Development and Screening of a Marker to Detect Activated Rainbow Trout Leukocytes

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by

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DECLARATION

I declare that this thesis has been completely compiled by myself, and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

Sandra M. Laffon Leal

A mis carenos: Avik y Enrique
por su amor y paciencia

A mis padres: Elsa y Miguel
y hermanos: Edgar, Pablo y Miguel
por su amor y confianza

"no solo no hubiera sido nada sin ustedes, sino con toda la gente que estuvo alrededor desde el comienzo……...algunos siguen hasta hoy.

Gracias……totales" **G.C.**

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ABBREVIATIONS

× g: multiples of gravity
1D: one dimensional
2D: two dimensional
APP: acute phase proteins
APR: acute phase response
bmHCA: basal multiple histocompatibility activation
BN-PAGE: blue native polyacrylamide gel electrophoresis
BSA: bovine serum albumin
CD: cluster of differentiation
Con A: concanavalin A
e.g.: example gratia
ELISA: enzyme-linked immunosorbant assay
emHCA: enhanced multiple histocompatibility activation
et al.: et alias (and others)
FCM: flow cytometry
FCS: foetal calf serum
FCS: forward scatter channel
FITC: fluorescein isothiocyanate
g: gram
h: hours
HCA: histocompatibility activation
HK: head kidney
HRP: horseradish peroxidase
i.e.: id est (that is)
Ig: immunoglobulin
IHC: immunohistochemistry
IP: immunoprecipitation
kDa: kilo Dalton
kg: kilogramme
L: litre
LPS: lipopolysaccharide
M1: immunised mouse with non activated cells
M1CA: immunised mouse with Con A activated cells
mAb: monoclonal antibody
MALDI-TOF: matrix-assisted laser desorption ionisation time of flight mass spectrometry
mg: milligram
MHC: major histocompatibility complex
mHCA: multiple histocompatibility activation
min: minutes
ml: millilitre
MLR: mixed leukocyte reaction
MW: molecular weight
pAb: polyclonal antibody
PAMP: pathogen associated molecular pattern
PBL: peripheral blood leukocytes
PBS: phosphate buffer saline
PHA: phytohaemagglutinin antigen
PKC: protein kinase C
PMA: phorbol miristate acetate
Poly I:C: polyribocytidylic acid
PRR: pattern recognition receptors
PWM: pokeweed mitogem
RAG: recombinant activator gene
RGran: gated granulocyte like cells
RLymph: gated lymphocyte like cells
rpm: rounds per minutes
SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP: spleen
SSC: side scatter channel
TBS: tris buffer saline
TcR: t cell receptor
TH: thymus
TTBS: tween 20 tris buffer saline
WB: western blot
µg: microgram
µl: microlitre

Abstract

Monoclonal antibodies (mAbs) have been essential tools in the elucidation of the immune system of mammals, and their application to identify surface molecules on leukocytes have allowed important functions of these cell to be identified (such as receptors that bind antigens, ligands involved in cell to cell signaling or in initiating immune response activity). Not only have mAbs been used to discriminate cells during different stages of cell development, but have also assisted in understanding the dynamics of molecules expressed during functional processes. Such molecules detected on human leukocytes are called human leukocyte differentiation antigens or HLDA. In order to group the antibodies that detect similar molecules and have similar patterns of reaction, immunologists have organised the mAbs that bind to these antigens into Clusters of Differentiation (CD). So far, there are about 350 leukocyte surface molecules detected by mAbs with a CD nomenclature for human leukocytes (www.hcdm.org).

In fish immunology there is a great need to produce mAbs that are able to differentiate the various components of the fish immune system to assist in the elucidation of the fish immune system. The present study was an endeavour to develop and characterise mAbs that could be accredited to such scheme. A better understanding of the fish immune system is urgently required so that effective strategies of control can be developed for significant diseases during fish farming.

