to pigmented myeloid cells called melano-macrophages (MMs). Previous *in vivo* work suggested that MMs have functions consistent with those of follicular dendritic cells (trapping antigen) and tingible body macrophages (phagocytosis of apoptotic cells) found in the GC. Quantitative PCR used to assess gene expression of pro- and anti-inflammatory cytokines in MMs under *in vivo* and *in vitro* conditions revealed that these cells may have different roles in the immune response depending on their anatomical location.

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α	Alpha/anti
Ab	Antibody
ACP	Acid-phosphatase
Ag	Antigen
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
β	Beta
BLAST	Basic local alignment search tool
CAF	Citrate-acetone-formaldehyde
cAMP	Cyclic adenosine monophosphate
cfu	Colony forming units
CXCR5	CXC-Chemokine receptor 5
D	Diversity
DAMP	Damage-associated molecular pattern(s)
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDCs	Follicular dendritic cells
γ	Gamma
GC	Germinal centre(s)
Ig	Immunoglobulin

IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IFN	Interferon
IL	Interleukin
J	Joining
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MFGE8	Milk fat globule-epidermal growth factor-factor 8
MM	Melano-macrophage
MMC	Melano-macrophage cluster
PAMP	Pathogen-associated molecular pattern(s)
PBLs	Peripheral blood leukocytes
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKM	Primary kidney derived macrophages
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
T_{FH}	Follicular helper T-cell
T_H	Helper T-cell
TBM	Tingible body macrophage
TGF	Transforming growth factor

TMS	Tricane methanosulfonate
TNF	Tumor necrosis factor
V	Variable
VDJ	Variable, diversity and joining segment

CHAPTER 1: INTRODUCTION

1.1. Overview

Immunoglobulins (Igs) are a central element of the adaptive immune response. These molecules have been described from higher vertebrates to cartilaginous fish, where the adaptive immune system is known to have first appeared (Reviewed in Litman, Rast et al. 2010). Igs or antibodies (Abs) are expressed on B-cells and can recognize to any pathogen; every B-cell has an Ab with a different antigen-binding site.

A defining feature of the adaptive immune response is its ability to improve the affinity and specificity of the antibody for a given antigen. During this process, the antibody undergoes somatic hypermutation and class switch recombination in order to achieve higher specificity and affinity for an antigen. Both somatic hypermutation (SHM) and class switch recombination (CSR) are mediated by the immunoglobulin mutator enzyme activation induced cytidine deaminase (AID) (Reviewed in Victora and Nussenzweig 2012).

In higher vertebrates, the antibody affinity maturation and AID expression occurs in highly organized microenvironments known as germinal centres (GCs). In the classical model for antibody affinity maturation, GCs develop when a few activated B-cells and T helper (T_H) cells are recruited to primary follicles where they proliferate. Primary follicles containing resident follicular dendritic cells (FDCs) that trap unprocessed antigen through antigen-antibody complexes on their surface via Fc and complement receptors. Perhaps the predominant model

for the affinity maturation system is that after undergoing AID mediated modification of their Ig genes the B-cells compete to bind the trapped antigen and successful B-cells can then process and present the antigen to the TH-cells which rescue the B-cell from pre-programmed apoptosis (Victora and Nussenzweig 2012).

The proliferation of B-cells undergoing affinity maturation creates the cell aggregate that histologists have long known as the germinal center. Lower vertebrates do not appear to have histologically distinct structures like GCs, but they do have the enzyme AID.

Previous studies in our laboratory used *in situ* hybridization of AID transcripts to identify sites of possible antibody affinity maturation. AID-expressing cells were found in close proximity to myeloid auto-fluorescent cells known as melano-macrophages (MMs) in the channel catfish spleen and kidney (Saunders, Oko et al. 2010). RT-PCR on laser-captured micro-dissected sections of MM aggregates or clusters (MMCs) also revealed that in addition to AID, Ig heavy chain (IgH), CD4, and TcRβ are expressed by cells within or proximal to MMCs, suggesting that B-cells and TH-cells exist in these cell clusters (Saunders, Oko et al. 2010). These findings are consistent with MMCs having GC-like cellular constituents, but the role of the melano-macrophages in immunity has not yet been established, though it has been postulated.

Past studies have shown the retention of soluble antigen within MMs for several weeks after fish vaccination. Because antigen retention was accompanied by high levels of antibody production, it was suggested that MMCs were crucial

in the development of immunological memory (Press 1996). However, it has also been shown that MMs can phagocyte particulate antigens such as bacteria, thus raising the possibility that MMs function as antigen presenting cells that activate T_H cells before these lymphocytes activate antigen specific B-cells.

We hypothesized that the cytokine profile of macrophages operating to simply trap antigen would anti-inflammatory rather than pro-inflammatory, characterized by the expression of Interleukin 10 (IL-10) and transforming growth factor β (TGF-β). And that this expression profile would be different from antigen presenting cells (APCs) which would exhibit a pro-inflammatory response characterize by tumor necrosis factor-alpha (TNF-α) and interleukin 1β (IL-1β). APCs like macrophages and dendritic cells (DCs) are capable of immediately responding to pathogenic insults by producing mediators of inflammation that activate other cells types for the development of the immune response (Reviewed in Batista and Harwood 2009). The up-regulation of pro-inflammatory cytokines by APCs after stimulation has been extensively characterized. Bovine DCs have been demonstrated to up-regulate the expression of pro-inflammatory cytokines namely TNF- α and IL-12 after stimulation with *Mycobacterium avium* spp. and *Escherichia coli* (Langelaar, Weber et al. 2005), and canine DCs were shown to up-regulate the expression of IL-1β, IL-10, IL-12, and TNF-α after stimulation with LPS or TNF-α (Wang, Chi et al. 2007). Furthermore, DCs play a central role in the balance of T helper 1 (T_H1) and T_H2 responses by providing T cells with differential antigenic and co-stimulatory signals (Reviewed in Kalinski and Moser 2005). DCs activated by viruses produce cytokines like IL-12, IL-15 and IL-18

that induce $T_H1$cell responses, which subsequently promote anti-viral activity (Reviewed in Kalinski and Moser 2005). APCs activated by extra-cellular pathogens produce IL-4, IL-25, and IL-33 that induce T_H2 responses, which result in the development of humoral immunity (Reviewed in Paul and Zhu 2010).

Our goal was to adapt a method to purify MMs from channel catfish to the goldfish, for which macrophage functions are much better characterized. The expression levels of both pro- and anti-inflammatory cytokines have been compared between MMs and other tissue macrophages isolated from the lymphoid tissues of 'healthy' and vaccinated goldfish. We further examined the cytokine profiles of the same cell populations following *in vitro* exposure to either heat-killed bacteria or apoptotic cells, to determine the potential of MMs to produce each of the cytokines we were examining.

1.2. The B-cell receptor primary repertoire

T- and B-lymphocytes are two major cell types of the adaptive immune response of vertebrates that detect and combat foreign particles through their antigen-specific receptors. While the receptor of the T-cell is membrane bound, the receptor of the B-cell can be retained at the membrane or can be secreted. The B-cell receptor, antibody or immunoglobulin (Ig), is formed of four polypeptide chains; two identical heavy chains encoded by the immunoglobulin heavy chain (IgH) genes, and two identical light chains encoded by the immunoglobulin light chain (IgL) genes (Figure 1). Each of the immunoglobulin genes comprises a constant (C) and a variable region (V). The variable region encodes the antigen-biding site and it is generated by the recombination of three types of sub-exon

called a variable-element (V), diversity-element (D) and Joining-element (J) in the IgH gene, or several V- and J-elements in the case of the IgL gene (Figure 2). The constant region constitutes the isotype of the antibody and determines its biological function; in higher vertebrates five isotypes have been described: IgG, IgM, IgD, IgE, and IgA.

Ig gene segments are scattered along the chromosome (Reviewed in Kalinski and Moser 2005) and are brought together to form a functional Ig gene during the B-cell development. This process results in B-cells expressing antibodies of a single specificity. In the IgH, the V, D, and J elements are randomly brought together in a process know as somatic recombination, and the resulting VDJ exon is then joined to a constant region to form a fully functional gene (Altfeld, Fadda et al. 2011). The IgL gene undergoes a similar process with the exception that there are no D elements.

In order to recognize and to mount specific immune responses against the huge variety of antigens vertebrates encounter, the adaptive immune system generates a highly diverse repertoire of antibody-expressing B-cells. Two rounds of genetic modifications to the immunoglobulin gene achieve this great diversity. The primary repertoire is generated during early stages of the B-cell development in the primary lymphoid organs (the adult bone marrow or the fetal liver) is estimated to generate over 10^{11} different antibody specificities in humans (Hozumi and Tonegawa 1976; Brack and Tonegawa 1977). Because the binding region of the antibody is randomly generated (Tonegawa 1983; Fanning, Connor et al. 1996), its ability to bind antigen is given by chance and its affinity and

specificity for an antigen are usually low. For this reason, a secondary antibody repertoire is generated by antigen-dependent modifications to the pre-arranged Ig gene through a process known as the antibody affinity maturation. The antibody affinity maturation occurs in highly organized microenvironments known as germinal centres (GC), which have evolved to improve the affinity and the specificity of the primary repertoire antibody for the challenging antigen.

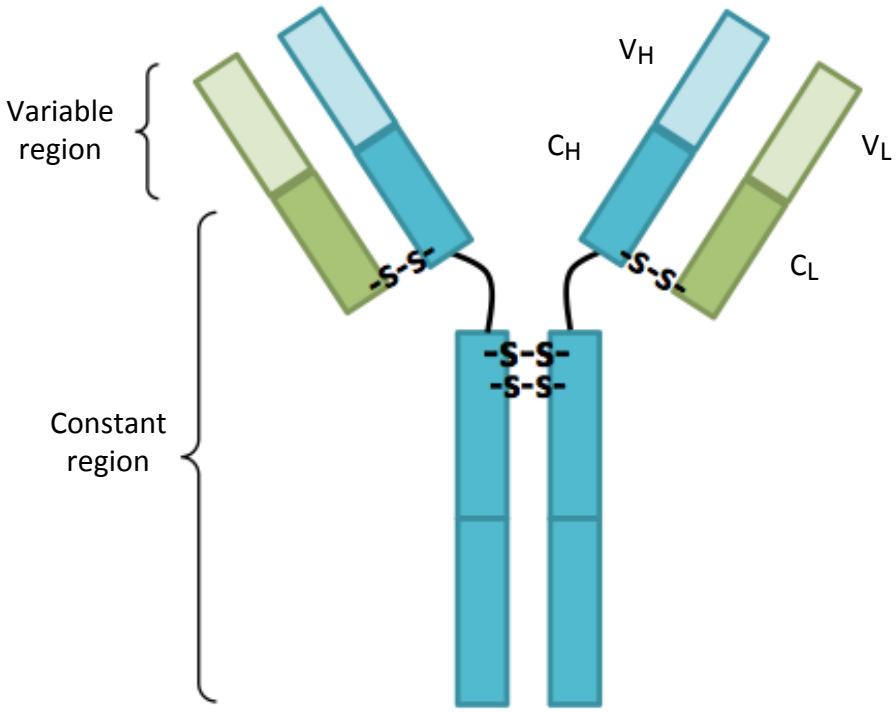

Figure 1.1. Simplified diagram of the immunoglobulin molecule.
The immunoglobulin molecule consists of two light chains (depicted in green) and two heavy chains (depicted in blue) attached by disulfide bonds (-s-s-). The heavy chain consists of one variable and a few constant domains. The light chain comprises one constant and one variable domain. The variable domains from the heavy and the light chains (depicted in light blue and green respectively) conform the variable region responsible for the antigen-binding site of the antibody. The constant domains of the heavy chain correspond to the isotype of the antibody, which determines its biological function.

1.3. The germinal centre (GC) reaction in mammals.

1.3.1. Initial stage of the B-cell activation and differentiation.

Germinal centres (GCs) develop in B-cell follicles as a response to antigen challenge. These follicles are mainly composed of naïve B lymphocytes and follicular dendritic cells (FDCs), and are located in secondary lymphoid organs such as the spleen and the lymph nodes (Reviewed in Market and Papavasiliou 2003; Janeway 2005).

Mature B-cells constantly recirculate through the follicles of secondary lymphoid organs searching for antigen. Initial activation of B-cells occurs by engagement of the BCR to a specific antigen. Following antigen encounter, B-cells internalize and process the antigen and migrate to the boundary between the B-cell follicle and the T-cell area (T-B boundary) in the search for T-cell help signals (Hozumi and Tonegawa 1976; Tonegawa 1983). In order to provide help signals, naïve T-cells need to become activated first. Activation occurs when the T-cell receptor (TCR) binds its cognate antigen, which is presented to the T-cell in the form of MHC class II-antigen complex by DCs. Activated T-cells migrate to the T-B boundary where they provide help signals to B-cells upon interaction of the antigen-specific TCR with the MHC class II-antigen complex on the B-cell (MacLennan 1994). The interaction between B and T_H lymphocytes leads to the proliferation of B-cells in the outer follicle before the appearance of the GC (Garside, Ingulli et al. 1998). The resulting B-cell population from the early proliferation is then divided into a fraction that forms the GC (along with a few

activated T_H-cells), and another that eventually differentiates into antibody-secreting plasma cells (Reviewed in King 2009).

Several factors play important roles in the differentiation of activated B-cells into the GC or the plasmablast pathway. These factors include but are not limited to: transcriptional control, interactions with T_H cells, and the strength of the interaction between the B-cell primary repertoire receptor and the antigen (Coffey, Alabyev et al. 2009; Chan, Gardam et al. 2010). GCs are heavily dependent on T_H signals. Especially, the interaction between CD40, a TNF family receptor constitutively expressed in B-cells, and its ligand CD40L (CD154), expressed by activated T_H cells has been found to be of particular importance in the GC formation (Blink, Light et al. 2005; Inamine, Takahashi et al. 2005; Chan, Gatto et al. 2009).

After T-dependent antigen B-cell activation, the first wave of antibody production is generated by short-lived plasma cells as a response to the host's need for immediate protection against invading antigen. These short-lived plasma cells result from B-cell rapid proliferation and differentiation in the extra-follicular areas of secondary lymphoid tissues (Gatto and Brink 2010).

Several studies indicate that when the primary repertoire antibody has a higher affinity for the antigen, B-cells tend to differentiate into short-lived plasma cells in extra-follicular. Contrary, when the antibody affinity for the antigen is weak, B-cells tend to undergo the GC pathway and differentiate into long-live memory B-cells and plasma cells (Foy, Laman et al. 1994; Han, Hathcock et al. 1995).

1.3.2. Germinal centre cell types.

Follicular dendritic cells (FDCs):

Little is known about the FDC origin and early development; a definite precursor of FDCs has not been identified yet, and it is unknown if FDCs differentiate from follicular stromal cells, or if the differentiate from migratory precursors (Jacob and Kelsoe 1992). FDCs are stromal, not hematopoiesis-derived cells, and unlike professional antigen presenting dendritic cells they do not express MHC class II molecules (O'Connor, Vogel et al. 2006; Paus, Phan et al. 2006; Chan, Gatto et al. 2009).

FDCs form networks that occupy most of the LZ in the GC (Allen and Cyster 2008). These networks trap and retain unprocessed antigen primarily through complement receptors CR1 and CR2 (Cyster, Ansel et al. 2000), but also through Fc receptors like FcγRIIb and FcεRII, (Cyster, Ansel et al. 2000). Antigen retention by FDCs serves as a long term antigen deposit important in the selection of high-affinity B-cell clones and in the establishment of immunological memory (Barrington, Pozdnyakova et al. 2002). Antigen presentation by FDCs is highly stimulatory for B-cells (Cyster, Ansel et al. 2000) and can induce AID expression, thereby leading to SHM and CSR (Mandel, Phipps et al. 1980).

FDCs also provide chemo-attractant signals for the recruitment of B-cells and T_{FH} cells to the LZ of the GC; FDCs are a source of CXCL13, the ligand for CXCR5 which is expressed by naïve B-cells, some GC B-cells, and T_{FH} cells (Tew, Wu et al. 2001). And provide high affinity B-cell clones with survival

signals that switch off the pre-program apoptotic machinery (Aydar, Sukumar et al. 2005).

Follicular T helper cells:

T_{FH} are a small, specialized population of $CD4^+$ T-cells that constitute about 5 to 20% of the GC cells (Forster, Mattis et al. 1996; Haynes, Allen et al. 2007). Little is known about the development and gene expression of T_{FH} cells; early studies with micro arrays indicate that the T_{FH} gene expression profile is different from T_H1 and T_H2 cells, suggesting that T_{FH} cells represent a distinct lineage or maturation state (MacLennan, Liu et al. 1990; Grouard, de Bouteiller et al. 1995).

T_{FH} are characterized by their follicular localization; the up-regulation of the chemokine receptor CXCR5, along with the production of its ligand by FDCs, allows for the migration of T_{FH} to the LZ of the GC (Arnold, Campbell et al. 2007; Haynes, Allen et al. 2007).

T_{FH} are essential for the GC reaction; when lymphocyte-signaling molecules provided by T_{FH} are absent, the GCs formed are abnormal in different ways and in different levels (Breitfeld, Ohl et al. 2000; Schaerli, Willimann et al. 2000). T_{FH} express the cytokine IL-21, which is has little autocrine function, but is very important in signaling GC B-cells. Il-21 directly regulates Bcl-6 expression, thereby allowing for GC B-cell proliferation and differentiation (Haynes, Allen et al. 2007).

Tingible body macrophages (TBMs):

TBMs are a specialized subset of macrophages that reside in the GCs and engulf apoptotic cells resulting from the affinity maturation process occurring in the GCs (Cannons 2010; Good-Jacobson and Shlomchik 2010; Vinuesa, Linterman et al. 2010). The phagocytosis of apoptotic cells by TBMs is facilitated by opsonins, and defects in opsonins or their receptors have been associated with autoimmunity in humans and mice (Linterman, Beaton et al. 2010; Zotos, Coquet et al. 2010). Milk fat globule epidermal growth factor 8 (Mfge8) is an opsonin that binds to phosphatidylserine on apoptotic cells and to integrins on macrophages (MacLennan 1994). Mfge8 deficiency impairs engulfment of apoptotic cells by TBMs in mice, and it has been associated with diseases like systemic lupus erythematous and autoimmune glomerulonephritis (Vinuesa, Sanz et al. 2009).

TBMs suppress the immune response by reducing their reactivity to apoptotic cells. In their absence, apoptotic cells accumulate in the T-cell zone and their clearance is relegated to red pulp macrophages which increase the production of pro-inflammatory cytokine thus enhance lymphocyte reactivity to apoptotic cells. Moreover, TBM absence accelerates the progression of systemic lupus erythematous in mice prone to the autoimmune disorder (Hanayama, Tanaka et al. 2002).

Centroblasts

Centroblasts are highly proliferative B-cells found in the DZ of the GC. Centroblasts down-regulate the expression of surface Ig and activate the expression of the immunoglobulin mutator enzyme, activation-induced cytidine deaminase (AID) (Hanayama, Tanaka et al. 2002). As the population of centroblasts increases, it continues to acquire AID mediated somatic point mutations in their respective Immunoglobulin variable region (IgV) in a process known as somatic hypermutation (SMH) (McGaha, Chen et al. 2011) (Figure 1.3).

Centrocytes

Centrocytes are B-cells found in the LZ of the GC. Centroblasts migrate to the LZ where they are known as centrocytes. Contrary to centroblasts, centrocytes are not proliferative and they express surface Ig. The binding region of the membrane Ig slightly varies from centrocyte to centrocyte due to the SHM process B-cells undergo in the DZ of the GC. Centrocytes are pre-programmed to undergo apoptosis and must receive survival signals in order to suppress their apoptotic program (Muramatsu, Kinoshita et al. 2000; Luo, Ronai et al. 2004)

1.4. The antibody affinity maturation and the B-cell selection in the germinal centre.

GCs are the main sites of diversification and affinity maturation of B-cells that have encountered antigen. The mature GC develops in the centre of the B-cell primary follicle where naïve B-cells are pushed aside to become the follicular

mantle surrounding the GC. The GC comprises two distinct compartments known as the dark zone (DZ) and the light zone (LZ) differentiated by their histological appearance (Neuberger, Lanoue et al. 1999).

The classical model that explains the antibody affinity maturation is known as the cyclic re-entry model, and it spatially separates proliferation and diversification in the DZ, from antigen binding mediated selection in the LZ. In this model, activated B-cells committed to the GC pathway differentiate into centroblasts (Reviewed in Victora and Nussenzweig 2012). As centroblasts proliferate, the growing population continues to acquire AID mediated somatic point mutations in their respective Immunoglobulin variable region (IgV) through SHM (Reviewed in Victora and Nussenzweig 2012) (Figure 1.3). The IgV of the heavy chain is germline assembled by recombination of the variable (V), diversity (D) and joining (J) elements (MacLennan 1994). Mutations occurring in this region happen at a rate as high as 10^3 per base pair per generation (which is 10^6-fold the normal rate of somatic mutation) (Neuberger, Lanoue et al. 1999), and result in random changes in the B-cell Ig affinity and specificity (Dudley, Manis et al. 2002). AID also mediates class switch recombination (CSR), the process of Ig isotype switching where the effector function of the antibody changes without changing its specificity (McKean, Huppi et al. 1984; Berek and Milstein 1987) (Figure 1.3). Although CSR can occur shortly after the initial interaction between the B-cell and the T-cell in the T-cell zone prior to entering the B-cell follicle, it can still occur within the GC (Gearhart and Wood 2001).

After centroblasts proliferate they differentiate into centrocytes and migrate from the DZ to the LZ. Centrocytes start re-expressing cell surface Ig when mutations resulting from the somatic hypermutation (SHM) process have not inactivated the Ig gene. Centrocytes are pre-programmed to undergo apoptosis, and in order to be rescued from death they must compete for antigen trapped on the surface of FDCs to receive survival signals from T_{FH} cells. The amount of antigen trapped by FDCs in the LZ is limited, therefore only B-cells with higher affinity are able to bind and receive survival signals (Honjo, Muramatsu et al. 2004). Centrocytes that are not rescued from death undergo apoptosis and are engulfed by TBMs present in the LZ.

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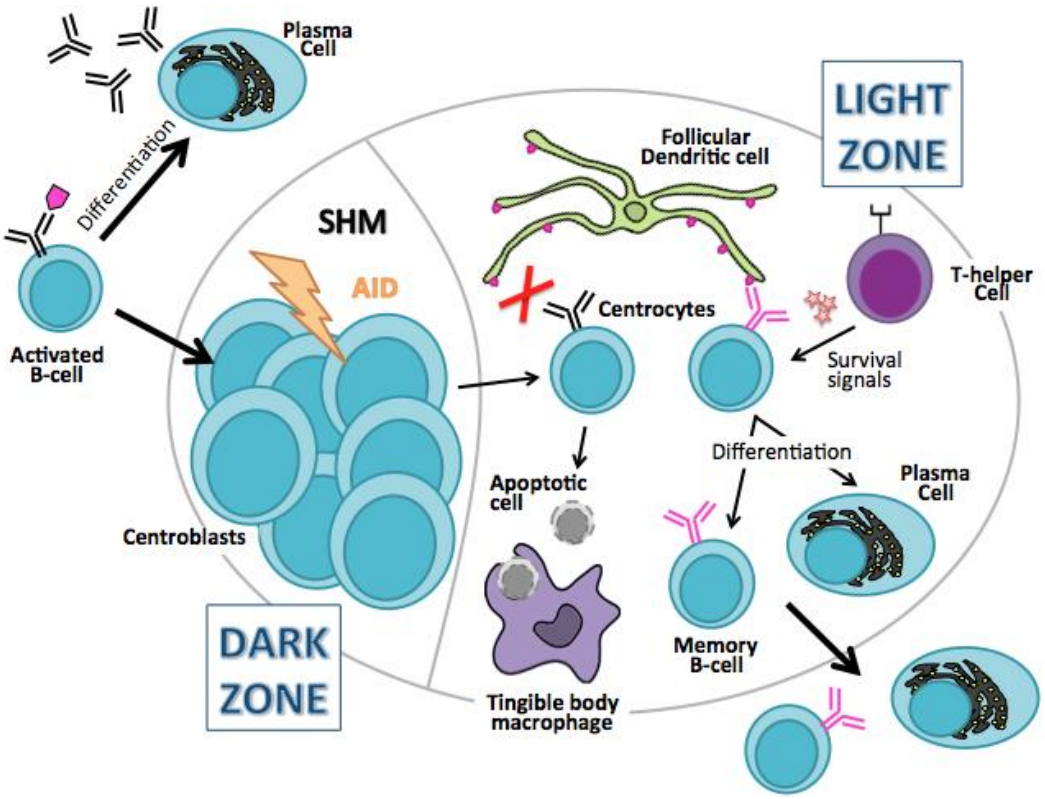

Figure 1.2. Schematic representation of the classical model for the germinal centre reaction in higher vertebrates.
A fully activated B-cell clonally expands in the dark zone of the germinal centre. As proliferation occurs cells (centroblasts) undergo Somatic hypermutation in their antigen-binding region. Follicular dendritic cells retain un-processed antigen in their surface, as the antigen amount is limited, B-cells (centrocytes) with newly mutated receptors compete for antigen binding in the light zone of the germinal centre in order to receive survival signals from follicular dendritic cells and follicular T helper cells. B-cells with disadvantageous mutations undergo apoptosis and are phagocytosed by tingible body macrophages. B-cells with the highest affinity for the antigen survive and differentiate into long lasting memory B-cells or antibody-secreting plasma cells.

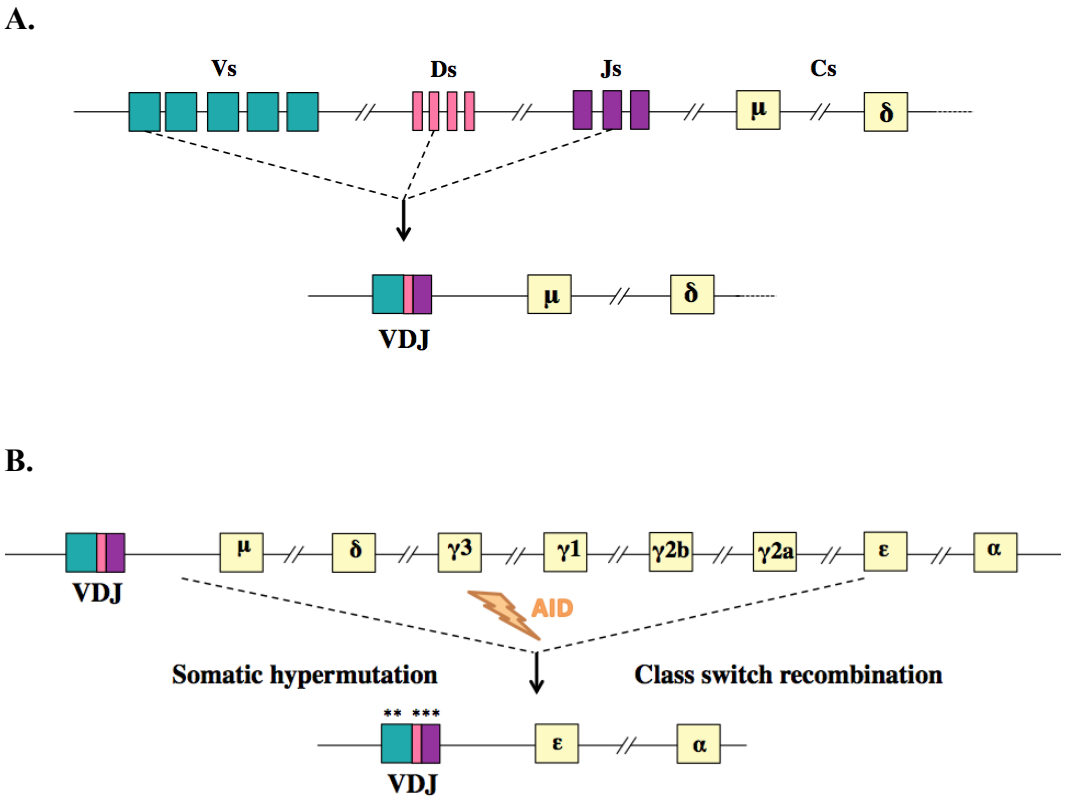

Figure 1.3. Simplified diagram of the hypothetical IgH gene.
(A) IgH gene formation by random recombination of V, D, and J sub-exons during primary antibody repertoire development. (B) Development of secondary antibody repertoire by AID-dependent somatic hypermutation and class switch recombination.

1.5. The antibody affinity maturation in teleost

1.5.1. The IgH and IgL genes of teleost fish

The fish kidney is considered to be the primary hematopoietic organ, analogous to the mammalian bone marrow. Evidence suggests that in fish, B-cells mature in the anterior kidney and then migrate to secondary lymphoid organs, the spleen and the posterior kidney (non-classical lymphoid organ), where cell activation and development of the secondary antibody repertoire take place (Toellner, Luther et al. 1998; Pape, Kouskoff et al. 2003).

Like in higher vertebrates, the IgH genes of teleost are arranged in a translocon organization (Goodnow, Vinuesa et al. 2010), where fish use the V, D, and J elements to assemble the variable region of the primary antibody repertoire. This assembly occurs by recombination of different Vs, Ds, and Js with each other, and further diversity is given by the addition and deletion of nucleotides, which generates junctional diversity (Zwollo, Cole et al. 2005).

The IgL genes of fish are arranged in clusters like Ig (both IgH and IgL) genes of sharks and other cartilaginous fish. These clusters comprise one V element, one or more D elements (no D elements for IgH), one J element, and one constant region (Hinds and Litman 1986), and mounting evidence suggests that the recombination process occurs within and not between clusters (Ventura-Holman and Lobb 2002). The organization of teleost Ig genes represents an evolutionary bridge that links the mechanisms of diversification of the variable region employed by higher and lower vertebrates (Dooley and Flajnik 2006).

Expression of both IgM and IgD isotypes, results from alternative splicing in higher vertebrates and teleost fish (Hinds and Litman 1986; Kokubu, Litman et al. 1988; Greenberg, Avila et al. 1995; Lee, Fitch et al. 2000). Although expression of IgG, IgA, and IgE equivalents has not been found in teleost, a different IgH isotype, IgZ or IgT, has been described in numerous fish species (Dooley and Flajnik 2006). IgZ expression occurs by alternative splicing of the VDJ, and not from CSR (Bengten, Quiniou et al. 2002; Bengten, Quiniou et al. 2006).

1.5.2. AID and SHM evidence in fish, but an apparent lack of conventional germinal centres.

To date, AID homologues have been described in all examined jawed vertebrates (Danilova, Bussmann et al. 2005; Hansen, Landis et al. 2005; Savan, Aman et al. 2005; Gambon-Deza, Sanchez-Espinel et al. 2010), and some of these homologues have been found capable of driving SHM and CSR in AID knockout mice (Danilova, Bussmann et al. 2005; Hansen, Landis et al. 2005). There is no evidence that fish undergo CSR, and this has been consistent with fish lacking the element targeted by AID during this process (Conticello, Thomas et al. 2005; Rogozin, Iyer et al. 2007). Additionally, although CSR makes its first appearance in amphibians and is only present in higher vertebrates (Barreto, Pan-Hammarstrom et al. 2005; Wakae, Magor et al. 2006), teleost AID has been shown to be able to perform this function in mouse cells; zebrafish AID is able to mediate CSR at levels comparable to the mouse AID, while the catfish and the

pufferfish AID exhibit significantly less activity (Flajnik 2002; Cannon, Haire et al. 2004; Stavnezer and Amemiya 2004).

Different groups have indicated that SHM occurs after repeated immunization in amphibian *Xenopus laevis*, (Diaz and Flajnik 1998; Flajnik 2002; Hsu, Pulham et al. 2006), the nurse shark (Barreto, Pan-Hammarstrom et al. 2005; Wakae, Magor et al. 2006), and fishes (Wilson, Hsu et al. 1992). Furthermore, more recent studies in the nurse shark demonstrated a highly antigen-specific Ig response after prolonged immunization, indicating that somatic hypermutation occurs *in vivo* (Dooley and Flajnik 2005).

1.5.3. Localization of AID in Catfish tissues.

Using AID as a marker for sites of affinity maturation, the location of AID-expressing B-cells has been identified through *In situ* hybridization. These B-cells were visualized in the catfish posterior kidney, in the classical secondary lymphoid organs: the spleen and to a very limited extent in the intestine (Russell, Voss et al. 1970; Voss, Groberg et al. 1978; Lobb 1985; Cain, Jones et al. 2002). These AID expressing B-cells were always found in close proximity to auto-fluorescent cells known as melano-macrophages (MMs). MMs are myeloid cells that express the colony-stimulating factor 1 receptor (CSF-1R) but not AID (Dooley and Flajnik 2006). MMs and AID expressing B-cells in the spleen and the kidney of the catfish were typically found in clusters known as melano-macrophages centres (MMCs) (Saunders, Oko et al. 2010).

1.6. Melano-macrophages and their pigments.

Melano-macrophages are pigmented cells located in the tissues of poikilothermic vertebrates (Saunders, Oko et al. 2010). Different types of pigments have been found in MMs through histochemistry techniques, namely, melanin, lipofuscin, and hemosiderin. The origin and function of these pigments is not well understood (Saunders, Oko et al. 2010), and presence of the pigments varies between different species (Roberts 1975), and within cells and tissues of the same organism (Wolke, Murchelano et al. 1985), making variable the MM morphological appearance.

1.6.1. Lipofuscin.

Lipofuscin is often the predominant pigment found within MMs (Roberts 1975; Agius 1980), and also the most widespread pigment in the MMCs of numerous fish species (Agius and Agbede 1984; Kranz and Peters 1984). Lipofuscin is thought to come from the degradation of damaged subcellular membranes by oxidation of polyunsaturated fatty acids (Agius 1979), and the main source of this pigment was postulated to be the degradation of degenerate mitochondria through lipid-peroxidation (Agius 1979; Agius, Robinson et al. 1979). Lipofuscin progressively accumulates with age (Tsuchida, Miura et al. 1987), but it also builds up to a greater extent in fish suffering from tissue decay associated with dietary deficiencies or disease caused by bacteria or virus (Agius and Agbede 1984).

1.6.2. Melanin.

Melanin is the least common pigment found in MMCs (Agius 1981). This pigment is a complex polymer of heavy molecular weight that varies in color from yellow to dark brown (Agius and Roberts 1981). It was originally believed that MMs were incapable of synthesizing melanin (Wolke 1992), and early studies in fish suggested that melanin in MMCs came from the phagocytosis of melanin-containing cells (Edelstein 1971). Later studies in the Gilthead Sea bream (Ellis 1974) among other marine bony fishes (Agius and Agbede 1984), and in different frog species (Zuasti, Jara et al. 1989), demonstrated that MMs are capable of synthesizing melanin, although this process differs from the melanogenesis occurring in melanocytes. Melanin is capable of neutralizing free radicals and toxic agents derived from the breakdown of phagocytosed material (Zuasti, Ferrer et al. 1990). For this reason, two possible functions were suggested for the melanin in MMs: First, to neutralize the free radicals from the breakdown of fatty acids of cellular membranes at low temperatures (Sichel, Scalia et al. 1997; Zuasti, Jimenez-Cervantes et al. 1998; Gallone, Guida et al. 2002), and second, to be of importance in the production of antibacterial compounds such as hydrogen peroxide (Zuasti, Jara et al. 1989).

1.6.3. Hemosiderin.

Hemosiderin is a brown, insoluble pigment involved in iron storage. In higher vertebrates, iron is stored in the body as ferritin. When the organism becomes saturated with iron, iron is then stored at intracellular level as hemosiderin (Agius and Agbede 1984). Hemosiderin is composed of iron in

ferric form and the protein apoferritin, which is derived from the break down of the hemoglobin of effete red blood cells (Wolke, Murchelano et al. 1985). Two possible roles of hemosiderin are iron storage, and its accumulation as a defense mechanism.

In teleost fish, hemosiderin is usually restricted to the melano-macrophages clusters of the spleen (Agius 1979). Hemosiderin concentration is found to be generally low, but it appears to be higher in melano-macrophage clusters of fish with hemolytic anemia (Kranz 1989), and fish exposed to prolonged starvation (Agius 1979; Agius and Roberts 1981).

1.7. Melano-macrophage clusters (MMCs).

Melano-macrophage clusters (MMCs) or macrophage aggregates are groupings of pigmented cells present in the tissues of poikilothermic vertebrates (Roberts 2001). The study of the hematopoietic organs of 72 different species representative of Agnatha, Chondrichthyes and Osteichthyes, revealed the presence of MMs in all species studies with the exception of the lamprey where MMs where not found (Agius and Roberts 1981). In addition, the study revealed the evolution in the organization of the MMs from a random distribution in Agnatha and Chondrichthyes, to organized centres in all Osteichthyes except salmonids. This tendency to organization was observed along with the change in the location of MMs. In cartilaginous and primitive bony fish MMs are mainly found in the liver, while in teleost fish, MMs are mainly found in the two major lymphoid organs, the spleen and the kidney (Roberts 1975). More so, MMCs are generally located in the main site of hematopoiesis of these two tissues: the

stroma of the spleen and the interstitium of the kidney (Agius 1980), although they may also occur in the gills, brain and gonads of these fishes, but not in the form of clusters (Agius 1980).

1.8. The macrophage role in immunity.

Mammalian macrophages are derived from bone marrow hematopoietic stem cells (Roberts 1975), and they are found throughout the body acting as initiators and effectors of the innate immune response (Kranz and Peters 1984; Macchi, Romanol et al. 1992; Meseguer, Lopez-Ruiz et al. 1994; Haaparanta, Valtonen et al. 1996). Macrophages are a heterogeneous group of cells with different functions, morphologies, and phenotypic characteristics, that are classified into subpopulations based on their anatomical location in the organism and their functional phenotype (Naito, Umeda et al. 1996; Takahashi, Naito et al. 1996).

Macrophages are professional phagocytes that help maintain homeostasis; they constitutively express a wide array of scavenger receptors to facilitate the removal of senescent cells, and tissue remodeling and repair after inflammation, thus being essential in maintaining tissue homeostasis (Ruco and Meltzer 1977; Meltzer, Occhionero et al. 1982; Held, Weihua et al. 1999). Additionally, macrophages also are key players of the innate immune response; they express pattern-recognition receptors that allow them to recognize pathogen-associated molecular patterns (PAMPs) expressed during microbial infections, and damage-associated molecular patterns (DAMPs) expressed during cellular stress (Gordon and Taylor 2005).

Macrophages are a heterogeneous group of cells divided into subpopulations based on their functional phenotype and location (Gordon 1986; Gordon 1998; Gordon and Taylor 2005). Some examples of these subpopulations are: macrophages in the gut which help maintain tolerance to food and gut flora, macrophages in immune privileged site which play a central role in tissue remodeling and maintenance of homeostasis, and macrophages in secondary lymphoid organs which suppress the innate and adaptive immune response to apoptotic cells, macrophages in the bone which are in charge of bone resorption, and macrophages in the lung in charge of removing dust, allergens and microorganisms (Gordon and Taylor 2005).

Macrophages subsets have been further characterized based on their gene expression profile to different stimuli (Gordon and Taylor 2005). This includes but is not limited to: classically activated macrophages (M1), alternatively activated macrophages (M2), and regulatory macrophages.

M1 macrophages exhibit a pro-inflammatory phenotype and are involved in mediating host defense from viruses, bacteria, and protozoa. M1 macrophages are characterized by the expression of inflammatory mediators including TNF-α, Interferon-gamma (IFN-γ), IL-1β, and IL-6. These macrophages also exhibit enhanced antimicrobial functions and often produce nitric oxide (NO) and reactive oxygen species (ROS) (Reviewed in Murray and Wynn 2011).

M2 macrophages antagonize inflammatory responses and promote wound healing (Biswas and Mantovani 2010). These macrophages are characterized by the production of growth factors that stimulate epithelial cells and fibroblasts,

including TGF-β and platelet-derived growth factor (PDGF). M2 macrophages engulf dead cells and debris that promote M1 macrophage responses and express the immune-regulatory cytokine IL-10 (Reviewed in Mosser and Edwards 2008; Murray and Wynn 2011).

Regulatory macrophages are potent inhibitors of inflammation. These cells retain the ability to produce pro-inflammatory cytokines, but are characterized by the production and secretion of large quantities of the immune regulator IL-10 in response to different stimuli including immune complexes, apoptotic cells, or IL-10 (Reviewed in Murray and Wynn 2011).

Deregulation of the different functional phenotypes leads to host illness; excessive response by pro-inflammatory macrophages, or switch from anti-inflammatory to pro-inflammatory phenotype can lead to chronic inflammation and auto-immunity diseases (Reviewed in Murray and Wynn 2011).

Polarization of macrophage activation has also been described in teleost species. Head kidney derived macrophages of the carp where found to display differential regulation of immune relevant genes upon stimulation with LPS, an inducer of classical activation, or cAMP, and inducer of alternative activation (Reviewed in Mosser and Edwards 2008). Regulation of immune relevant genes has also been described in the trout. *In vitro* exposure to LPS was shown to up-regulate the expression of IL-1β (Reviewed in Murray and Wynn 2011), TNF-α (Joerink, Ribeiro et al. 2006), and IL-10 (Brubacher, Secombes et al. 2000).

Macrophages are key mediators of the immune response and they secret a number of signaling molecules depending on their state of activation. During

both the pro- and anti-inflammatory response, a wide array of cytokines is expressed in order to extend the response to other cells. TNF-α and IL-1β are two cytokines characteristic of the pro-inflammatory response. TNF-α is crucial in inflammation and host defense (MacKenzie, Planas et al. 2003); it has been shown to enhance cellular functions including phagocytosis and the production of reactive oxygen and nitrogen intermediates, and also to up-regulate the production of other pro-inflammatory cytokines (Harun, Costa et al. 2011). IL-1β is involved in the response to microbial infection and tissue injury; it enhances phagocytic activity, macrophage proliferation, and leukocyte migration (Beutler, Greenwald et al. 1985).

Two hallmark cytokines of the anti-inflammatory response are IL-10 and TGF-β). IL-10 has been linked to the inhibition of TNF-α synthesis (Pfeffer 2003; Goetz, Planas et al. 2004), and has been shown to down-regulate inflammatory processes including the production of NO and ROI, and the pro-inflammatory cytokine production by monocytes/macrophages in mammals (Dinarello 1997). The goldfish IL-10 has been previously identified and characterized (Oswald, Wynn et al. 1992). Functional analyses of IL-10 demonstrated its role in the down-regulation of pro-inflammatory cytokines production, and the down-regulation of the ROI response in goldfish monocytes, thus indicating the conservation of IL-10 function through evolution (Bogdan, Vodovotz et al. 1991; Oswald, Wynn et al. 1992). TGF-β is a pleiotropic cytokine that has important roles in the regulation of cell proliferation, differentiation, survival, activation and de-activation (Grayfer, Hodgkinson et al.

2011). TGF-β has been identified and characterized in the goldfish. Functional analyses showed that recombinant TGF-β down-regulated the production of reactive nitrogen intermediates in activated cells (Grayfer, Hodgkinson et al. 2011).

1.9. Rationale for the Research.

The germinal centre is a highly regulated microenvironment that supports the antibody affinity maturation process that activated B-cells undergo in higher vertebrates. Mounting evidence suggests that lower vertebrates undergo antibody affinity maturation; although modest, increase in the antibody affinity has been observed after repetitive immunization in amphibians (Li, Wan et al. 2006) and fish (Haddad, Hanington et al. 2008). It has been thought that the reason for the poor increase in the antibody affinity is the lack of conventional GCs in lower vertebrates. Because AID is essential for affinity maturation, previous work in our laboratory used this enzyme as a marker for SHM, and found AID-expressing B-cells in close proximity to MMs in cell aggregates, which by RT-PCR, were shown to contain Ig heavy chain, TcR□ and CD4 expressing cells (Wilson, Hsu et al. 1992). These findings are consistent with a GC-like structure.