Monoclonal antibodies were prepared by immunizing mice with thymic leukocytes from rainbow trout. The leukocytes were activated with the lectin Concanavalin A to promote the activation and proliferation of the target T-cell population. The selection of clones producing antibodies during screening was performed on the basis of the response of the supernatant from hybridomas using three consecutive assays. First, selection was determined by the positive staining of cells from the thymus in a Dot blot assay. Secondary screening was performed by means of flow cytometry (FCM) and the criterion for selection was the preferential detection of leukocytes gated in the lymphocyte region. Finally, the positive supernatants from hybridomes were evaluated to determine their effectiveness in the detection of modifications in the labelled cells during a multiple way activation by detection of foreign histocompatibility complex enhanced with mitogens.

Monoclonal antibody TcOm15 was selected from 564 hybridomas produced and then used to stain cells from various Rainbow Trout tissues. It was clear from FCM, microscopy and Western blot analysis that mAb TcOm15 not only reacted with thymic cells

but also with cells from other tissues. Differential staining of cells with mAb TcOm15 was observed with 27.1 ±1.4 % of leukocytes from peripheral blood leukocytes (PBL) stained in comparison to 2.0 ±0.2 % from the thymus, 13.8 ±0.4 % from the spleen, and 5.6 ±0.6 % cells stained from head kidney. The labeled cells showed characteristics of lymphocytes and monocytes, presenting a distinctive staining in immunohistochemistry and confocal microscopy.

Western blot analysis, using electrophoresed proteins under denaturing conditions with leukocytes from several different tissues, showed that mAb TcOm15 did not detect a single protein. At least three proteins appeared to be identified by the mAb at 105, 160 and 200 kDa. The proteins were identified as α Actinin-4, non-erythroid Spectrin αII chain or Ig-like protein and non-muscle Myosin (MYH10) by MALDI-TOF analysis. Three of these identities are for compositional molecules for the cytoskeleton of different types of cells, and one it is associated to immunoglobulin superfamily. The identification of these proteins by mAb TcOm15 suggests an ability of this mAb to detect a specific function, possibly related with the synchronicity of expression or interaction of cytoskeleton-membrane proteins forming a multiprotein complex. Another possibility is as a carrier role for a protein during interactions.

Colocalization of the mAb with F actin from the cytoskeleton was also observed suggesting the possibility that mAb TcOm15 detects a specific site in a multi-protein complex from the cytoskeleton. The molecule detected showed down-regulation in a dose dependant way with Concanavalin A and the expression was almost lost following stimulation of cells with phorbol 12-myristate 13-acetate stimulation. Leukocytes from the PBL and thymus up-regulated the expression of the TcOm15 molecule under mitogenic conditions *in vitro*, and results from *in vivo* experiments suggested the possibility of up-regulation on thymic cells.

In conclusion, the results obtained in the present study provide information on a potentially useful marker (mAb TcOm15) for a cytoskeleton-membrane antigen that is modulated during stimulation of teleost lymphocytes. Additionally, this may enable insights into the relationship between cytoskeletal proteins and membrane associated immunoglobulin. Future research is necessary in order to explain this relationship and to determine the functional participation of the TcOm15 molecule during the activation of rainbow trout cells.

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Chapter 1 General Introduction

1.1 Overview

Aquaculture production has expanded during recent years as a result of increased food demands. Given the artificial conditions under which fish are cultivated for this purpose, the organisms suffer a number of physiological constraints that result in regular infectious disease outbreaks. Therefore, one of the major concerns and a significant investment cost to the industry is related to preserving the health of the fish stocks. Avoidance of stress, immunostimulation and vaccination (among others) play an important part in the biosecurity programs used to help prevent the negative impacts of infective organisms in fish rearing systems (Yoshimizu, 2003; Pruder, 2004). In order to implement effective disease control strategies, efforts are being made to develop ways of enhancing the processes that enable the fish to limit or eliminate the infectious agents. The immune response is the process by which organisms, including fish, preserve homeostasis and defend themselves against disease. However, practical approaches to improve the health management of fish require fundamental knowledge on the immune response of fish (Siwicki *et al.,* 1994; Lorenzen *et al.,* 2002; Scapigliati *et al.,* 2002; Moore & Hawke, 2004). There is therefore great interest in developing immunological techniques and tools to elucidate the immune system of fish and establish methods for modulating the immune response of fish biology. Not only the health of fish under culture would benefit from studies of immune response, but also in general the knowledge gained will improve the understanding of vertebrates. Teleost are organisms that share characteristics with major vertebrates and as experimental organisms are easy to manage in practical terms, making fish good model organisms.