For three specific reasons it was hypothesized that if melano-macrophages were involved in the antibody affinity maturation of the goldfish, after exposure to *A. salmonicida* or to apoptotic cells they would adopt an anti-inflammatory or regulatory functional phenotype characterized by IL-10 and TGF-β, rather than a pro-inflammatory one characterized by TNF-α and IL-1β. First, the similarities

found between the MMC tissue architecture and aberrant GCs found at the site of auto-immunity and cancer in humans, suggesting that under this disorders the GC reverts to a primitive structure similar to fish MMCs. Second, MMs have been previously shown to trap and retain antigen and to up-take apoptotic cells, resembling follicular dendritic cells and tingible body macrophages respectively. And third, the absence of suppressant GC macrophages precipitates autoimmune disease progression (Cain, Jones et al. 2002; Kaattari, Zhang et al. 2002). A different possibility is for MMs to acquire the functional phenotype of `classically activated macrophages after exposure to *A. salmonicida*. This phenotype is characterized by the up-regulation of the pro-inflammatory cytokines TNF-α and IL-1β, and would indicate that MMs are acting as antigen presenting cells involved in the innate antimicrobial response.

Understanding the dynamics of the MMC will give information on how the antibody affinity maturation process first originated in lower vertebrates, and it could also help improve the immunization techniques used in aquaculture. Additionally, MMCs resemble ectopic germinal centres found during some autoimmune disorders and some types of cancers in humans, thus fish could potentially be used as a model to study these diseases.

1.10. Aims and Objectives.

Our aim is to understand the role of MMs in the adaptive immune response of fish, in the context of the MMC. The objectives of the present study were to:

1. Adapt the auto-fluorescence based cell sorting method for isolating MMs from channel catfish to the goldfish.
2. Verify that the histochemical characterization of MMs done previously *in situ*, holds true for isolated MMs.
3. Use histochemistry to determine how heterogeneous MMs are within and between the spleen and kidney.
4. Establish the 'basal' expression levels of select cytokines from MMS of relatively healthy goldfish spleen and kidney.
5. Establish the cytokine gene expression profile of melano-macrophages when treated under both *in vivo* and *in vitro* conditions; *in vivo* with the heat-killed pathogen *Aeromonas salmonicida*, and *in vitro* with heat-killed *A. salmonicida* and apoptotic bodies.
6. Compare the gene expression profile of MMs to the expression profile of pro-inflammatory macrophages, primary kidney derived macrophages.

CHAPTER 2: MATERIALS AND METHODS

2.1. Fish.

Goldfish (*Carassius auratus* L.) were obtained from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and kept at the aquatic facility of the Department of Biological Sciences, University of Alberta. Fish were maintained in tanks with continuous flow water system at 17^0C, simulated natural photoperiod (Edmonton, Alberta), and fed daily to satiation with trout pellets. After purchased, fish were acclimatized to this environment for at least 3 weeks before used. The numbers of goldfish used for each experiment are listed in Table 2.1.

2.2. Goldfish macrophage culture medium.

The incomplete medium used in all the studies for the culture of goldfish leukocytes (MGFL-15) was previously described (Saunders, Oko et al. 2010). The components of the medium are shown in Table 2.2 and 2.3. Complete medium (C-MGFL-15) consisted of MFGL-15 (incomplete medium) containing 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 10% newborn calf serum (Gibco) and 5% carp serum. All goldfish cell cultures were grown and maintained at 20^0C without additional CO_2.

Fish serum used for tissue culture was obtained by collecting blood from common carp (*Cyprinus carpio*). Carp were anesthetized with tricane methanosulfonate (TMS, Syndel Laboratories) and bled from the caudal vein every 4-6 weeks using a 3mL syringe and a 23-gauge needle. Blood was pooled,

allowed to clot overnight at 4^0C, and centrifuged at 1000 x *g* for 30 minutes. Serum was collected, heat-inactivated at 56^0C for 30 minutes, filter sterilized using a 0.22 μm filter, and stored at -20^0C until used in the culture media.

2.3. Establishment of primary kidney macrophage (PKM) cultures.

PKM cultures were established as previously described (McGaha, Chen et al. 2011). Briefly, freshly isolated leukocytes from the kidney were seeded at a density of ~ 2 x 10^6 cells/mL into tissue culture flasks (Corning) containing 75% of complete medium (C-MGFL-15) and 25% of cell-conditioned medium (CCM) from previous cultures. Cell-conditioned medium was obtained by centrifugation of PKM cultures at days 6-8 of cultivation at 230 x *g* for 10 minutes and 4^0C. The supernatants from individual cultures were pooled, filter sterilized using a 0.2 μm filter and stored at 4^0C until used for tissue culture.

2.4. Isolation of cell populations from goldfish tissues.

2.4.1. Tissue collection.

Fish were anesthetized with TMS (Syndel Laboratories) and sacrificed by severing their spines. The kidneys and the spleens were aseptically removed, placed in ice-cold MGFL-15 with heparin (50,000 U/mL, Sigma) and antibiotics (100 U/mL penicillin / 100 μg/mL streptomycin, Gibco).

2.4.2. Isolation of kidney leukocytes and splenocytes.

After tissue collection the kidneys and spleens were gently passed through sterile 40 μm cell strainers (BD Falcon) in preparation for melano-macrophage isolation, or through sterile stainless steel screens for primary kidney macrophage cultures. In both cases, tissues were processed using sterile plungers from 3 mL syringes and MGFL-15 containing heparin and antibiotics. The resulting single cell suspensions were layered on 51% Percoll (Sigma) and centrifuged for 25 minutes at 400 x *g*. Cells at the Percoll/medium interface were removed and washed twice with MGFL-15 by centrifugation for 10 minutes at 230 x *g* and 4^0C.

2.4.3. Isolation of peripheral blood leukocytes.

Goldfish were anesthetized with TMS (Syndel Laboratories) and blood was drawn from the caudal vein using 1 mL syringes and 25-gauge needles. The blood from individual fish was diluted 1:5 with MFGL-15 containing heparin and antibiotics. For removal of erythrocytes, blood was layer on 51% Percoll (Sigma) and centrifuged for 25 minutes at 400 x *g*. Cells at the percoll/medium interface were removed and washed twice with MGFL-15 by centrifugation for 10 minutes at 230 x *g* and 4^0C.

2.4.4. Isolation of tissue macrophages.

Freshly isolated leukocytes from the spleen and the kidney of the goldfish were seeded into 25 and 75 cm^2 tissue culture flasks (Corning) respectively, with 75% complete medium (C-MGFL-15) and 25% PKM cell-conditioned medium

(CCM). Cells were incubated overnight at 20^0C to allow tissue macrophages to adhere to the tissue culture flasks. Supernatants were removed and the flasks were washed twice with 0.9 X PBS to remove all non-adherent cells. Cells in the supernatant were collected by centrifugation at 230 x *g* for 10 minutes at 4^0C and taken to the flow cytometry unit for melano-macrophage isolation. Flasks containing adherent cells were incubated at 4^0C for 30 minutes with 0.9 X PBS – 0.5 mM EDTA and gently tapped to dislodge tissue macrophages, these cells were collected by centrifugation at 230 x *g* for 10 minutes at 4^0C and re-suspended in complete medium (C-MGFL-15).

2.4.5. Isolation of leukocytes from the peritoneal cavity.

Fish were anesthetized with TMS (Syndel Laboratories) and sacrificed by severing their spine. The peritoneal cavity was flushed with 10 mL of ice cold 0.9 X PBS and cells were collected by centrifugation at 230 x *g* for 10 minutes at 4^0C.

2.5. Melano-macrophage cell sorting.

Melano-macrophages were isolated from the spleen and the kidney based on size, complexity and their auto-fluorescence properties. Isolated peripheral blood leukocytes (PBLs) were use to set the fluorescence parameters for melano-macrophages sort as previously described (Wang 1995; Barreda, Hanington et al. 2005). Isolation of leukocytes and removal of red blood cells (RBCs) from tissues was performed as described in section 2.4. In preparation for cell sorting, leukocytes from blood, spleen and kidney were centrifuged at 230 x *g* for 10

minutes and 4^0C and re-suspended at a concentration of ~ 5-10 x 10^6 cells/mL in 0.9 X PBS – 0.05 mM EDTA. Cells were sorted using a FACSAria flow cytometer (BD Biosciences) at the flow cytometry unit from the Department of Medical Microbiology and Immunology, University of Alberta. A sorting gate was set based on cell on size and complexity. From this gate, cells with fluorescence intensity higher than the background in both the green and the red channels were isolated as MMs. Cells were isolated into tubes coated with 5% BSA containing 5 mL of ice-cold MGFL-15 medium, and were counted with a haemocytometer and trypan blue to asses viability. After the sort, MMs from vaccinated fish were collected by centrifugation at 230 x *g* for 10 minutes at 4^0C, placed in TRIzol reagent, and stored at -80^0C until used for RNA extraction and subsequent cDNA synthesis. Cells intended for *in vitro* studies were collected and re-suspended in C-MGFL-15 (complete media used for tissue culture described in section 2.2).

2.6. Fish vaccination for *in vivo* challenge of melano-macrophages.

In order to study the cytokine production of MMs challenged under *in vivo* conditions, goldfish ranging from 60 to 70 g were anesthetized with TMS and injected intra-peritoneally with heat-killed *A. salmonicida* using 1 mL syringes and 25 gauge needles. Treatments consisted of fish injected 1 time or fish that received a booster injection 21 days after the first vaccination. In both cases, 2 different bacterial concentrations were used: 1 x 10^6 or 1x 10^9 heat killed *A. salmonicida* in 100 µL of 0.9 X PBS. Fish that received only one injection were

sacrificed 21 days later to study the MM during the process of antibody affinity maturation, and fish that received the booster injection were sacrificed 3 days after the boost (24 days after the primary vaccination) to study the response of MMs to a more recent encounter with the antigen. Fish injection with 1 x 10^9 heat killed *A. salmonicida* was based on previous literature (Neumann, Barreda et al. 1998; Neumann, Barreda et al. 2000). The 1 x 10^6 heat killed *A. salmonicida* was chosen in order to compare the effect of the low and high bacterial concentration in the response of MMs to *in vivo* challenge. The low bacterial concentration experimental groups (1 x 10^6 with or without booster injection) consisted of ten individual fish each, and the high bacterial concentration experimental groups (1 x 10^9 with or without booster injection) consisted of five individual fish each. All experimental groups were compared to a control group that consisted of five individual fish that were not vaccinated with the heat-killed bacteria. After vaccination, fish were kept at 17 ^oC until sacrificed. The spleen and the kidney of the goldfish were processed as described in section 2.4, and melano-macrophages from both tissues were isolated as described in section 2.5. Isolated MMs were stored in TRIzol reagent (Invitrogen) at -80^0C until used for RNA extraction and subsequent cDNA synthesis.

2.7. Exposure of leukocytes to *A. salmonicida* or apoptotic cells for *in vitro* challenge of melano-macrophages, tissue macrophages, and primary kidney derived macrophages.

In order to compare the cytokine production of MMs and tissue macrophages under *in vitro* conditions, freshly isolated melano-macrophages and tissue macrophages from the spleen and the kidney of six individual fish were seeded into 96 well plates at a concentration of 3-5 X 10^4 cells/well in complete medium (C-MGFL-15). Cells were incubated for 16 hours at 20^0C in media alone, media containing a 1:200 dilution of heat killed *A. salmonicida* (Saunders, Oko et al. 2010), or in media with a 5:1 (apoptotic cells: macrophage) ratio of apoptotic cells (Katzenback, Karpman et al. 2011). After incubation, cells were harvested, placed in TRIzol reagent and stored at -80^0C until used in RNA extraction and subsequent cDNA synthesis. The 16-hour exposure was chosen based on the PKM *in vitro* studies; the largest regulation of the cytokines analyzed was observed at this time point.

2.8. Heat-killed *Aeromonas salmonicida* A449.

The *Aeromonas salmonicida A449* was a kind gift from Dr. Miodrag Belosevic (Department of Biological Sciences, University of Alberta). Heat-killed *A. salmonicida* was prepared as previously described (Katzenback and Belosevic 2009; Katzenback and Belosevic 2012). Briefly, *A. salmonicida* was streaked onto Tryptic Soy Agar (TSA) plates, incubated at 20^0C for 48 hours, and stored at 4^0C. A single colony was used to inoculate 5 mL of tryptic soy broth

(TSB) and was allowed to grow for 24 hours at 18 °C with shaking to stationary phase. Bacteria were washed twice and re-suspended in sterile 0.9× PBS to the original volume of the culture. *A. salmonicida* was killed by heat exposure in a circulating water bath at 60 °C for 45 minutes and stored at −20 °C until used. After heat-killed, bacteria were streaked on a TSA plate and incubated at 20^0C for 48 hours to ensure the cells were not viable.

2.9. Generation of apoptotic cells and confirmation of apoptosis by flow cytometry.

Because clonal goldfish leukocytes are not available, the catfish 3B11 B-cell line was selected to generate apoptotic cells for all experiments in order to maintain consistency. The 3B11 B-cells were maintained in catfish medium (50% AIM V-50% Leibovitz's L-15) with 10% fetal bovine serum and 1% carp serum. Cells were incubated at 27^0C, 5% CO_2 and were passed once a week at a 1:40 dilution. Apoptotic cells were generated as previously described (Rieger, Konowalchuk et al. 2012). Briefly, cycloheximide (Sigma-Aldrich) was added to the cell culture for 24 hours at a concentration of 10 μg/mL. After exposure, cells were washed 3 times in 0.9 X PBS by centrifugation at 230 x *g* for 10 minutes and 4^0C, to remove all traces of cycloheximide. Fresh apoptotic cells were always prepared previous to use in all the experiments required.

To determine cell viability and the percentage of induced apoptosis, cells were stained following manufacture's instructions. Briefly, cells were harvested and washed once with 0.9x PBS and once with 1× Annexin V binding buffer (BD

Biosciences) by centrifugation 230 x *g* for 10 minutes and 4^0C. Cells were re-suspended at a final concentration of 2×10^7 cells/mL and a 1:20 dilution of Annexin V-Alexa Fluor 488 (Molecular Probes) was added at to 100 µL (2×10^6 cells) of the cell suspension. Cells were incubated for 15 min at room temperature. After staining, cells were diluted in 0.9x PBS and were confirmed to be apoptotic using a FACS Calibur. Fluorescence parameters were set using live cells. Live cells were also stained with the Annexin V conjugate to confirm induction of apoptosis by the cycloheximide.

2.10. Survival of spleen and kidney melano-macrophages.

To study the longevity of MMs in spleen and kidney cell cultures, freshly isolated leukocytes from the spleen and the kidney of three individual fish were seeded into 24 well plates at a concentration of 2.5 X 10^4 cells/well in 75% of complete medium (C-MGFL-15) and 25% of PKM cell-conditioned medium (CCM). Enough cultures were started in order to use a different well for a period of 5 days. The supernatant of the culture was collected and the wells washed twice in 0.9X PBS for MM recovery. Cells were collected by centrifugation at 230 x *g* for 10 minutes and 4^0C, and counted with a haemocytometer and trypan blue to assess viability of the non-adherent population. Cells were also passed through a FACSCalibur flow cytometer (BD Biosciences) to determine the percentage of MMs present in the non-adherent portion of the culture represented by events positive for red and green fluorescence. Flow cytometer settings were determined as previously described in section 2.5.

2.11. Cytochemical staining.

In order to determine how heterogeneous MMs are within and between the spleen and the kidney of the goldfish, several cytochemical staining's were performed to reveal morphological properties and functional characteristics of these cells.

In preparation for cytochemical staining's, freshly sorted melano-macrophages from the spleen and the kidney of 3 individual fish were washed twice in 0.9X PBS and re-suspended in 0.9X PBS – 1% BSA at a concentration of 1 X 10^6 cells/mL. 100 µL of the cell solution (1 X 10^5 cells/slide) was spun down onto a clean glass slide at 55 *g* for 8 minutes using a cytospin (Shandon Instruments).

For Hematoxylin and Eosin stain, MM cytospins were fixed in 70% methanol for 1 minute and rinsed with distilled water. Cells were stained with Hematoxylin for 30 seconds and rinsed with distilled and tap water. Cells were counterstained with eosin for 45 seconds and rinsed with tap water. Slides were dehydrated, cleared, mounted with DPX and allowed to dry overnight at 37^0C.

For Sudan Black B staining, MM cytospins were fixed and stained according to manufacture's (Sigma-Aldrich) instructions. Briefly, cells were fixed for 1 minute at 4^0C in the glutaraldehyde fixative solution and rinsed with distilled water. Cells were stained with Sudan black by immersion, rinsed with 70% ethanol and water, counterstained with Hematoxylin solution, Gill No. 3 (Sigma) for 1 minute, and rinsed with tap water. Slides were dehydrated, cleared, mounted with DPX and allowed to dry overnight at 37^0C.

For α-naphthyl acetate esterase (non-specific esterase) staining, MM cytospins were fixed and stained according to manufacture's (Sigma-Aldrich) instructions. Briefly, cells were fixed for 30 seconds in citrate-acetone-formaldehyde (CAF) solution and rinsed with distilled water. Cells were stained by immersion in α-naphthyl acetate esterase for 30 minutes at 37^0C protected from light, and rinsed with distilled water. Cells were counterstained with Hematoxylin solution, Gill No. 3 (Sigma) and rinsed with tap water.

For acid phosphatase (ACP) stain, MM cytospins were fixed and stained according to manufacture's (Sigma-Aldrich) instructions. Briefly, cells were fixed for 30 seconds in citrate-acetone-formaldehyde (CAF) solution at room temperature and rinsed with distilled water. Cells were stained by immersion in acid-phosphatase solution without tartrate for 1 hour at 37^0C and rinsed with tap water. Cells were counterstained with methylene blue and rinsed with distilled water.

For Perls' Prussian blue, MM cytospins were fixed for 10 minutes with 10% formalin and rinsed with distilled water. Briefly, fixed cells were immersed in a solution containing equal parts of 5% Potassium ferrocyanide and 5% hydrochloric acid. After 30 minutes, slides were washed with distilled water and counter stained with nuclear red. Slides were dehydrated, cleared, mounted with DPX and allowed to dry overnight at 37^0C.

For fluorescence microscopy, cytospins of peritoneal lavage cells and MMs were fixed for 10 minutes with methanol at 4^0C and rinsed with tap water. Cells were stained and mounted with 5 μL of VECTASHIELD HardSet

containing DAPI blue nucleic acid stain. Slides were sealed and allowed to dry overnight at 4^0C in the dark.

Following cytochemical staining, cells were visualized and photographed using a compound (Zeiss Ax 10 scope. A1), or a fluorescence microscope (Leica DMRXA). Hematoxylin and Eosin, Sudan Black B, α-naphthyl acetate esterase, ACP and Perls' Prussian blue slides were visualized and photographed under bright field using a compound microscope. Cytospins stained with DAPI blue were visualized and photograph under bright field, and the green, red and blue channels using a fluorescence microscope.

2.12. RNA isolation from goldfish.

2.12.1 Isolation of RNA from spleen and kidney leukocytes.

Total RNA was isolated from spleen and kidney leukocytes using the TRIzol reagent (Invitrogen) according to manufacture's instructions. During RNA isolation, 150 μg of glycogen were added to the samples to improve the yield of RNA. After isolation, the concentration and purity of the RNA was determined by reading the absorbance of the samples at 230, 260 and 280 nm using a NanoDrop (ND-1000 spectrophotometer, NanoDrop). RNA samples were stored at -80^0C until used in cDNA synthesis.

2.12.2. Isolation of RNA from goldfish spleen and kidney.

50 mg of spleen and kidney were individually homogenized in a glass Teflon with 1 mL of TRIzol reagent (Invitrogen) according to manufacture's

instructions. After isolation, the concentration and purity of the RNA was determined by reading the absorbance of the samples at 230, 260 and 280 nm using a NanoDrop. RNA samples were stored at -80^{0}C until used in cDNA synthesis. Total RNA isolated from tissue was used for sequencing of goldfish genes and for validation of qPCR primers.

2.13. cDNA synthesis.

Isolated total RNA was reverse transcribed into cDNA using anchored Oligo $(dT)_{16}$ primer and the SuperScript III™ First-Strand cDNA Synthesis System (Invitrogen™). cDNA synthesis from whole spleen and kidney was performed using 1 μg of total RNA and 200 U of enzyme according to the manufacture's instructions. cDNA synthesis for qPCR from isolated leukocytes was performed using 200-500 ng of RNA and 100 U the enzyme. In all cases, 20 μL reactions were incubated at 50^{o}C for 2 hours, heat inactivated at 70^{o}C for 15 minutes and stored at 4^{0}C for qPCR, or at -20^{0}C for PCR.

2.14. *In silico* analysis of goldfish genes.

Primers for sequencing the genes for goldfish AID, MFGE8, MHC class II α 2, and the ribosomal protein L13a were designed using Gene runner 3.01. Available gene sequences from other fish species were obtained from NCBI and aligned using the Clustal W software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). All primer sequences used are listed in Table 2.4.

2.15. DNA sequencing of goldfish genes.

Primers designed for AID, MFGE8, MHC class II alpha 2, and the ribosomal protein L13a were used in conventional PCR reactions with cDNA from goldfish spleen or kidney. 20 μL PCR reaction was set using 1 X PCR buffer (GibcoBRL®), 0.2 mM dNTP (Invitrogen™), 1.4 mM $MgCl_2$, 5pmol forward primer, 5 pmol reverse primer, and 1.25 U of "in-house" produced recombinant Taq polymerase. PCR products were run on an agarose gel, excised and DNA was extracted using the QIAquick® Gel Extraction Kit (Qiagen®), according to manufacture's instruction. Purified DNA fragments were cloned into the vector pCR4-TOPO (Invitrogen™) according to manufacture's instruction and used to transform DHα5 *Escherichia coli* chemical competent cells. Bacteria were then plated on Luria-Bertani (LB) + Carbenicillin (50 μg/mL, Sigma-Aldrich) plates with IPTG and x-gal, and incubated for 24 hours at 37^0C. Colonies were selected based on blue/white screening and sequenced using the DYEnamic™ ET Terminator Cycle Sequencing Kit (GE Healthcare) and vector specific primers listed on Table 2.5.

2.16. Quantitative PCR primers.

All primers for qPCR were designed using the Primer Express software (Applied Biosystems). Primers were designed using reference sequences or sequences generated in the laboratory and all primer sequences are listed on Table 2.5. For validating the primers, 1:5 serial dilutions of cDNA were run with the primers, a standard curve was generated, and the R^2 value, the efficiency and the

melt curves were analyzed with the 7500 Fast software. The primers used for qPCR have a R^2 value ≥ 0.997, an efficiency of 85% or higher, and a single melting peak. Additionally, primer products were run on an agarose gel, extracted and sequenced to verify the identity of the amplicon.

2.17. Quantitative PCR

All quantitative PCR was performed using a 7500 Fast Real Time machine (Applied Biosystems) and SYBR green reagent. The elongation factor 1 alpha (Ef-1α) was used as the endogenous control to compare the expression of all genes analyzed. Thermocycling parameters were 95^0C for 2 minutes, 40 cycles of 15 seconds at 90^0C and 1 minute at 60^0C. Fold difference of gene expression was determined using the ΔΔCt method ($2^{-(\Delta Ct, \text{ experimental sample} - \Delta Ct, \text{ reference sample})}$). ΔCt = Ct (target gene) – Ct (endogenous control). The fold change in qPCR represents the change in expression in an experimental sample compared to the calibrator or control group. The calibrator has a relative quantification (RQ) of 1. Experimental samples are compared to the calibrator and are expressed as an n-fold difference relative to the calibrator.

2.18. Statistical analysis.

The results of quantitative gene expression analysis were assessed using one-way analysis of variance (ANOVA). Post hoc testing was done using Tukey's or Games-Howell's tests. Probability level of $P < 0.05$ was considered to be significant.

Table 2.1. Number of goldfish used in the different experiments.

Type of experiment	Number of goldfish used
Cytochemistry of melano-macrophages	3
Cytochemistry of peritoneal lavage	3
Melano-macrophage survival	3
PKM *in vitro* challenge	5
Low dose single vaccination	10
Low dose vaccination with boost	10
High dose single vaccination	5
High dose vaccination with boost	5
Control group for *in vivo* challenge	5
In vitro challenge of MMs and tissue macrophages	6

Table 2.2. Composition of MGFL-15 medium (pH 7.4).

Components	Amount per 1 L
HEPES	3.5 g
KH_2PO_4	0.344 g
K_2HPO_4	0.285 g
NaOH	0.375 g
$NaHCO_3$	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 4^0C.

*GFL-15 medium is made by mixing 1 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 4^0C

Table 2.3. Composition of nucleic acids precursor solution.

Components	Amount per 100 mL
Adenosine	0.067 g
Cytidine	0.061 g
Hypoxanthine	0.034 g
Thymidine	0.061 g
Uridine	0.061 g
Milli-Q Water	100 mL
Not filtered. Store at 4^0C	

Table 2.4. List of PCR primer sequences.

Primer	Sequence (5'-3')
AID FWD	AGT GTG CTC ATG ACC CAG AAG
AID REV	AAG GCG TGA CAC AAA GAT TCT C
MFGE8 FWD	GCC GAG TTC ATC AAA GCC TTC
MFGE8 REV	CCG TCG TCA CTG TGA GCC AC
MHC II FWD	GAW TGG WKW TGA TGG AGA GGT
MHC II REV	CAG TTG CAS TKG TTT CCT TTA
L13a FWD	CCY AGC ARR ATC TTC TGG AGG AC
L13a REV	GTG ATR GCC TGG TAY TTC CAG CC

Table 2.5. List of qPCR primer sequences.

Primer	Sequence (5'-3')
MFGE8 FWD	CTG GTG GGC TGT GAA CTC AAC
MFGE8 REV	CAT CGA TCA GCC GTG ACT TG
AID FWD	GTT ACG CAG TGA CCT GGT TCT G
AID REV	CAG GAA GTG GGC AAG TTG TTC
TNFα-2 FWD	TCA TTC CTT ACG ACG GCA TTT
TNFα-2 REV	CAG TCA CGT CAG CCT TGC AG
IL-1β-1 FWD	GCG CTG CTC AAC TTC ATC TTG
IL-1β-1 REV	GTG ACA CAT TAA GCG GCT TCA C
MHC II FWD	TCC AAA CCC ACA GTT GAG CAA
MHC II REV	CAC CCA GCA CCA CGT CAT T
IL-10 FWD	CCT TTG AGT TCG CCA GCA TAA
IL-10 REV	GAT GCC AAA TAC TGC TCG ATG T
TGF-β FWD	AGG CAG AGC AGG GAT TTC AA
TGF-β REV	TGT GAG GCT TTG GCA TAT GGT
CSF-1R FWD	CCT GCT GGG TGC CTG TAC A
CSF-1R REV	TGC GCA GGA AGT TCA GAA GAT

CHAPTER 3: RESULTS

3.1. FACS Isolation, survival, and cytochemistry of melano-macrophages from the spleen and the kidney of the goldfish.

The first part of this work was to study the survival of MMs in culture, and to examine morphological and functional characteristics of isolated cells through cytochemistry techniques. To date, MMCs have been extensively described in tissue sections, but MMs as single cells have not been characterized. The auto-fluorescence property of MMs was used to isolate these cells from the spleen and the kidney of the goldfish through flow cytometry (Figure 3.1). In our studies, MMs were found to represent approximately 12 to 20% (SD=3.18) of the cells isolated from the spleen, and 9 to 16% (SD=3.01) of the kidney. MM fluorescence dot plots revealed the presence of two separate fluorescence peaks in the kidney that are not observed in the spleen (Figure 3.1). The two separate peaks along with low fluorescence intensity cells were gated separately in order to see if these differences correlated with MM subpopulations. It was observed in the case of the kidney that cells located in the Gate 3 were similar in size and internal complexity to those located in the Gate 2, and that cells located in the Gate 4 were bigger and of less internal complexity when compare to Gates 2 and 3 (Figure 3.2). Spleen MMs were divided into low and high fluorescence intensity. The two separate gates were back analyzed and it was observed that neither the low or the high fluorescence intensity corresponded to a defined population, instead, the cells were found to be dispersed on the dot plot indicating

variations of both size and internal complexity within MMs of the spleen (Figure 3.3).

Survival assessment of MMs in culture revealed that MMs die shortly after being placed in culture. It was observed that the number of MMs in culture is reduced to half by the second day in the kidney and third day in the spleen (Figure 3.4).

Isolated cells were observed under a fluorescence microscope and it was found that approximately 63% of the FACS isolated spleen cells, and 60% of the kidney cells contained auto-fluorescent pigments visible under both the red and the green auto-fluorescence channels. Fluorescence microscopy also revealed that auto-fluorescent pigments in goldfish melano-macrophages, vary in size and shape between cells and tissues (Figure 3.5 and 3.6). Additionally, leukocytes obtained through goldfish peritoneal lavage did not show any fluorescence in the green or the red channels (Figure 3.7), revealing that MMs are no found in the peritoneal cavity and that auto-fluorescent pigments are a unique characteristic of these cells.

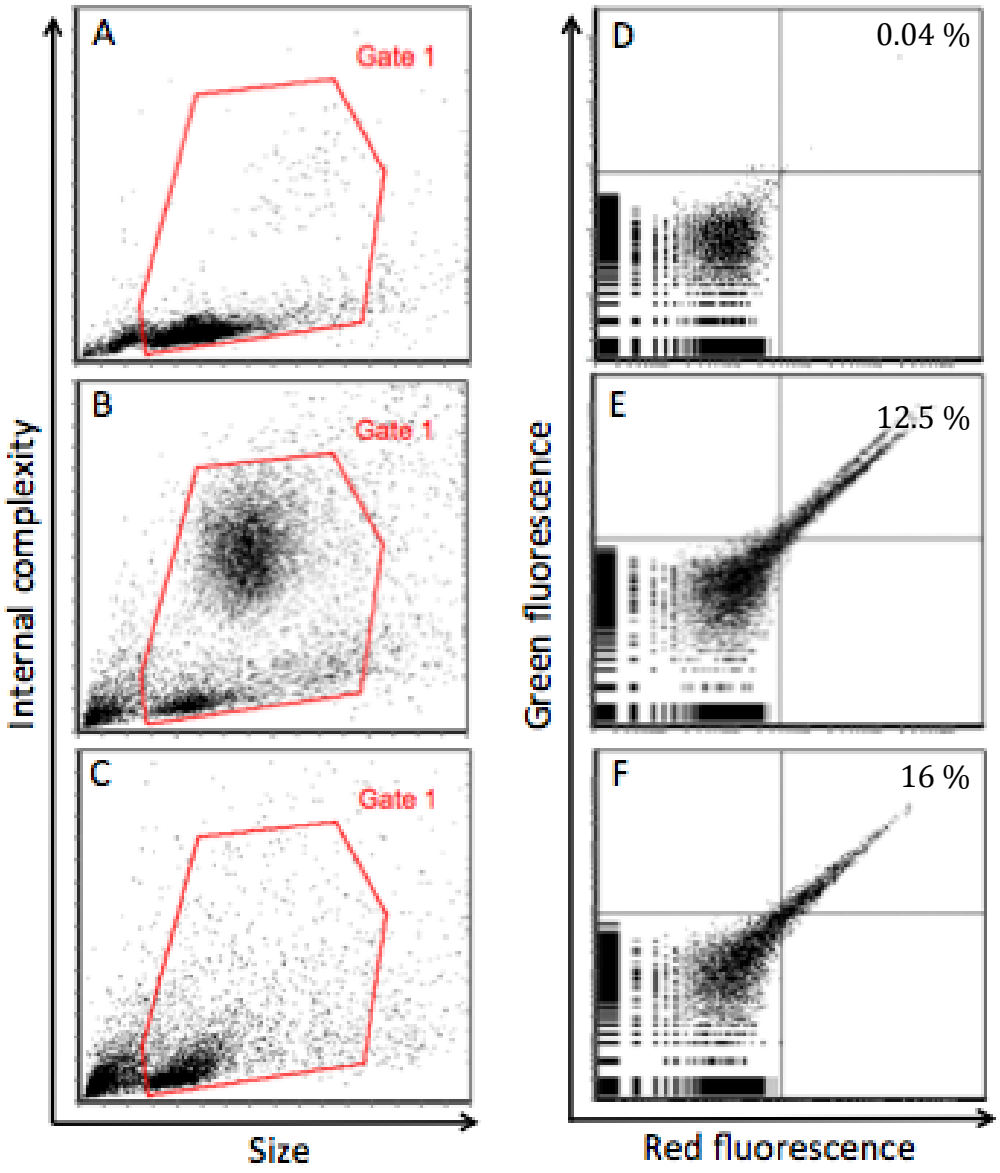

Figure 3.1. Flow cytometric analyses of melano-macrophages from the spleen and the kidney of the goldfish.
MMs were analyzed by flow cytometry based on their size, complexity, and auto-fluorescence properties in the green and the red channels. Peripheral blood leukocytes were used to establish MM sorting parameters. Size and internal complexity dot plot of (A) PBLs, the entire cell population of the spleen (B) and the kidney (C). Green and red fluorescence dot plot of cells belonging to Gate 1 from the PBLs (D), the spleen (E) and the kidney (F).

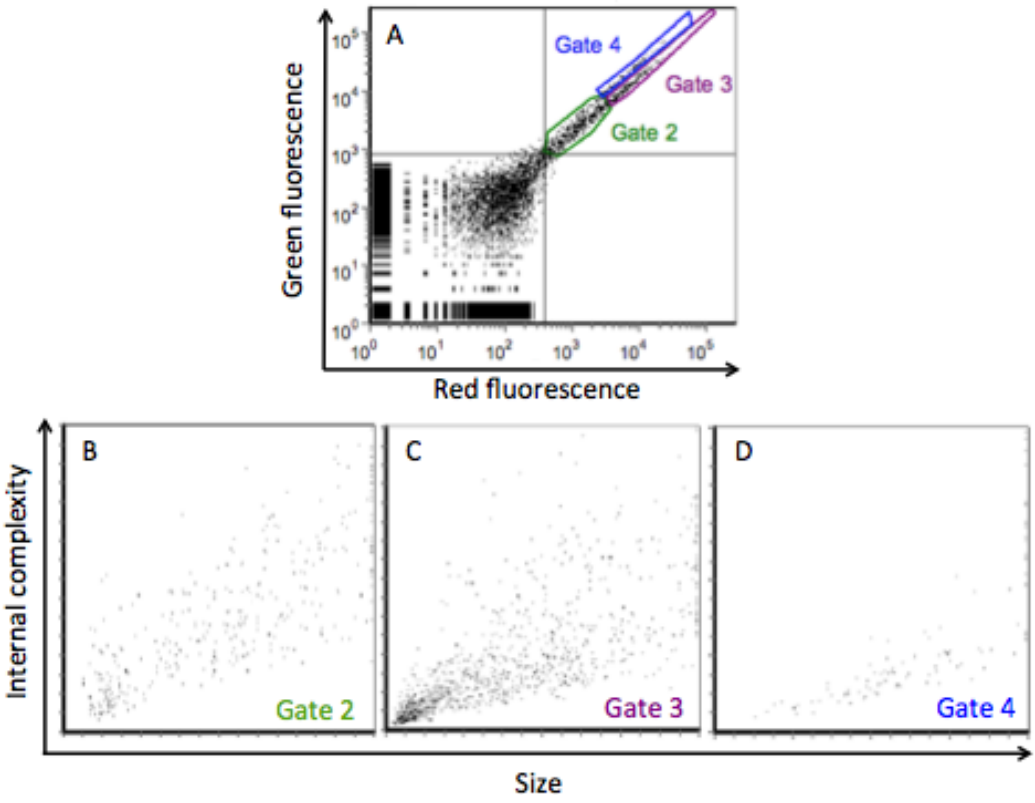

Figure 3.2. Flow cytometric back analyses of goldfish kidney melano-macrophages.

Back analyses were performed by dividing the kidney fluorescence dot plot (A) into low fluorescence (Gate2) and high fluorescence (Gate 3 and 4) cells. Dot plots of Gate 2 (B), Gate 3 (C), and Gate 4 (D) reveal the relative size and complexity of the low and high fluorescence populations.

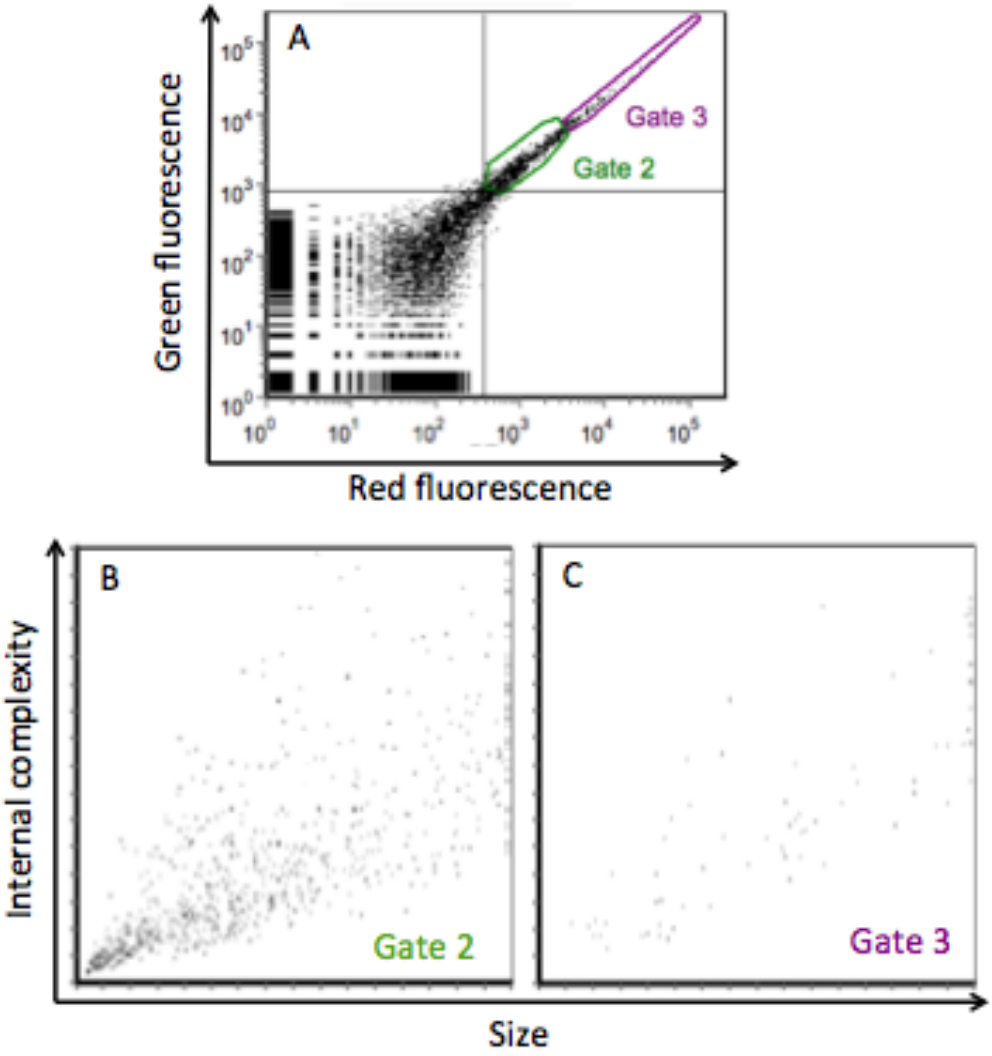

Figure 3.3. Flow cytometric back analyses of goldfish spleen melano-macrophages.

Back analyses were performed by dividing the spleen fluorescence dot plot (A) into low fluorescence (Gate2) and high fluorescence (Gate 3) cells. Dot plots of Gate 2 (B) and Gate 3 (C) reveal the relative size and complexity of the

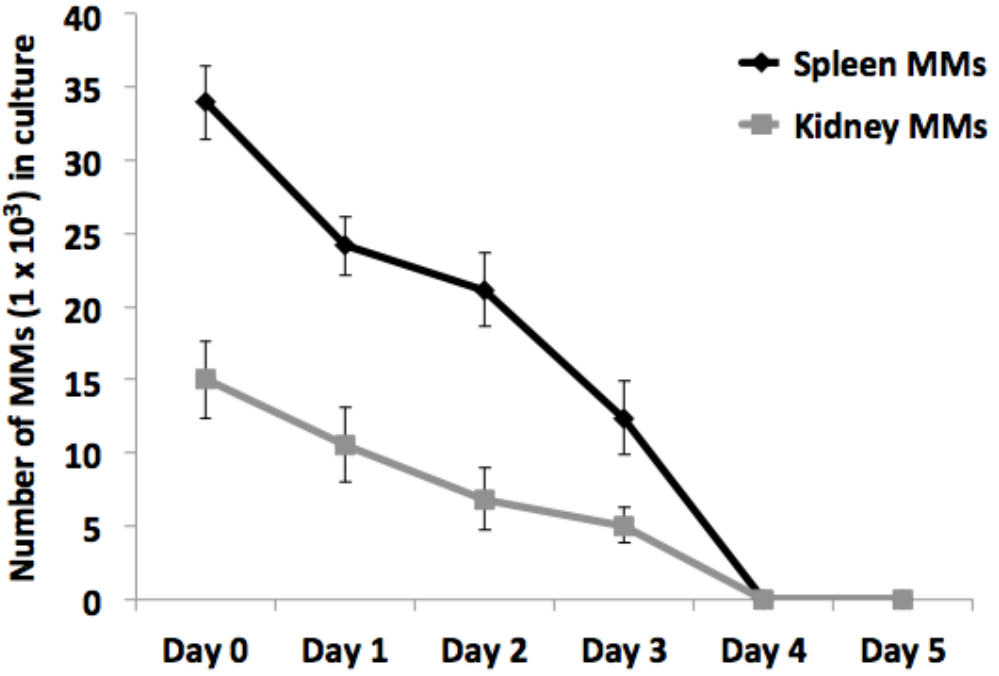

Figure 3.4. Survival of melano-macrophages in culture over the period of 5 days.
Spleen and kidney cell cultures were started with 2.5 x 104 cells/well. The number of MMs in culture was estimated daily from haemocytometer cell counts and flow cytometry.

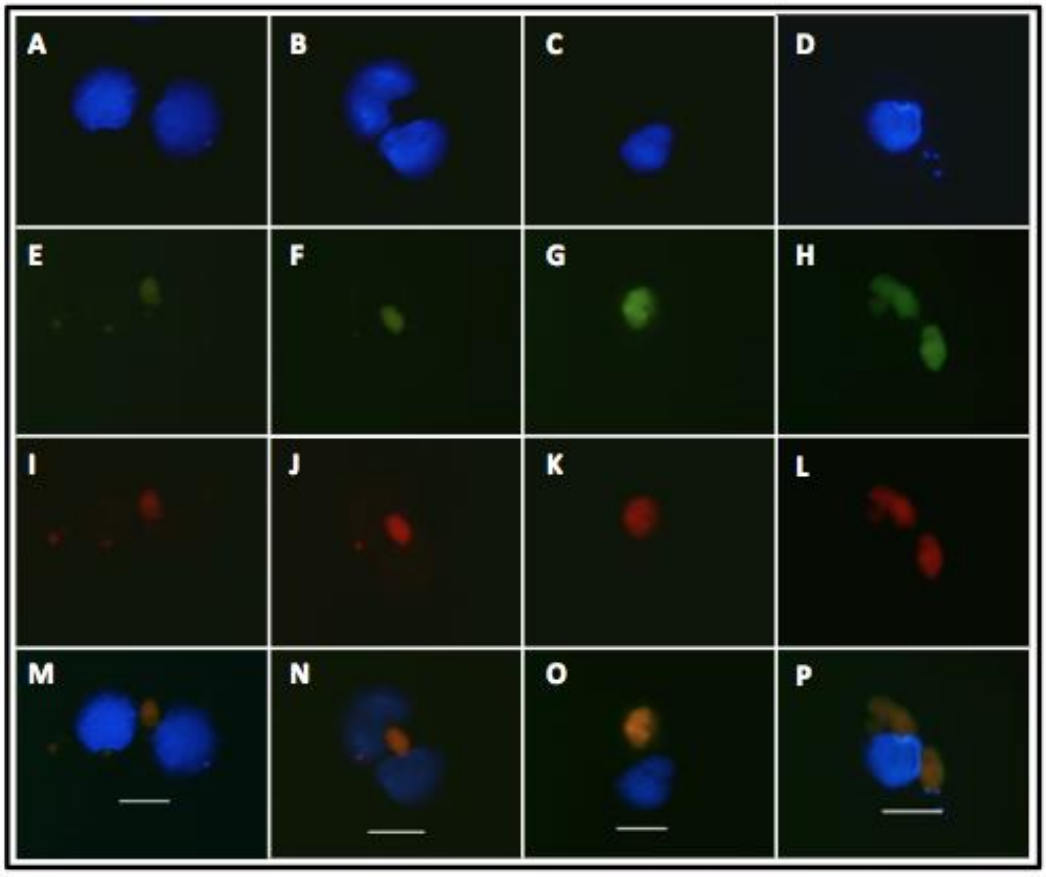

Figure 3.5. Fluorescence microscopy images of FACS isolated spleen MMs. Cytospins of spleen MMs were mounted with VECTASHIELD containing DAPI blue nuclear stain. Cells were observed and photographed at 1000x magnification under the blue (A-D), green (E-H) and red channels (I-L), and an overlay of each field of view was generated (M-P). Each column contains the pictures of one field of view under the different channels plus the overlay. The scale bar = 10 μm.