From a comparative immunology perspective, the work performed on this subject could contribute with information about fish that can be applied to major invertebrates (Trede *et al.,* 2004).

1.2 Understanding the teleost immune system

Immunology is a complex subject continuously being enriched with new information. Experimentation in this field is frequently being redesigned based on these new findings, and new hypotheses to explain how the immune system functions in mammals, are presented and modified accordingly (Matzinger, 1994; Medzhitov & Janeway, 2000). Teleost immunologists have applied these novel results to fish immunology based on the evolutionary relationship between fish and mammals, but have done so with caution (Magor & Magor, 2001; Whyte, 2007), and in general have focused on generating information by comparing analogous immune response mechanisms between fish and mammals (Dixon & Stet, 2001; Magor & Magor, 2001; Whyte, 2007). The immunological response is a complex interaction of mechanisms that have evolved in invertebrate and vertebrate organisms to preserve homeostasis. Some aspects of this defence system are triggered by the detection of foreign patterned molecules, or danger signals. The processes activated in response to these are divided, artificially, by researchers into innate (unspecific or natural) and adaptive (specific or acquired) (Table. 1.1). However, these are not separate events but a group of effectors interacting to communicate danger and defend according to the insult (Matzinger, 1994; Dixon & Stet, 2001; Janeway & Medzhitov, 2002).

Table 1.1 Cells and molecules of the adaptative and innate arms of vertebrate immune systems (modified from Bayne & Gerwick, 2001, page 726).

	ADAPTIVE	*INNATE*
Tissues	Lymphoid and mucosal	Skin, liver, spleen, pronephros
Cells	T, B cells, APC	B cells, NK cells, monocytes/macrophages, granulocytes
Regulators	Cytokines	Cytokines/kemokines
Humoral components	Igs	Complement system, clotting system, anti-proteases, metal-binding proteins, lectins, lysozymes, antimicrobial peptides, opsonins
Kinetics	Slow	Fast

The innate immune responses are preserved through evolution from invertebrates to vertebrates, with the gnathostomata being the first class to have developed not just innate mechanisms but also acquired mechanisms too. The immune responses of all jawed organisms show a highly conserved innate system, ability to rearrange genes for an adaptive immune function, and immunity with interacting unspecific and specific effectors. Through years of research it is known that the teleosts are capable of mounting immune responses (analogues and/or homologues) similar to mammals (with highly specialised adaptive immunity), however most of the processes are not well understood (Figure 1.1) (Dixon & Stet, 2001; Du Pasquier, 2001; Bowden *et al.,* 2005).