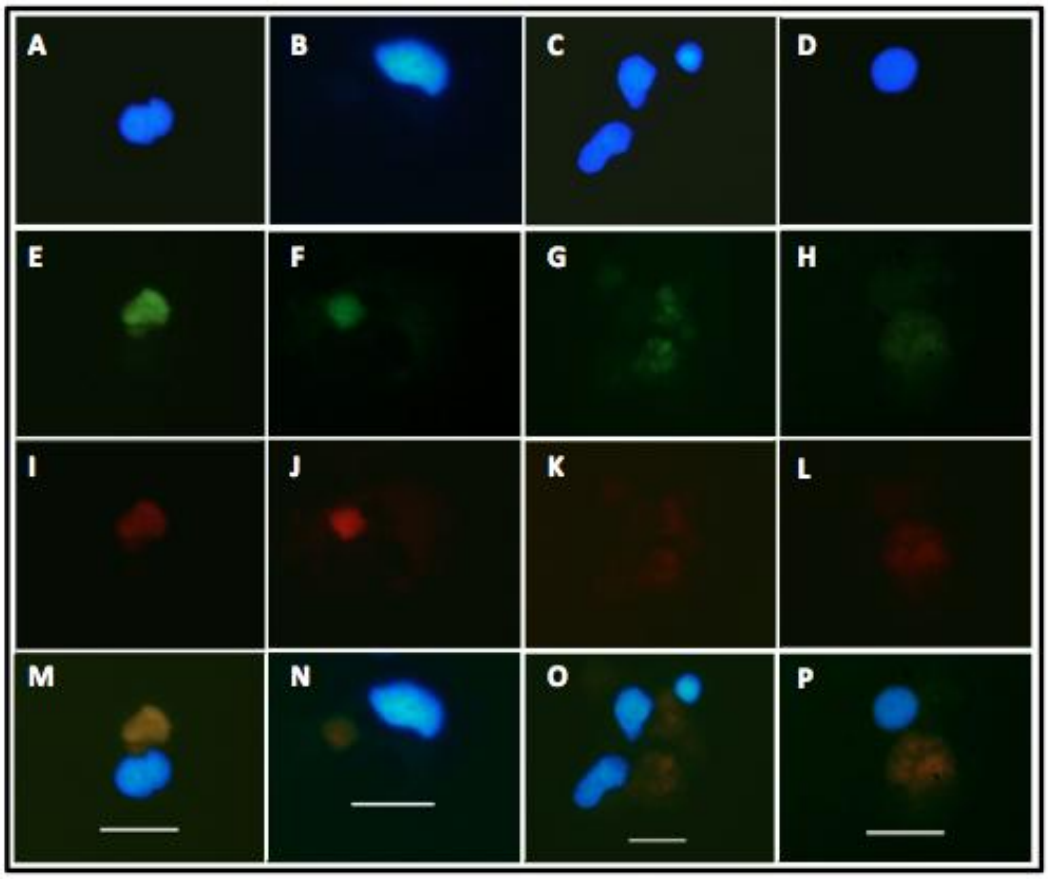

Figure 3.6. Fluorescence microscopy images of FACS isolated kidney MMs. Cytospins of kidney MMs were mounted with VECTASHIELD containing DAPI blue nuclear stain. Cells were observed and photographed at 1000x magnification under the blue (A-D), green (E-H) and red channels (I-L), and an overlay of each field of view was generated (M-P). Each column contains the pictures of one field of view under the different channels plus the overlay. The scale bar = 10 μm.

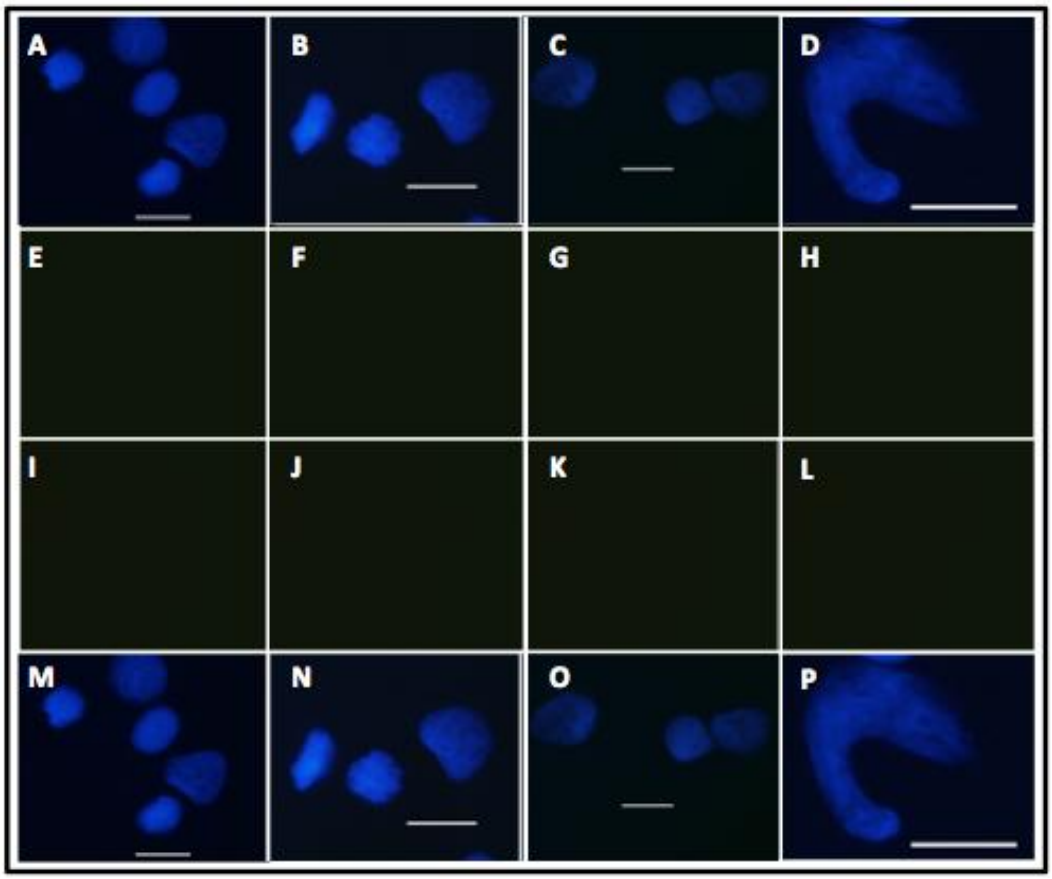

Figure 3.7. Fluorescence microscopy images of cells harvested from the peritoneal cavity reveal lack of auto-fluorescent pigments in macrophages and neutrophils.
Cytospins of cells harvested by peritoneal lavage were mounted with VECTASHIELD containing DAPI blue nuclear stain. Cells were observed and photographed at 1000x magnification under the blue (A-D), green (E-H) and red channels (I-L), and an overlay of each field of view was generated (M-P). Each column contains the pictures of one field of view under the different channels plus the overlay. The scale bar = 10 μm.

Isolated MMs were found to be approximately 7 to 11 μm in size with a round morphology and a round or indented nuclei. Hematoxylin/eosin staining revealed the presence of basic granules stained with hematoxylin in the cytoplasm of some spleen MMs (Figure 3.8 A, D-E). These granules were not seen in any of the kidney cells observed (Figure 3.8 F-J).

Both spleen and kidney MMs stained positive for Sudan Black B (Figure 3.9). Although Sudan Black B is typically used as an indicator of mammalian neutrophils, it stains all intracellular neutral lipids including lipofuscin. Sudan Black B has been widely used to stain the lipofuscin present in the tissues of different organisms including eel (Katzenback and Belosevic 2009), clam (Rieger, Konowalchuk et al. 2012), monkey, human, and rat (Ravaglia and Maggese 1995).

Acid-phosphatase activity was present in isolated MMs from both the spleen and the kidney of the goldfish (Figure 3.10), this enzyme has also been reported in MMCs of the spleen and the kidney of other fish species including the Atlantic salmon (Lomovasky, Morriconi et al. 2002), the Atlantic halibut (Schnell, Staines et al. 1999).

Isolated MMs from the spleen and the kidney of the goldfish were also found to be positive for non-specific esterase (Figure 3.11). This enzyme is characteristic of monocytes and macrophages, and its presence indicates that these cells are not neutrophils.

Additionally, approximately 10% of the spleen MMs (Figure 3.10 A-D), and non of the kidney MMs (Figure 3.12 F-J) stained positive for Perls' Prussian

blue, indicating the presence of hemosiderin in spleen MMs and its absence in kidney MMs. Hemosiderin is the least common of the MMC pigments and its presence is usually limited to the spleen of fish where the recycling of hemoglobin from effete red blood cells occurs (Falk, Press et al. 1995).

In mammalian systems the iron level is highly regulated through its acquisition and storage. Most of the iron is found in the hemoglobin of erythrocytes. Senescent red blood cells are phagocytosed by macrophages, which recycle the iron and depending on the organism's requirements, can either store or release the iron to the plasma to be re-used (Grove, Johansen et al. 2006). Macrophages constantly release the iron from the degradation of red blood cells. Rapid and excessive accumulation of intracellular iron can result in both cell and tissue damage. Deregulation in the iron metabolism can lead to diseases involved with iron deficit or iron overload. Accumulation of iron occurs mainly in the liver, the heart and the pancreas, which damages the tissue and can over time lead to chronic diseases like cirrhosis and diabetes (Agius 1979; Agius 1980).

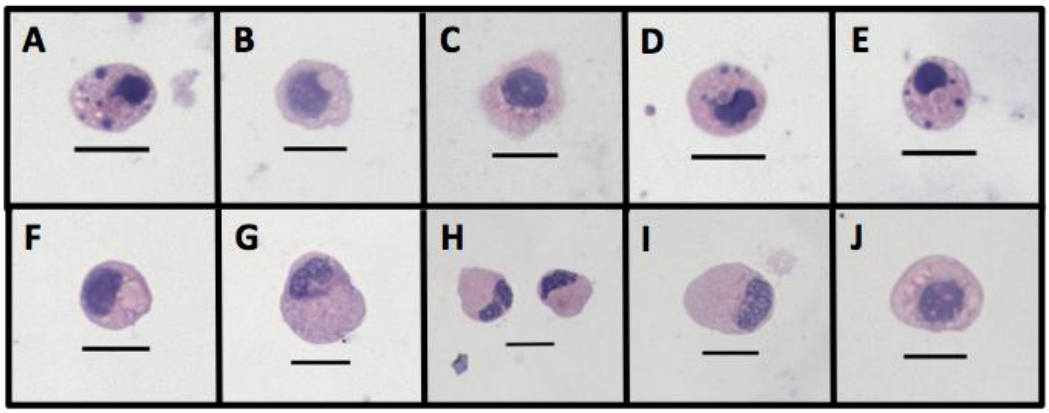

Figure 3.8. Hematoxylin and eosin bright field images of goldfish spleen and kidney melano-macrophage.
Cytospins of freshly isolated spleen (A-E) and kidney (F-J) MMs were stained for H&E. Scale bar = 10 μm.

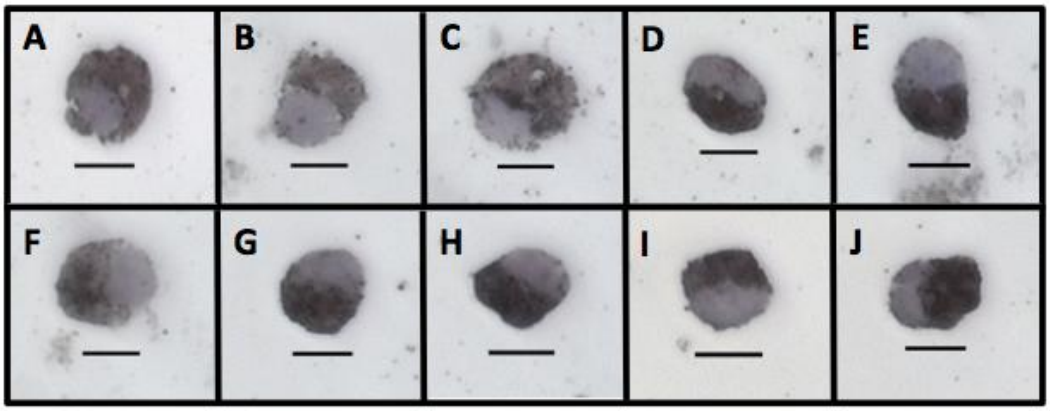

Figure 3.9. Sudan black bright field images of goldfish spleen and kidney melano-macrophages.
Cytospins of freshly isolated spleen (A-E) and kidney (F-J) MMs were stained for Sudan Black B. Scale bar = 10 μm.

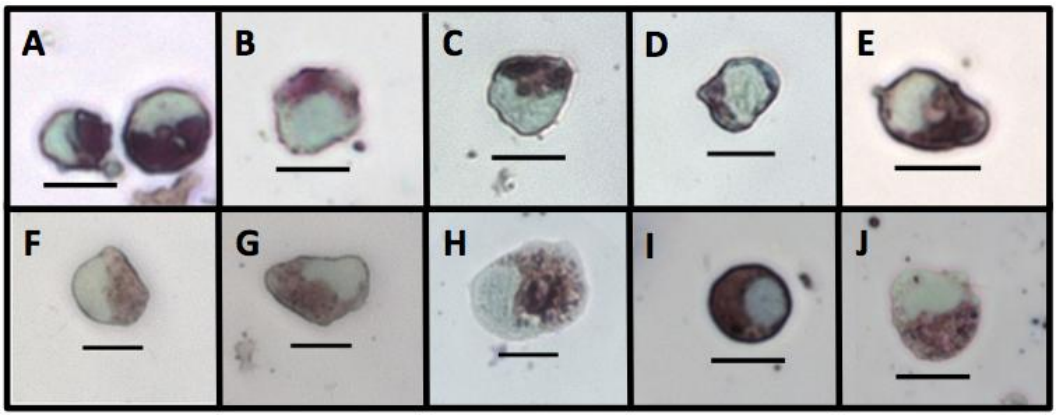

Figure 3.10. Acid phosphatase bright field images of goldfish spleen and kidney melano-macrophages.
Cytospins of freshly isolated spleen (A-E) and kidney (F-J) MMs were stained for acid phosphatase. Scale bar = 10 μm.

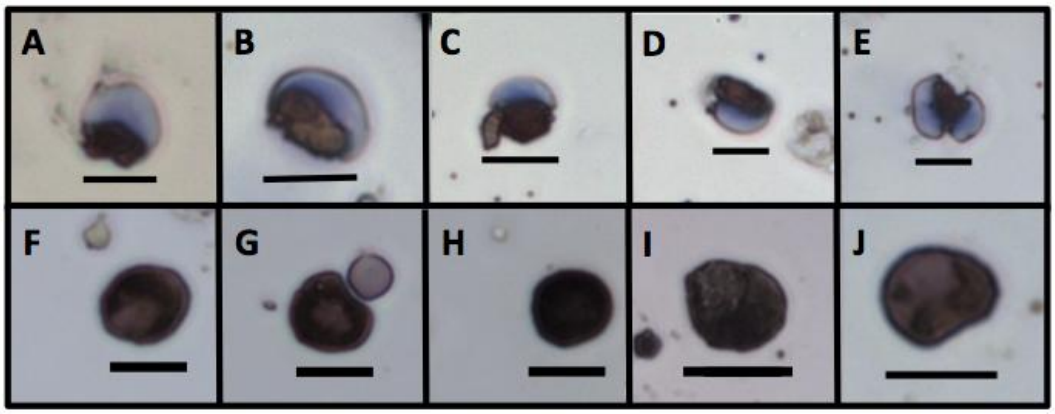

Figure 3.11. Non-specific esterase bright field images of goldfish spleen and kidney melano-macrophages.
Cytospins of freshly isolated spleen (A-E) and kidney (F-J) MMs were stained for non-specific esterease. Scale bar = 10 μm.

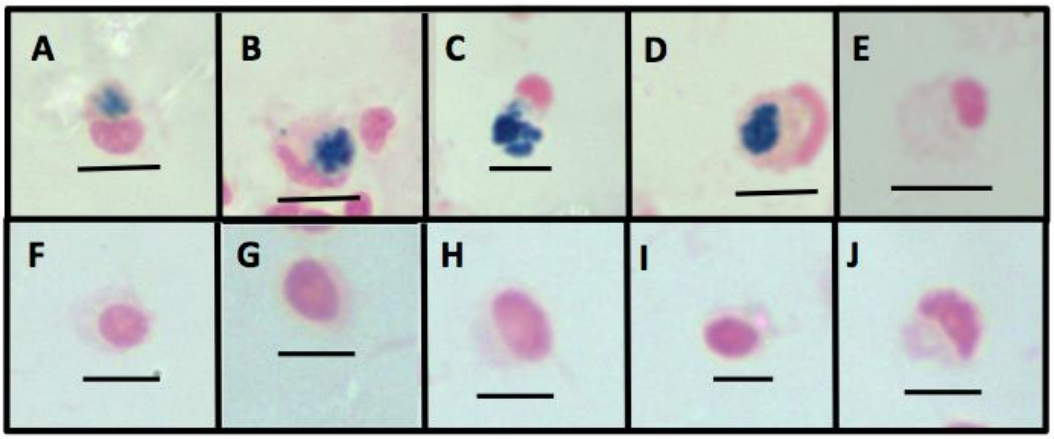

Figure 3.12. Perl's Prussian blue bright field images of goldfish spleen and kidney melano-macrophages.
Cytospins of freshly isolated spleen (A-E) and kidney (F-J) MMs were stained for Perl's Prussian blue. Scale bar = 10 μm.

3.2. Primary kidney derived macrophage gene expression of pro- and anti-inflammatory cytokines after stimulation with *A. salmonicida* evidences a classical pro-inflammatory macrophage role in the immune response.

The second part of this study focused on establishing a cytokine gene expression profile for classical role macrophages involved in fighting and clearing off infection. Primary kidney derived macrophages have been extensively characterized in the goldfish in the laboratory of Dr. Miodrag Belosevic, and we used this system in order to compare the response of PKM cultures stimulated with either *A. salmonicida* or apoptotic cells to the response of MMs against the same stimuli.

Stimulation of PKM cultures with *A. salmonicida* elicited a pro-inflammatory response evidenced by the statistically significant up-regulation of TNFα, IL-1β and IL-10 that peaked after 16 hours of stimulation with the fish pathogen (Figure 3.13 A-C). After stimulation of PKMs with apoptotic cells, we only observed modest changed in the regulation of the cytokines studied.

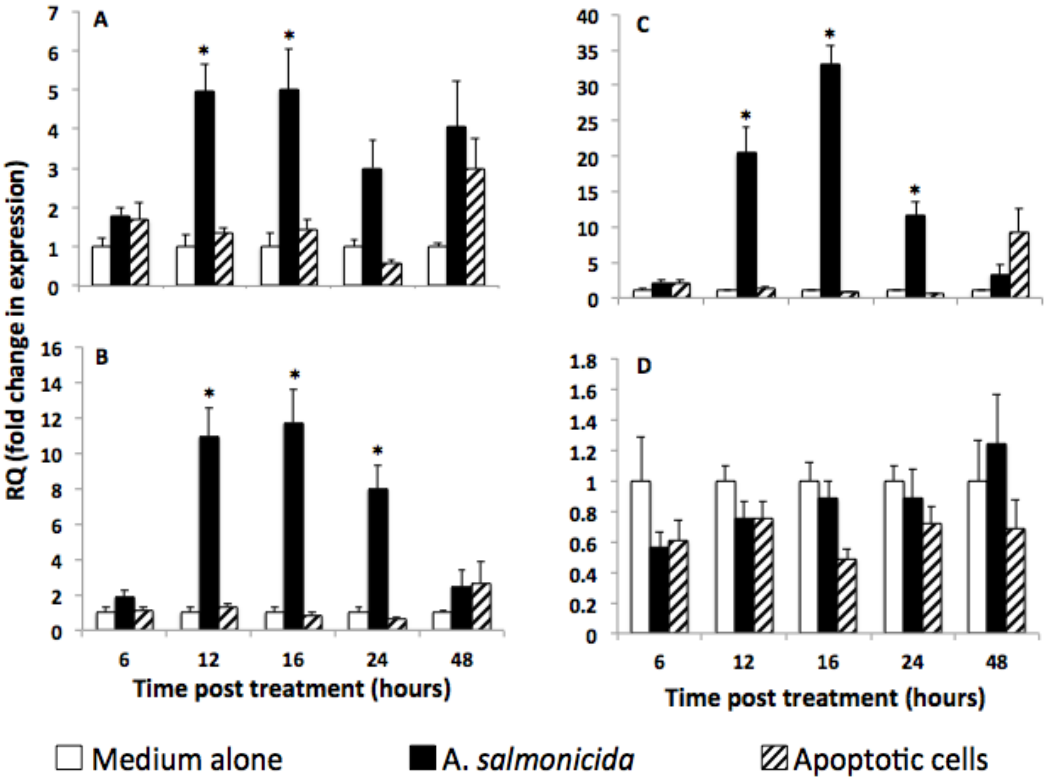

Figure 3.13. Quantitative gene expression analysis of goldfish pro- and anti-inflammatory cytokines in stimulated primary kidney derived macrophages. PKM were stimulated with either medium alone, 2µg/mL heat-killed *A. salmonicida*, or apoptotic cells at a 5:1 apoptotic cell: macrophage ratio, and the expression of (A) TNF-α, (B) IL-1β, (C) IL-10, and (D) TGF-β was analyzed. The expression of the four cytokines was examined relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for medium alone at each time point. The results are mean ± SEM of primary macrophage cultures established from five to eight individual fish ($5 \leq n \leq 8$). One-way ANOVA was performed, and (*) indicates statistically significant difference ($P < 0.05$) from the control group (medium alone) for each individual time point.

3.3. Spleen tissue macrophages were found to have a higher basal level of cytokine expression.

To analyze the basal expression level of the different cytokines, un-stimulated tissue macrophages and MMs from *in vitro* studies along with MMs from healthy fish from *in vivo* studies were compared. All cells used in the in vivo studies were freshly isolated and processed from healthy fish, and all cells used in the *in vitro* experiments were isolated and incubated for 16 hours with medium alone.

It was found that tissue macrophages from the spleen had the highest basal level of all cytokines analyzed and this was found to be statistically significant in the case of IL-1β and TGF-β (Figure 3.14).

Kidney tissue macrophages, kidney and spleen MMs from healthy fish, and kidney and spleen MMs used in the *in vitro* studies were found to have similar basal expression levels of both pro- and anti-inflammatory cytokines, and no statistically significant differences were observed (Figure 3.14).

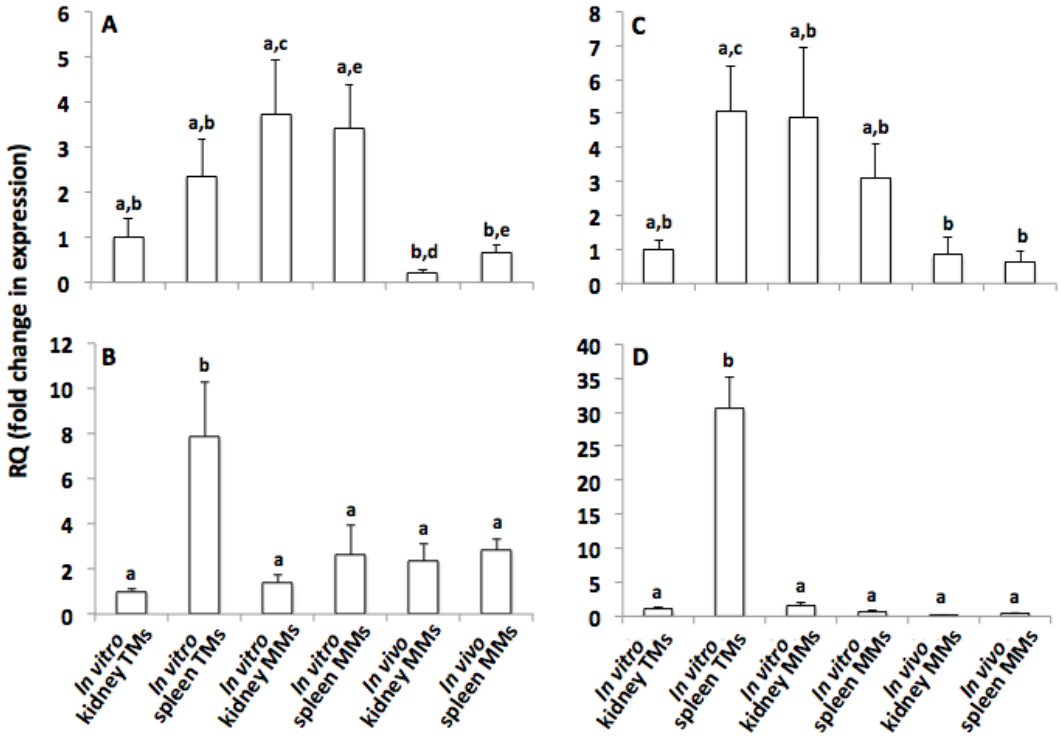

Figure 3.14. Quantitative gene expression analysis of goldfish pro- and anti-inflammatory cytokines basal levels in spleen and kidney *in vitro* tissue macrophages and melano-macrophages, and *in vivo* melano-macrophages. The basal level of expression was analyzed from un-stimulated tissue macrophages (TMs) and MMs for (A) TNF-α, (B) IL-1β, (C) IL-10, and (D) TGF-β. Gene expression was analyzed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for *in vitro* kidney TMs. The results are mean ± SEM from three to six individual fish ($3 \leq n \leq 6$). One-way ANOVA was performed. Letters denote significant differences among groups. Groups denoted by the same letter are not significantly different from each other ($P < 0.05$).

3.4. Cytokine gene expression profile of MMs from goldfish vaccinated with heat-killed *A. salmonicida.*

In order to analyze the cytokine gene expression profile of MMs during the adaptive immune response, fish were injected once and allowed to develop a response for 21 days, or they received a second injection after these 21 days to examine the response of MMs to a recent encounter with the antigen.

It has been previously demonstrated that there is a relation between temperature and immunity; after goldfish DNA-immunization, maintaining the fish at 15^{o}C allowed for antigen to be retained for longer period of 4 weeks which led to a higher antibody production when compared to fish that were kept at 25^{o}C that retained antigen for only 2 weeks and had a lower antibody production (Reviewed in De Domenico, McVey Ward et al. 2008). For this reason fish were kept at 17^{o}C during the incubation period after immunization with the heat-killed bacteria.

For all genes analyzed, the injection of the two concentrations of bacteria induced a different regulation in the expression of cytokines within the tissue analyzed and between the spleen and the kidney.

The 1 x 10^{6} injection elicited the up-regulation of the cytokines analyzed except for IL-β in spleen MMs when compared to the control group. This increase in cytokine expression is the down-regulated by the boost injection, except for IL-β (Figure 3.15). For these two treatments, kidney MMs follow the same pattern of increase/decrease in all cytokines analyzed. However, the fold

changes in cytokine expression observed in spleen MMs are higher than for kidney MMs.

The 10^9 injections induced only modest changes in the expression profile of both spleen and kidney MMs after 21 days. However, after the administration of the boost injection, up-regulation of IL-10 and TGFβ occurred for both spleen and kidney MMs. Additionally, after the boost injection, spleen MMs showed to increase TNF-α and decrease IL-1β, while kidney MMS showed to decrease TNF-α and increase IL-1β (Figure 3.15).

In addition to the cytokines analyzed, we also studied the expression profile of membrane CSF-1R, MFGE8, and MHC class II. We only observed modest changes in the expression of MFGE8, and the expression of CSF-1R and MHC class II was very similar in the spleen and the kidney MMs, with the exception of the statistically significant increase in the expression of CSF-1R in spleen MMs after the 10^9 boost injection, and the statistically significant up-regulation of MHC class II by kidney MMs after one injection with 10^6 bacteria and an incubation period of 21 days (Figure 3.16).

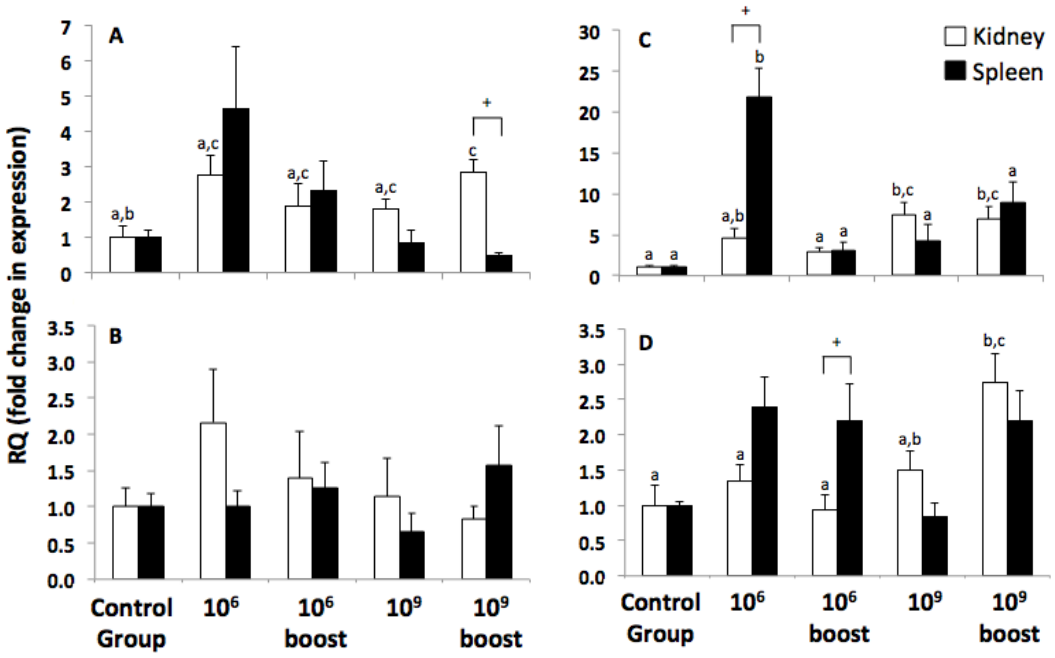

Figure 3.15. Quantitative gene expression analysis of goldfish pro- and anti-inflammatory cytokines in spleen and kidney melano-macrophages from vaccinated fish.

Goldfish were injected one time with 10^6 or 10^9 heat-killed *A. salmonicida* and cells were isolated 21 days later, or fish received a booster injection 21 days after the first vaccination and cells were isolated 3 days after the last vaccination. The expression of (A) TNF-α, (B) IL-1β, (C) IL-10, and (D) TGFβ was analyzed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for cells from un-vaccinated fish (control group). The results are mean ± SEM of MMs from five to ten individual fish ($5 \leq n \leq 10$). Statistical analysis was performed using one-way ANOVA. Groups denoted by the same letter are not significantly different from each other ($P < 0.05$). (+) above lines denote statistical differences ($P < 0.05$) between indicated experimental bars.

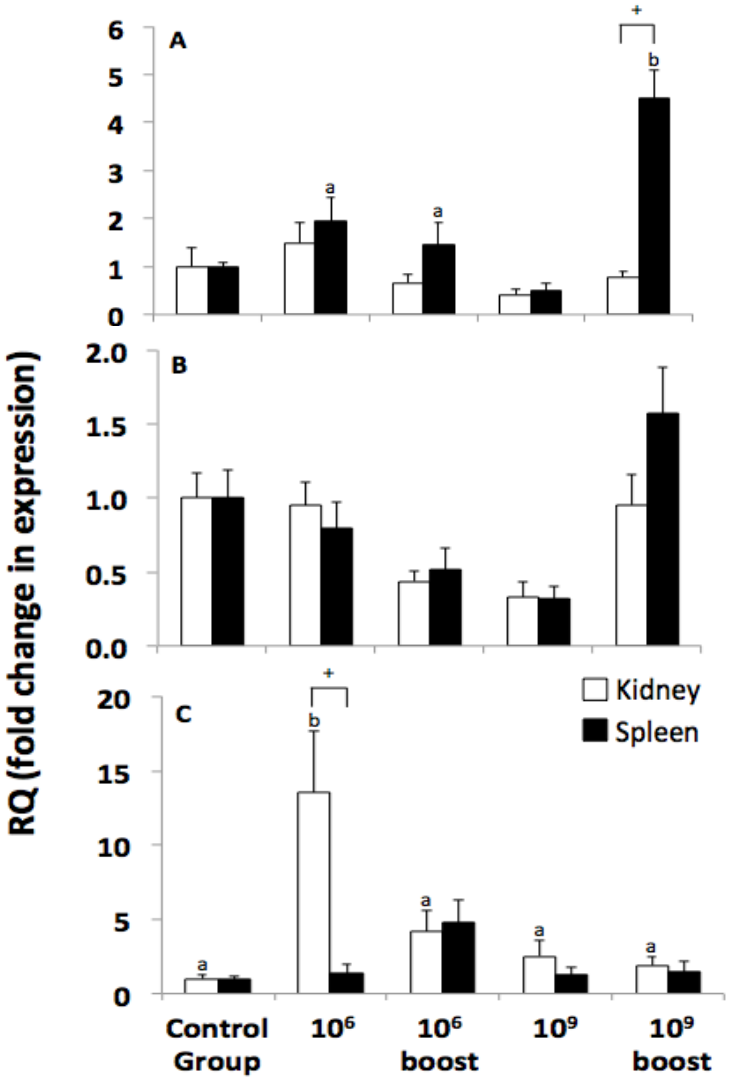

Figure 3.16. Quantitative gene expression analysis of goldfish genes in spleen and kidney melano-macrophages from vaccinated fish.
Goldfish were injected one time with 10^6 or 10^9 heat-killed *A. salmonicida* and cells were isolated 21 days later, or fish received a booster injection 21 days after the first vaccination and cells were isolated 3 days after the last vaccination. The expression of (A) membrane bound CSF-1R, (B) MFGE8, and (C) MHC II was analyzed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for cells from un-vaccinated fish (control group). The results are mean ± SEM of MMs from five to ten individual fish ($5 \leq n \leq 10$). Statistical analysis was performed using one-way ANOVA. Different letters above each bar denote significant differences ($P < 0.05$), the same letter indicate no statistical difference between groups. (+) above lines denote statistical differences ($P < 0.05$) between indicated experimental bars.

3.5. *In vitro* stimulation of tissue macrophages and MMS reveals differences in the cytokine gene expression profile of these two cell populations after stimulation.

Melano-macrophages and tissue macrophages isolated from the tissue and were stimulated for 16 hours to two different stimuli in order to analyze the changes in the cytokine gene expression, and to compare the response elicited in these cells by the two different stimuli. Tissue macrophages were isolated from the tissue by allowing adherence of the cells to the tissue culture flasks. MMs stick to the culture flask but easily dislodge by washing the cells and are FACS isolated.

The heat-killed pathogen *A. salmonicida* elicited a more pronounced response in the tissue macrophages from the kidney than in the tissue macrophages from the spleen; kidney macrophages showed statistically significant up-regulation of TNF-α, IL-1β, and IL-10 when compared to the control and to the group exposed to apoptotic cells, while changes of these cytokines in the spleen were only modest (Figure 3.17). The stimulation of cells with apoptotic cells elicited the down-regulation of the cytokines analyzed for both tissues, this down-regulation was only found to be statistically significant for IL-1β and TGFβ in spleen tissue macrophages (Figure 3.17). Additionally, statistically significant differences were observed in the regulation of pro-inflammatory cytokines between tissue macrophages of the kidney and the spleen when exposed to the heat-killed pathogen, and IL-1β and TGFβ when stimulated with the apoptotic cells (Figure 3.17).

MMs of the kidney were found to up-regulate the gene expression of TNF-α, IL-1β, and IL-10 (Figure 3.18 A-C) after being stimulated with *A. salmonicida*, and to down-regulate all the genes analyzed after stimulation with apoptotic cells (Figure 3.18). Spleen MMs were found to up-regulate the expression of all cytokines studied after stimulation with *A. salmonicida*, and to down-regulate pro-inflammatory cytokines of the genes analyzed in response to the bacteria except for IL-1β (Figure 3.18). Spleen MMs also exhibited down-regulation of the pro-inflammatory cytokines, and up-regulation of the anti-inflammatory cytokines (Figure 3.18). Additionally, spleen and kidney MMs revealed a statistically significant differential regulation of anti-inflammatory cytokines when exposed to the heat-killed pathogen and to the apoptotic cells.

Altogether the results from this section show that tissue macrophages and melano-macrophages from the kidney exhibit a similar response after *in vitro* stimulation with *A. salmonicida* and apoptotic cells, while tissue macrophages and melano-macrophages from the spleen have a different behavior after exposure to the same stimulus.

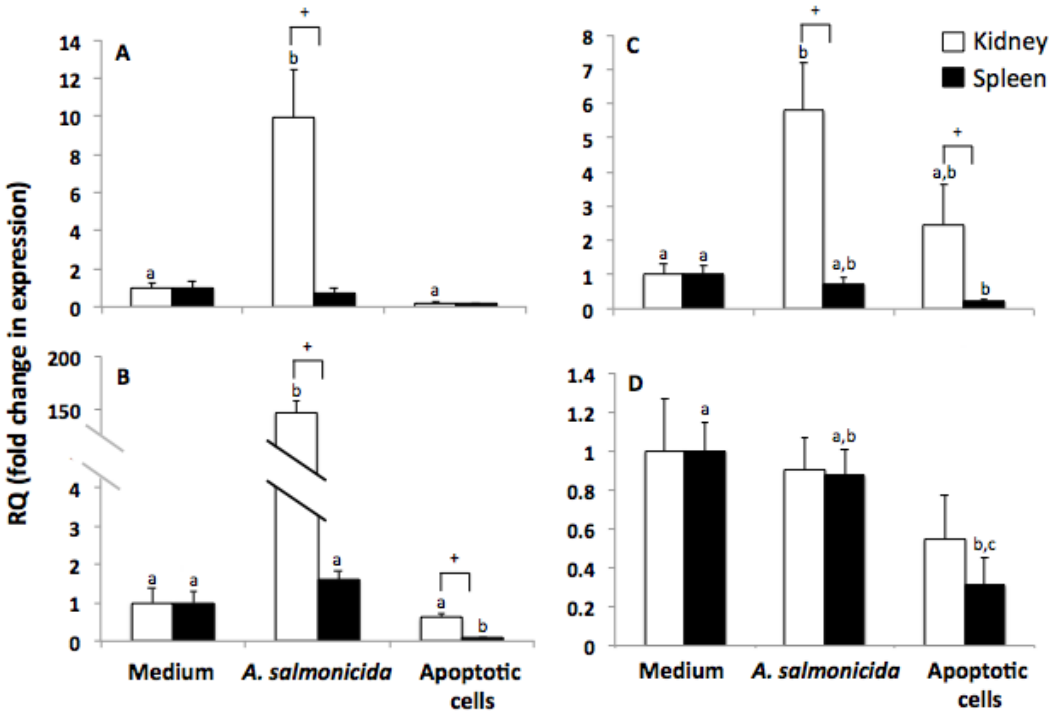

Figure 3.17. Quantitative gene expression analysis of goldfish pro- and anti-inflammatory cytokines in spleen and kidney tissue macrophages after in vitro stimulation.

Tissue macrophages were stimulated with either medium alone, 2µg/mL heat-killed *A. salmonicida*, or apoptotic cells at a 5:1 apoptotic cell: macrophage ratio, and the expression of (A) TNF-α, (B) IL-1β, (C) IL-10, and (D) TGF-β was analyzed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for cells with medium alone. The results are mean ± SEM of tissue macrophages from three to six individual fish ($3 \leq n \leq 6$). Statistical analysis was performed using one-way ANOVA. Different letters above each bar denote significant differences ($P < 0.05$), the same letter indicate no statistical difference between groups. (+) above lines denote statistical differences ($P < 0.05$) between indicated experimental bars.

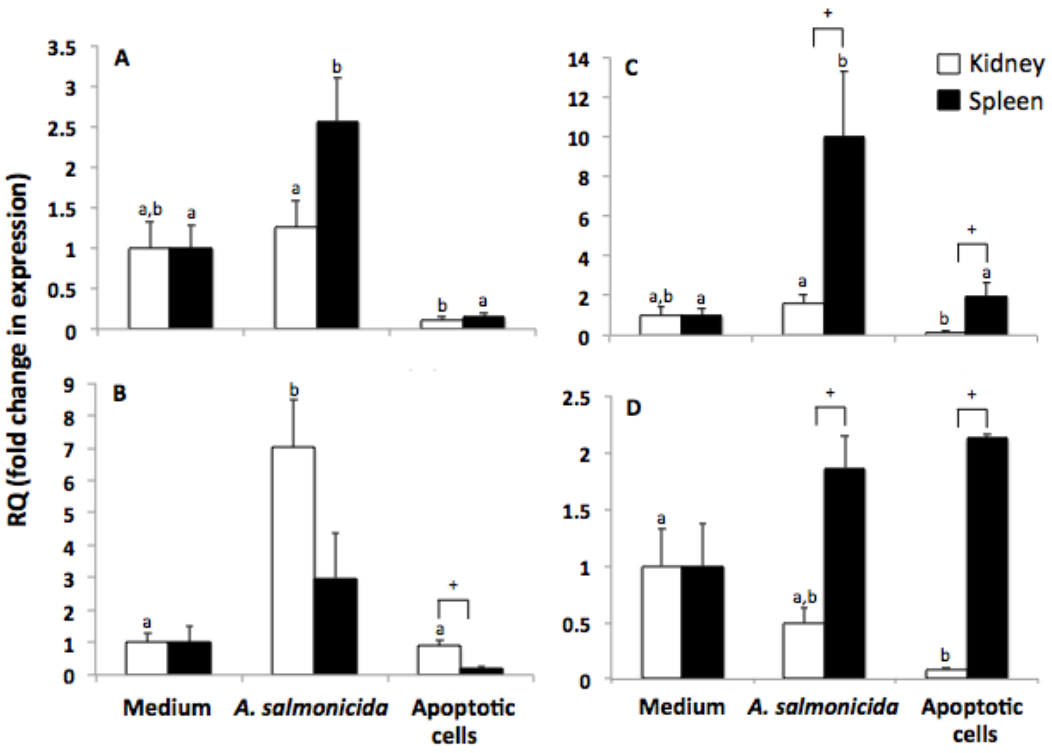

Figure 3.18. Quantitative gene expression analysis of goldfish pro- and anti-inflammatory cytokines in spleen and kidney melano-macrophages after in vitro stimulation.

MMs were stimulated with either medium alone, 2µg/mL heat-killed *A. salmonicida*, or apoptotic cells at a 5:1 apoptotic cell: macrophage ratio, and the expression of (A) TNF-α, (B) IL-1β, (C) IL-10, and (D) TGF-β was analyzed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for cells with medium alone. The results are mean ± SEM of MMs from three to six individual fish ($3 \leq n \leq 6$). Statistical analysis was performed using one-way ANOVA. Different letters above each bar denote significant differences ($P < 0.05$), the same letter indicate no statistical difference between groups. (+) above lines denote statistical differences ($P < 0.05$) between indicated experimental bars.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1. Discussion.

AID expression in mammals is generally restricted to the germinal centre reaction. GCs are well-characterized microenvironments and their structure has been extensively studied in tissues through histological staining. It was previously believed that fish completely lacked GCs based on the low affinity maturation of their antibodies and the absence of GCs in their tissues under histological staining. Using AID as a marker for somatic hypermutation in catfish (*Ictalurus punctatus*) tissues, our laboratory was able to localize AID-expressing B-cells in clusters of fluorescent melano-macrophages. MMs were previously shown to trap and retain antigen on their surface, and to phagocytose apoptotic cells (Reviewed in Ganz and Nemeth 2011), roles akin to FDCs and TBMs of the GC. In addition to AID expression, melano-macrophage clusters were also found to share some other features with the GC; Ig, CD4 and TcR□-expressing cells (Russell, Kanellos et al. 2000). Altogether these findings suggest that MMCs in fish tissues are analogous to GCs in mammalian tissues. Although the dynamics and the functional relationship of the MMC cells is yet to be determined.

The aim of the present study was to examine the gene expression profile of MMs under different conditions to help further understand MMCs possibly as germinal centre-like structures in fish. Different characteristics make the goldfish a suitable model for studying melano-macrophages; first, it is closely related to the zebrafish whose genome has already been sequenced, thus making *in silico*

analyses feasible. Second, it is larger than the zebrafish, thus it allows for the recovery of cells for *in vitro* studies. And third, goldfish macrophages have been extensively characterized by the laboratory of Dr. Miodrag Belosevic, which gives us a better understanding of the model system, namely the role of monocytes and primary kidney derived macrophages in the immune response of the goldfish.

It was hypothesized that if MMs in fish had equivalent roles to FDCs or TBMs in mammals, they would up-regulate the production of anti-inflammatory cytokines rather than pro-inflammatory ones when exposed to *A. salmonicida* under *in vivo* and *in vitro* conditions, or to apoptotic cells under *in vitro* conditions**.** We originally thought that MMs from the spleen and the kidney of the fish would yield very similar results, but our main finding indicates that the same stimulus elicits different responses in these two cell populations, thus suggesting that they may perform different functions in the spleen and the kidney of the goldfish.

4.1.2. Cytochemistry of FACS isolated melano-macrophages reveals morphological and functional characteristics of these cells.

To date, description of MMs is available from histological staining of MMCs in fish tissue sections, but there is no information of these cells isolated from the clusters, which would allow us to assess heterogeneity within the MM population. To further understand and characterize melano-macrophages, cytochemistry of isolated cells was performed. Our cytochemistry results

revealed some heterogeneity among MMs from the spleen and the kidney, but also from cells within the same tissue. Differences observed between cells were: intensity of the Sudan Black B staining, the presence of hemosiderin determined by Perl's Prussian blue, and the morphology and size of the auto-fluorescent pigments found inside the cells. The differences seen for the Sudan Black B staining could be explained by different concentrations of lipofuscin present in the cells. The accumulation of this pigment occurs because of the aging and the decay of tissues (Reviewed in Agius and Roberts 2003). Lipofuscin granules present in fish MMs are proposed to be polyunsaturated fatty acids of effete cellular membranes that have been denaturated by strongly oxidative substances (Saunders, Oko et al. 2010). Although lipofuscin is generally the most common pigment found in MMCs, it has been shown that its presence varies among clusters within the same tissue, different tissues, and among fish species (Dayan, Abrahami et al. 1988; Subramaniam and Chaubal 1990; Hashemzadeh-Bonehi, Phillips et al. 2006). Lipofuscin deposition is linked to tissue deterioration and it has been previously shown that starvation of fish dramatically increases the presence of lipofuscin granules in the spleen, the kidney and the liver of different fish species (Agius and Roberts 2003).

The presence of hemosiderin, as assessed by Perl's Prussian blue was also found to be variable in the MMs of the goldfish spleen and kidney; this pigment was observed in a few spleen MMs, but it was not observed in any of the kidney MMs stained. Observations of hemosiderin in only approximately 12% of the spleen MMs could be explained by the presence of a MM subpopulation in charge

of clearing effete red blood cells circulating through the spleen of the fish. To date, the anatomical location of hemosiderin positive cells within the tissues of the fish is currently unknown, but consistent with our findings, previous studies in the rainbow trout revealed that storage of iron occurred in the spleen and rarely in the kidney or the liver of fish. This observation was made in fish under different health status including healthy, diseased, and fish undergoing induced erythrocyte degradation by starvation (Agius and Agbede 1984; Leknes 2007). It was subsequently found that after splenectomy and starvation, the storage of iron was diverted to the kidney of the trout indicating that under abnormal conditions, the recycling of hemoglobin may take place in the kidney (Agius and Roberts 1981).

The largest difference observed among goldfish melano-macrophages was the size of the auto-fluorescent pigments found in the cells. Pigments found inside MMs are derived from the metabolism of phagocytosed particles, thus the presence of pigments varying in size could be explained by the age of a cell and the life quality of the fish; it would be expected that older MMs would have phagocytosed more particles than a younger ones, and that fish in stressful environments would undergo a higher rate of tissue decay than fish in healthy environment, thus leading to a greater accumulation of pigments in older cells and stress or diseased fish.

4.1.3. *A. salmonicida* elicits a classical pro-inflammatory response in PKM cultures and kidney macrophages that is not observed as well defined in spleen macrophages or MMs from either tissue.

Macrophages play different roles in the maintenance of tissue homeostasis; these professional phagocytes can rapidly change their functional phenotype in response to micro-environmental signals. During the process of inflammation and its resolution, macrophages undergo changes in their activation state, switching polarized functional phenotypes from classically activated to function as antimicrobial killer cells to non-classically activated cells to heal and repair tissue (Reviewed in Murray and Wynn 2011).

Cytokine regulation is pivotal in the progression and resolution of the inflammatory response; these signaling molecules bind specific receptors at the cell surface and trigger a chain of molecular events that lead to the induction, modulation or suppression of cytokine-regulated genes. The main goal of this thesis was to generate profiles of the cytokine expression of MMs and other tissue macrophages exposed to different stimuli, and to compare these profiles to that expected of classically activated, regulatory, and wound-healing macrophages.

Numerous cytokines have been identified in several teleost species. In the goldfish, cytokines key in the progression and resolution of inflammation have been identified and their expression levels have been characterized in monocytes/macrophages from the kidney. The kidney of the bony fish is analogous to the mammalian bone marrow, working as the principal source of pluripotent cell populations. The Belosevic group has previously demonstrated

that leukocytes isolated from the kidney are able to selectively develop into myeloid progenitor cells *in vitro* by producing endogenous growth factors under specific conditions. At different stages of development, these kidney primary cultures are composed of at least three cell populations: myeloid progenitors, monocytes, and macrophages (Neumann, Barreda et al. 1998; Neumann, Barreda et al. 2000). Using this unique system, the Belosevic group has extensively used monocytes and macrophages in the characterization of the goldfish inflammatory process during antimicrobial responses. Because these cells were shown to exhibit a classical pro-inflammatory profile of cytokine expression when exposed to *A. salmonicida* (Grayfer, Hodgkinson et al. 2011), we used these cells to compare their expression profile to that of other tissue macrophages and MMs. In our studies, cultures of primary kidney derived macrophages displayed a classical pro-inflammatory response against *A. salmonicida*, characterized by the up-regulation of TNF-α, IL-1β, and IL-10, and evidenced by the 5, 35, and 12-fold increase in the expression of TNF-α, IL-1β, and IL-10 respectively. Similar observations have been made in other fish species when exposed to the pathogen *A. salmonicida*. Intestinal epithelial cells of the rainbow trout where shown to up-regulate the production of TNF-α and IL-1β through semi-quantitative PCR (Komatsu, Tsutsui et al. 2009). In a different study, up-regulation in the expression of TNF-α, IL-1β, and IL-10 was observed in the head kidney (whole tissue) of carp after intra-peritoneal injection with *A. salmonicida.* Up-regulation in gene expression was observed 6 hours post-injection, and it was evidenced by a 10, 20, and 2-fold increase of TNF-α, IL-1β, and IL-10 respectively (Falco, Frost

et al. 2012). Furthermore, similar observations were made in the zebrafish after intra-peritoneal injection with a different fish pathogen, the Gram-negative *Listonella anguillarum.* In this study, the regulation of immune relevant genes was analyzed trough qPCR from the whole fish, and it was observed a 10-fold induction of IL-1β by 4 hours post-injection, and a 2.5-fold increase of TNF-α 6 hours post-injection (Rojo, de Ilarduya et al. 2007)

In our studies, *in vitro* stimulation of tissue macrophages isolated from the kidney revealed that these cells undergo classical activation when exposed to *A. salmonicida*, tissue macrophages from the spleen did not show the same pattern of activation. Instead, these cells were found to maintain the expression level of the different cytokines with only very subtle changes when stimulated *in vitro* with *A. salmonicida*. Although the constitutive expression of different cytokines including TNF-α (Grayfer, Walsh et al. 2008), IL-10 (Grayfer, Hodgkinson et al. 2011), and TGF-β (Haddad, Hanington et al. 2008)has been demonstrated in goldfish splenocytes, very little information is available on fish isolated splenic macrophages and their immune response against microbial challenge. Instead, studies available assess the immune response of the spleen as a whole without isolating different cell types. Goldfish intra-peritoneal injection with the pathogen *Mycobacterium marinum* elicited a pro-inflammatory response in the spleen observed 7 days post vaccination, evidenced by a 3, and 15-fold induction of TNF-α and IL-1β respectively (Hodgkinson, Ge et al. 2012). A different study investigated the cytokine production in the spleen of the rainbow trout after intra-peritoneal injection with the Gram-negative fish pathogen *Yersinia ruckeri*, by

qPCR. Up-regulation (2.6-fold increase) of IL-1β was observed 8 hours post vaccination, up-regulation of INF-γ (22-fold increase) and IL-10 (396-fold increase) was observed by 3 days post vaccination, and no regulation of TGF-β was observed during the study (Raida and Buchmann 2007). These studies demonstrate a classical pro-inflammatory response in the spleen elicited by bacterial challenge, and the observations made differ from our experiments with spleen tissue macrophages. Because of the subtle changes observed in the spleen tissue macrophages, it is possible that the differences observed are a product of the time point analyzed in which case this could be resolved by studying the cytokine production of tissue macrophages at different time points of stimulation. Although expanding the time points of stimulation could show differences in the regulation of the different cytokines, our results also raise the possibility that resident macrophages of the spleen are not the major producers of these cytokines in this tissue. Macrophages are very heterogeneous and differences between spleen and kidney tissue macrophages could be explained based on the anatomical location, the physiological environment, and the role of the tissue in which these two cell populations reside.

In vitro stimulation of isolated MMs with *A. salmonicida* elicited different responses in cells from then kidney and the spleen. Kidney cells showed statistically significant up-regulation of IL-1β, modest up-regulation of TNF-α and IL-10, and modest down-regulation TGF-β. Spleen MMs showed statistically significant up-regulation of TNF-α and IL-10, and modest up-regulation of IL-1β and TGF-β. The balance of pro- and anti-inflammatory cytokine production by

each of these two cell populations suggests a pro-inflammatory profile for kidney MMs and an anti-inflammatory profile for spleen MMs when challenged with *A. salmonicida*.

The teleost kidney can be divided in anatomical and functional regions into the anterior, the mid- and the posterior kidney. The mid- and posterior kidney has both immune and renal functions, while the anterior kidney is lymphopoietic/hemopoietic and lacks renal function (Zapata 1979; Grassi Milano, Basari et al. 1997). The teleost spleen represents a major secondary lymphoid organ during bacterial infections. The spleen is believed to be an important reservoir of red blood cells (Grace and Manning 1980; Secombes and Manning 1980), and a blood filter that removes effete erythrocytes from circulation (Agius and Agbede 1984).

Localization of antigen in the fish lymphoid tissue has been studied in different models using a wide variety of antigens. In these studies antigen is initially removed from the blood stream by sinusoidal macrophages and it becomes gradually located in or near MMCs. Plaice BSA injection revealed the initial localization of the antigen to be in scattered cells that with time tended to group near MMCs of both the spleen and the kidney, and in some cases, the antigen was found in the MMCs. Additionally, antigen and Ig distribution led to the possibility that like in mammals, antigen trapping in teleost occurred in the form of immune complexes (Ellis 1980). Similar observations in antigen localization were made in carp with *Aeromonas hydrophila* bacterin; bacterial antigen was initially found in single phagocytic cells, but as time progressed,

antigen gradually concentrated in or near MMCs of the spleen, the anterior and posterior kidney. Furthermore, antigen retention was extracellular and it remained in and near MMCs for up to a year (Agius and Agbede 1984). Supporting the idea of antigen trapping in the form of immune complexes occurring in fish, injection of carp with pre-formed antigen-antibody complexes led to a faster retention of the antigen compared to when carp was injected with the antigen alone. A similar observation was made in fish that received a prior vaccination with the antigen, suggesting its retention occurred in complex with circulating antibodies, perhaps through some form of Fc receptor (Lamers and De Haas 1985).

During the course of this study, the entire kidney was used for all the experiments. It is well know that the anatomical regions in the teleost kidney have different functions, thus studying the response of MMs by anatomical region would allow us to see if there are any differences in the cytokine production of these cells, indicating if there are any differences in the MM response that correlates with their anatomical location within the kidney.

4.1.4. Although in vitro exposure to apoptotic cells did not elicit a significant increase in the production of anti-inflammatory mediators, it did not induce a classical pro-inflammatory response in the different macrophage populations.

Cell apoptosis shapes the immune systems by triggering anti-inflammatory programs in mammals. Phagocytosis of apoptotic cells has been demonstrated to

have immunosuppressive and anti-inflammatory effects. Furthermore, clearance of apoptotic cells has been shown to promote the production of anti-inflammatory mediators specially TGF-β and IL-10, and also to decrease the production of pro-inflammatory cytokines including TNF, IL-1, and IL-12 (Secombes, Manning et al. 1982).

Exposure to apoptotic cells mostly led to the induction of modest changes in the expression of the different cytokines in our experiments. Statistically significant changes in the PKM culture cytokine expression was not observed at any of the time points for any of the cytokines studied. Furthermore, although not statistically significant, up-regulation of TNF-α, IL-1β, and IL-10 was observed at 48 hours of incubation. Lack of clearance of apoptotic cells in mammals has been demonstrated to have pro-inflammatory effects. Furthermore, failure to clear apoptotic cells can lead to necrosis and potentially disruption of self-tolerance, and it has been associated to autoimmune diseases like systemic lupus erythematous (Reviewed in Savill, Dransfield et al. 2002; Underhill and Goodridge 2012).

Exposure of tissue macrophages to apoptotic cells mostly induced modest changes in the regulation of cytokine production. Apoptotic cells were found to have a suppressive effect in the expression of both pro- and anti-inflammatory cytokines. Furthermore, the only statistically significant changes observed were the down-regulation of IL-1β and TGF-β by tissue macrophages of the spleen.

Isolated MMs from the kidney and the spleen revealed different expression profiles after being exposed to apoptotic cells. We observed a modest

up-regulation of anti-inflammatory cytokines paired with a subtle down-regulation of pro-inflammatory cytokines in spleen MMs akin to what has been observed in mammals after exposure to apoptotic cells. In kidney MMs, IL-1β remained with no change, while there was a modest down-regulation of TNF-α and IL-10 and also a statistically significant down-regulation of TGF-β.

Altogether, our results suggest a general suppression of the immune cytokines TNF-α and IL-1β after macrophage exposure to apoptotic cells. Furthermore, it was observed that PKMs, kidney MMs, and spleen and kidney tissue macrophages had a 'neutral' phenotype that was not characterized by either anti- or pro-inflammatory cytokines.

Phagocytosis of apoptotic cells has been demonstrated to down-regulate the production of the pro-inflammatory cytokines TNF-α and IL-1β in human monocyte-derived macrophages that have ingested apoptotic cells and are treated with LPS. Additionally, this treatment also down-regulated the production of IL-10, but up-regulated the production of TGF-β (Reviewed in Ravichandran and Lorenz 2007).

In addition to the cytokines studied, apoptotic cells in mammals have been demonstrated to extensively down-regulate the production of pro-inflammatory immune mediators like IL-6 IL-8 and platelet activating factor, but also to up-regulate the anti-inflammatory immune mediator prostaglandin E2 (Eming, Hammerschmidt et al. 2009). Including these immune mediators in the study of the effect of apoptotic cells on fish macrophages would broaden our knowledge

towards the understanding of how apoptosis shapes the immune response in lower vertebrates.

4.1.5. *In vivo* challenge by fish vaccination elicited a differential regulation of the genes studied. However, MMs did not appear to mount a classical pro-inflammatory response.

Goldfish vaccination was performed in order to study the *in vivo* response of MMs to *A. salmonicida*. Although we observed some differences between tissues and doses, none of our experimental treatments revealed a clear pro-inflammatory profile. Furthermore, regulation of IL-1β and TNF-α was in all cases very subtle with no statistically significant changes observed with the exception of the high dose booster vaccination in the spleen. This study provided us with insight into the immune response of MMs when challenged with a bacterial pathogen. Throughout the experiment it was observed that IL-10 was the cytokine undergoing the largest amount of regulation and in all cases, its expression was found to be greater than the control group. Additionally, TGF-β expression was found to be similar within tissues for the low dose single and booster vaccination, but we observed some up-regulation of this gene in both tissue induced by the high dose booster injection.

Other genes analyzed after goldfish vaccination were membrane bound CSF-1R, MHC class II, and MFGE8. Changes observed in the expression of these genes where for the most part modest, yet some differences between tissues were observed.

CSF-1R appeared to be down regulated in the kidney and up regulated in the spleen. CSF-1R is the receptor for CSF-1, a key growth and differentiation factor for both mammalian and fish macrophages. Mammalian CSF-1 has been involved in different macrophage activation processes including the up-regulation of pro-inflammatory cytokines and the enhancement of antimicrobial activity (Reviewed in Hamilton 2008). . In the goldfish, CSF-1 has been identified and characterized, and it was demonstrated that recombinant CSF-1 is able to activate cells and up-regulate pro-inflammatory genes (Hanington, Wang et al. 2007; Haddad, Hanington et al. 2008).

Regulation of MHC class II was observed to some extent in MMs isolated from the spleen and the kidney. Expression of this gene tended to be up-regulated especially in the kidney cells, suggesting that these cells are involved in antigen presentation.

MFGE8 expression was found to remain almost invariant from the control group in all treatments. In mammals, MFGE8 is an opsonin that plays a critical role in the engulfment of apoptotic cells. Different phagocytes including TBMs secrete MFGE8. Furthermore, MFGE8 mediates the TBM phagocytosis of apoptotic cells resulting from the antibody affinity maturation process in the GC (Kranich, Krautler et al. 2008). Additionally MFGE8-deficient mice have been demonstrated to break self-tolerance and develop different autoimmune disorders (Hanayama, Tanaka et al. 2002; Hu, Wu et al. 2009) . MFGE8 expression and function have yet to be characterized in fish. Perhaps *in vitro* assessment of MFGE8 expression in macrophages exposed to apoptotic cells would have offered

us a better insight into the role of this opsonin in fish engulfment of apoptotic cells. Unfortunately, the number of tissue macrophages and therefore the quantity of RNA available was very small, thus limiting the extent of *in vitro* analyses.

Altogether, we found intra-peritoneal injection to elicit gene expression changes in MMs under the different treatments and these changes did not exhibit a classical pro-inflammatory response. To better understand the response of these cells to recent pathogen encounter, the time line of the study needs to be adjusted in order to analyze gene expression hours after stimulation and not 3 days post injection as we did. Additionally, adding time points to a longer time frame experiment would give us a better idea of how the different cytokines are being regulated *in vivo*.

4.2. Conclusions.

As a step towards understanding the role of MMCs in the antibody affinity process in the fish, we focused our study in MMs from the kidney and the spleen of the goldfish.

We were able to adjust the process of MM isolation from the catfish to the goldfish. Fluorescent microscopy of isolated MMs coupled with cells from peritoneal lavage revealed that these cell populations were different and that FACS sorting of MMs did not isolate different cell types from tissue other that MMs.

The study of the expression profile of MMs and other macrophages revealed differences among these cell populations. Furthermore, MMs did not

appear to mount a classical pro-inflammatory response to *A. salmonicida* like PKMs did evidenced by the fold-induction of pro-inflammatory cytokines. Additionally, we observed differences between spleen and kidney MMs that could be explained by the different functions of the anterior, mid- and posterior kidney, and the anatomical location of MMs within the kidney.

Altogether, our results suggest a role for MMs akin to the one of FDCs or TBMs based on the lack of a classical pro-inflammatory response of MMs under the *in vivo* and *in vitro* conditions analyzed.

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APPENDICES

Appendix 1. *In silico* analyses of the goldfish MFGE8 gene and qPCR primer location.

Zebrafish MFGE8 NM_001080990.1 (mfge8b1)

Lab sequenced mfge8:
Tgnccgagcccctcaangcnttcaaggtggcatccagtttagatgggcgaacctacaccatgtacagacccgagggccagagcaaagatg
tgatctttgttggtaatgtggacaacgatgggacaaagactaatttgttcgatcctccaatcatagctcagtacatacgaatcattcctgttgtgtgc
cgtaaagcctgtacactgagaatggagctggtgggctgtgaactcaacggttgctccgagccgctaggtatcaagtcacggctgatcgatgac
aggcagctgacagcttccagcacttttcgcacctgggggatcgagtcgttcacctggcacccgcattacgctcgtctggacaagcagggcaa
gatcaacgcctggacagcagccaccaataaccgctccgagtggctccaggtggacctgctcagaccaaagcgcatcacagggattatcacg
caaggggccaaagactttggcaatgtgcaatttgtgtccgcctttaaagtggctcacagtgacgacgga

Highlight = primers for qPCR
Highlight = alternating exons

Alignment of the goldfish and zebrafish MFGE8 sequences:

```
Goldfish        ------------------------------------------------------------
Zebrafish       ACCTCTGCAACGCATGACAAAAACCCCTGGATTGAGGTCAACCTGCAGAGGAAGATGCGC 660


Goldfish        ----------------------------------TG------NCCGAGCCCCTCAANGCN 20
Zebrafish       TTCACTGGCATCATTACCCAGGGTGCCAGTCGCATGGGCACAGCCGAGTTCATCAAAGCC 720
                                                **      .*****  *.**** **

Goldfish        TTCAAGGTGGCATCCAGTTTAGATGGGCGAACCTACACCATGTACAGACCCGAGGGCCAG 80
Zebrafish       TTCAAGGTGGCGTCCAGTTTGGATGGAAGAACCTACACCATGTACAGACCTGAGGGCCAG 780
                ***********.********.******..********************** *********

Goldfish        AGCAAAGATGTGATCTTTGTTGGTAATGTGGACAACGATGGGACAAAGACTAATTTGTTC 140
Zebrafish       AGCAAAGATATGATTTTTGTTGGTAACATGGACAATGATGGGACAAAGACGAATTTGTTC 840
                *********.************** .*****************************

Goldfish        GATCCTCCAATCATAGCTCAGTACATACGAATCATTCCTGTTGTGTGCCGTAAAGCCTGT 200
Zebrafish       GAGCCTCCAATCATCGCTCAGTTCCTCCGGATTGTTCCTGTGGTGTGCCGTAAAGCCTGT 900
                ** ***********.*******:*.*.**.** .******* *****************

Goldfish        ACACTGAGAATGGAGCTGGTGGGCTGTGAACTCAACGGTTGCTCCGAGCCGCTAGGTATC 260
Zebrafish       ACTCTGAGAATGGAGCTGGTCGGCTGTGAACTCAACGGTTGCTCCGAGCCGCTCGGCGTC 960
                **:***************** ********************************.** .**

Goldfish        AAGTCACGGCTGATCGATGACAGGCAGCTGACAGCTTCCAGCACTTTTCGCACCTGGGGG 320
Zebrafish       AAGTCTCGGCTCATCAGTGACCGGCAGATGACATCGTCCAGCGCTTTCCGCACCTGGGGC 1020
                *****:***** ***..****.*****.***** * ******.**** ***********

Goldfish        ATCGAGTCGTTCACCTGGCACCCGCATTACGCTCGTCTGGACAAGCAGGGCAAGATCAAC 380
Zebrafish       ATTGAGGCCTTCACCTGGCACCCGCATTACGCCCGTCTGGACAAACAGGGCAAGACCAAC 1080
                ** *** * ********************** **********.********** ****

Goldfish        GCCTGGACAGCAGCCACCAATAACCGCTCCGAGTGGCTCCAGGTGGACCTGCTCAGACCA 440
```

```
Zebrafish       GCCTGGACGGCAGCTACAAACAACCGCTCCGAGTGGCTGCAGGTGGATCTGCTCAGACCA 1140
                ********.***** **.** ***************** ******** ************

Goldfish        AAGCGCATCACAGGGATTATCACGCAAGGGGCCAAAGACTTTGGCAATGTGCAATTTGTG 500
Zebrafish       AAGCGCATTACAGGAATCATCACACAAGGAGCCAAAGACTTCGGCAATGTGCAGTTTGTA 1200
                ******** *****.** *****.*****.*********** ***********.*****.

Goldfish        TCCGCCTTTAAAGTGGCTCACAGTGACGACGGA--------------------------- 533
Zebrafish       TCTGCCTATAAAGTGGCTCACAGTGACGACGGCCAACACTGGACGATACTCAAAGAAGAC 1260
                ** ****:**********************.

Goldfish        ------------------------------------------------------------
Zebrafish       AAGACCAAAACAGATAAGATCTTCCCTGGCAACAGTGACAACAACGTGCACAAAAAGAAT 1320

Goldfish        ------------------------------------------------------------
Zebrafish       GTGTTTGACCCGCCCTTCTACGCGCGATTCGTCCGCGTGCTGCCGTGGGCGTGGCATGAG 1380

Goldfish        -------------------------------------------
Zebrafish       CGCATCACTCTGCGAATGGAGCTGCTGGGCTGCGACGAGTAA 1422
```

Appendix 2. *In silico* analyses of the goldfish MCH class II gene and qPCR primer location.

Zebrafish NP_001005975.1

Lab sequenced MHC class II

gattggagttgatggagaggtactgtaccattcagacttcattagacaagaaggagtagtgacagcgcctgactttgca
gatcccgtcagctttactggattttatgagagtggtgttgctaatatagaggtctgcaaacaaaacttagccctagacatt
aagctttccaaacccacagttgagcaactagccccccagatgcttccatctattcaaaaaatgacgtggtgctgggtg
tcgagaacgccctcatctgtcttgtgtctggactcttccctccacctgtcaaagtctcctggactaaaaacaatcagattg
tgacagaggacgtgagtctaagccagtaccgcataaaaaatgctggcaccttcagcatcttctcctcgctgaagttcac
acctgcagagggagacatttacagctgcactgtgtcccacaaagctctggagagccgtggtttaaccaaaacatggg
gtgagacactgcttcatgtatattcagtggataggtggatgttgctgtgcccggcatcggtccagctgtgttctgtggag
tgggtctgtctctggggctgc

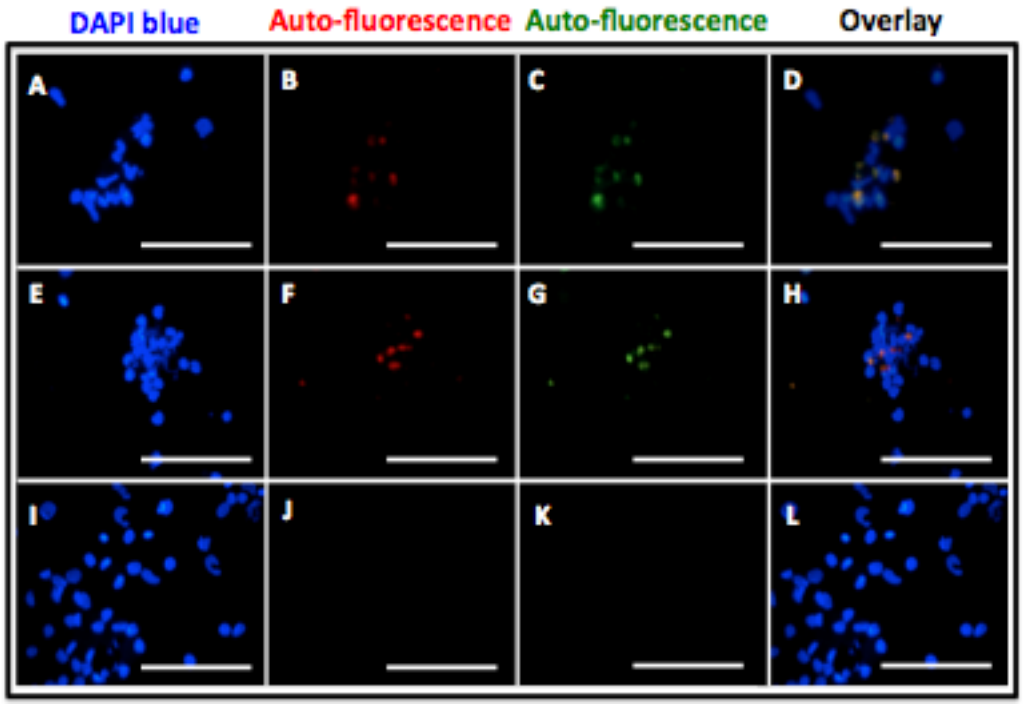
Highlight = primers for qPCR
Highlight = alternating exons

Alignment of the goldfish and zebrafish MHC class II sequences:

```
Goldfish        ------------------------------------------------------------
Zebrafish       ATGGATCTGTTTGGTTTTCTGCTGATATTCACTGTAATTGTGAGTAATGGGGCTCAAGCT 60

Goldfish        ----------------------------------------------------GATTGGA 7
Zebrafish       GAGCACAGGGATTTTCAGTTTACTGGATGTTCTGATACAGAGAAAGATGATTTGAATGGA 120
                                                                   **:****

Goldfish        GTTGATGGAGAGGTACTGTACCATTCAGACTTCATTAGACAAGAAGGAGTAGTGACAGCG 67
Zebrafish       TTTGATGGAGAGGTGCTGTACTATACAGACTTCATTAGAAAAGAAGGAGTAGTGATAGCG 180
                 *************.****** **:***************.*************** ****

Goldfish        CCTGACTTTGCAGATCCCGTCAGCTTTACTGGATTTTATGAGAGTGGTGTTGCTAATATA 127
Zebrafish       CCTGACTTTGCAGATCCTCTCAGCTTTCCTGGAGGTTATGAGACTGGTGTTGCTCAAATG 240
                ***************** ********.*****  ******** **********.*:**.

Goldfish        GAGGTCTGCAAACAAAACTTAGCCCTAGACATTAAGCTTTCCAAACCCACAGTTGAGCAA 187
Zebrafish       GAGGTCTGCAAACAAAATTTAGCCACAGACATTAAGGCATACAACTCCCCACAAGAGCAA 300
                ***************** ******. **********  :*.***. **.** ::******

Goldfish        CTAGCCCCCCCCAGATGCTTCCATCTATTCAAAAAATGACGTGGTGCTGGGTGTCGAGAAC 247
Zebrafish       CTAGACCCTCCTGTGACATCCATTTATTCAGAAGATGAAGTGGTTCTGGATGAAAAGAAC 360
                ****.*** **:*: .*:***** ******.**.****.***** ****.**:..*****

Goldfish        GCCCTCATCTGTCTTGTGTCTGGACTCTTCCCTCCACCTGTCAAAGTCTCCTGGACTAAA 307
Zebrafish       ACACTCATATGTTATGTGACTGGATTCTTCCCTCCACCTGTCAATGTCTCCTGGACGAAA 420
                .*.*****.*** :****:***** ********************:*********** ***

Goldfish        AACAATCAGATTGTGACAGAGGACGTGAGTCTAAGCCAGTACCGCATAAAAAATGCTGGC 367
Zebrafish       AACAATGATATTGTGACAGAGGACATTAGTTTTAGCCAGTACCGTGACAACAGTGACGGC 480
                ****** * ***************.* *** *:*********** .:.**.*.**. ***

Goldfish        ACCTTCAGCATCTTCTCCTCGCTGAAGTTCACACCTGCAGAGGGAGACATTTACAGCTGC 427
Zebrafish       ACCTTCAACATGTTCTCCGCTCTGAAGTTCACACCTGCTGAAGGAGACATTTACAGCTGC 540
```

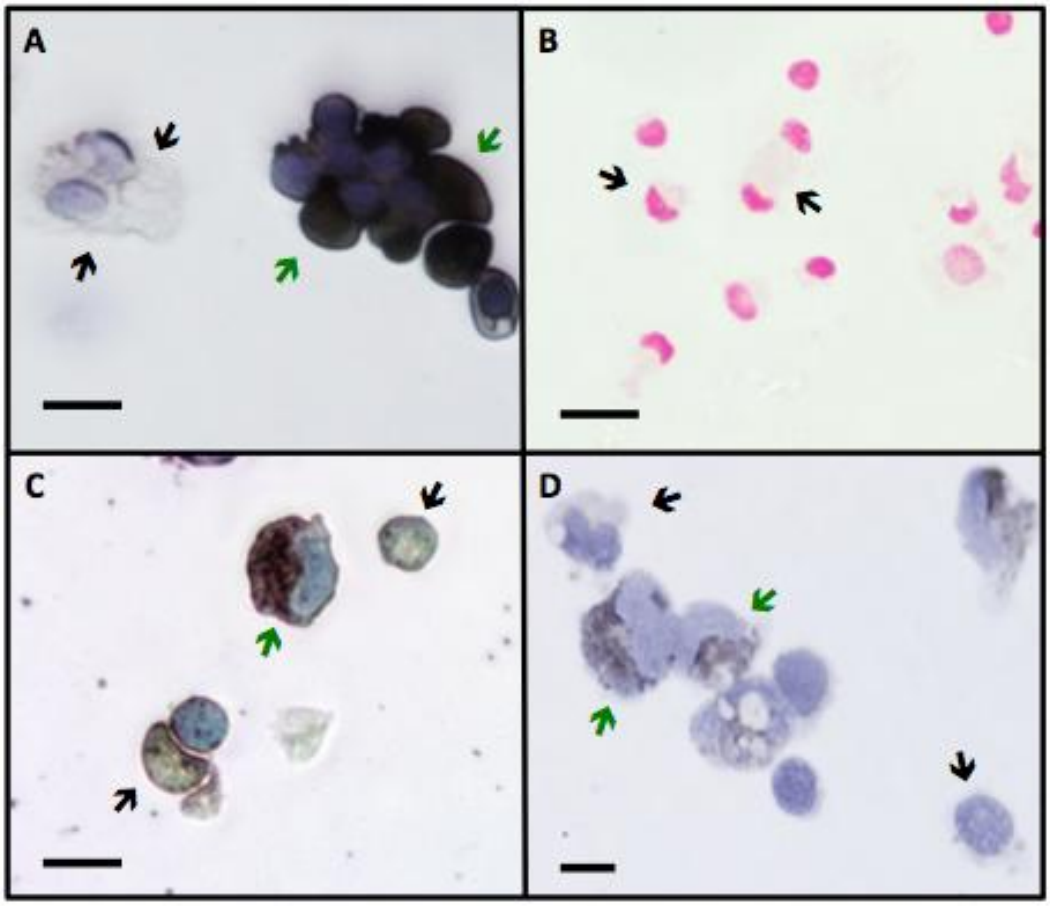
```
               *******.*** ****** * *****************:**.******************


Goldfish       ACTGTGTCCCACAAAGCTCTGGAGAGCCGTGGTTTAACCAAAACATGGGGTGAG-AC--- 483
Zebrafish      ACAGTGAAACACAGATCCATTCAAGGGCAACCCAACACCAAAACATGGGAGGTGGACGTT 600
               **:***:..****.* * .*  *..* *.:   ::.*************. *:* **

Goldfish       --ACTGCTTCATGTATATTCAGTG--GATAGGTGGA-------TGTTGCTGTGCCCG--- 529
Zebrafish      GAGCTGCCCAGTGTTGGTCCAGCAGTGTTCTGTGGAGTGGGTCTGGTTCTGGGGCTGTTG 660
                 .****  ..***: .* *** .  *:*. *****       ** * *** * * *

Goldfish       -GCATCGGTCCAG---CTGTGTTCTGTGGAGTGGGTCTGTCTCTGGGGCTGC 577
Zebrafish      AGAGTGGCTGCTGGAACTTTCTTCCTCATTAAAGG-AAACAACTGCAACTGA 711
                *..* * * *:*   ** * ***   . :.:.** .:. .:*** ..***.
```

Appendix 2. Fluorescent microscopy of FACS isolated MMs and peritoneal lavage cells.



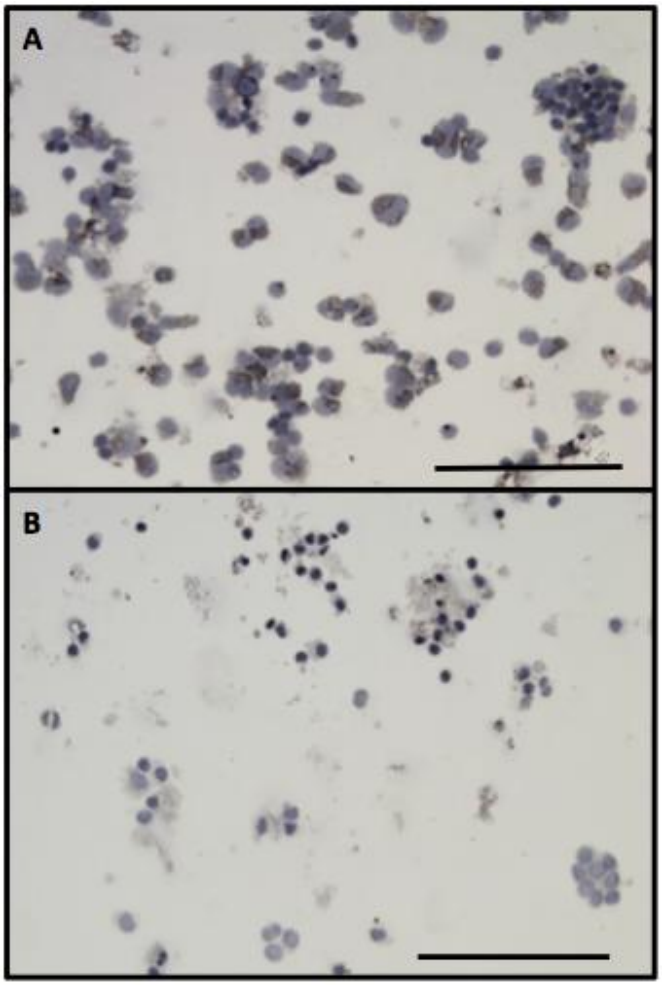
Appendix 2. Fluorescence microscopy images of isolated kidney MMs (A-D), isolated spleen MMs (E-H), and macrophages and neutrophils obtained through peritoneal lavage (I-L) reveal the presence of auto-fluorescence pigments in spleen kidney and spleen MMs absent in cells found in the peritoneal cavity. Pictures were taken at 400X magnification. Scale bar = 50 μm.

Appendix 3. Bright field images of cytochemistry negative control using kidney mixed leukocytes.



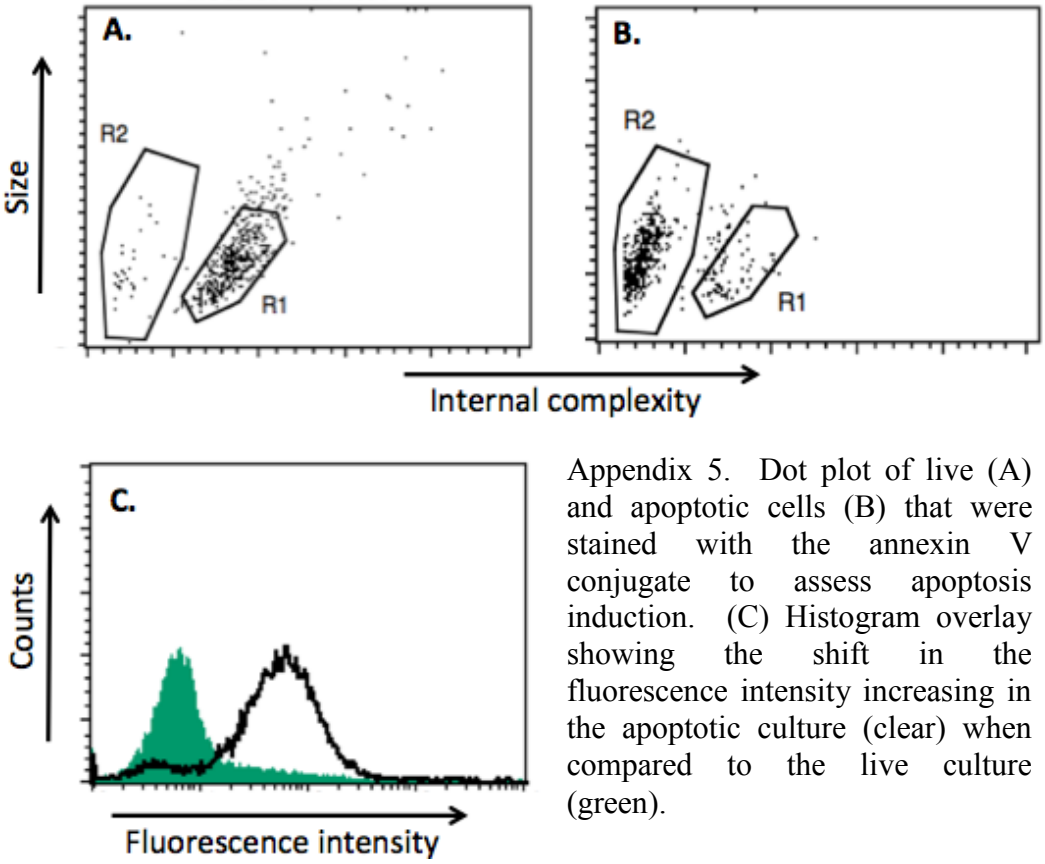
Appendix 3. Bright field images of kidney mixed leukocytes cytochemistry reveal the presence of negative cells. Cytospins of kidney leukocytes were stained for (A) non-specific esterease, (B) Perl's Prussian blue, (C) acid phosphatase, and (D) Sudan black. Images reveal the presence of positive stained cells (pointed by the green arrow) and negative stained cells (pointed by the black arrows). Pictures were taken at 600X magnification. Scale bar = 10 μm.

Appendix 4. Bright field images of Sudan black cytochemistry of isolated kidney and spleen MMs.



Appendix 4. Bright field images of isolated kidney (A) and spleen (B) MMs stained with. Images were taken at 400X magnification and the scale bar =100 μm

Appendix 5. Flow cytometric assessment of apoptosis induced in the 3B11 catfish cell line.



Appendix 5. Dot plot of live (A) and apoptotic cells (B) that were stained with the annexin V conjugate to assess apoptosis induction. (C) Histogram overlay showing the shift in the fluorescence intensity increasing in the apoptotic culture (clear) when compared to the live culture (green).