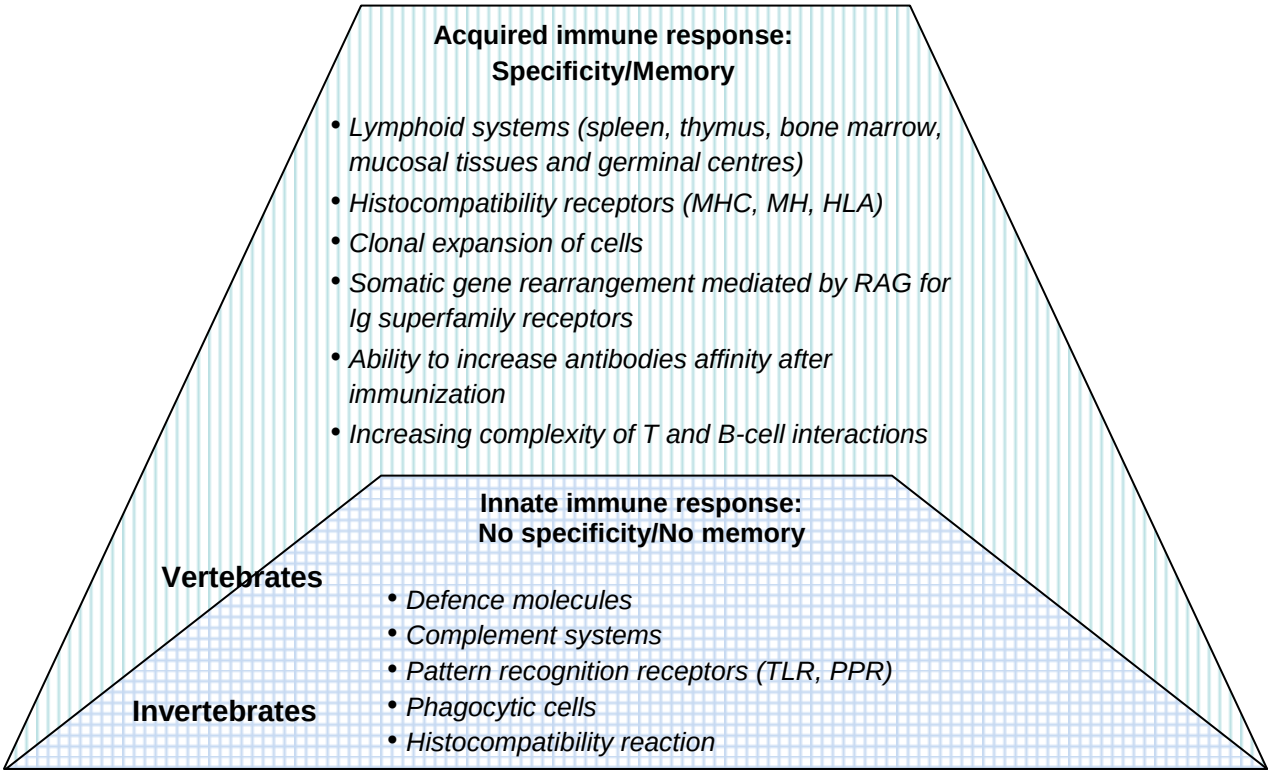


Figure 1.1 Innate and acquired immunity through the animal kingdom (based on Tort, Balasch & Mackenzie, 2003; Flajnik & Du Pasquier, 2004; Danilova, 2006).

2.1.1 The innate immune response

Invertebrate and vertebrate animals are born with a series of immune capabilities that enable them to react immediately against invading pathogens or other damaging processes. These inborn defences are the first line of attack, be it by anatomical, humoral or cellular means, and are characterised by protecting non-specifically, *i.e.* recognising pathogens or signals of danger (pathogen and stress related molecules) with no differentiation between the source of danger.

2.1.1.1 **Enhancing the attack**

Physical barriers such as skin, scales, gastro-intestinal villi, and peristalsis are essential to hinder pathogen penetration as they offer a mechanical deterrent to the

entry of invading microbes and toxins. These external tissues are covered with a glycoprotein containing a mucus layer that blocks the adherence of microorganisms by physically trapping them and preventing them from penetrating their host. In addition, mucus contains defence molecules such as lectins, pentraxins, lysozymes, anti-bacterial peptides, complement proteins, and natural antibodies that neutralise microbial enzymes, suppress growth or directly kill the microorganisms (Ellis, 2001; Saeij *et al.,* 2002). If the micro-organisms overcome the external barriers then they must confront the humoral and cellular defensive mechanisms (both innate and adaptive) of the immune system.

2.1.1.2 **Detecting invasion**

According to Medzhitov & Janeway (1997), based on studies with mammals, the signals that are detected as a threat or danger are molecules intrinsic and physiologically necessary for the invading organisms to infect, but which are not made by the host organism themselves. These molecules are conserved between groups of related microbes; *e.g.* teichoic acid in Gram negative bacteria. These molecules are not recognised by receptors as a specific structure, but rather as a patterned molecule (pathogen-associated molecule pattern, PAMP). The response against the threat is initiated by the detection of PAMPs by proteins that are able to function as recognition receptors (pattern-recognition receptors, PRRs) and have broad specificity. Depending on the type, PRRs can be expressed externally (cell surface), internally (cytoplasm) or secreted (mucus, serum, *etc*) (Magnadottir, 2006).

Another function is attributed to the recognition receptors (PRR's) in mammals, and this is related to Anderson-Matzinger's hypothesis (2000) by which PRRs also have the ability to detect self-molecules synthesised and/or released by stressed or injured

cells (heat shock proteins, double stranded DNA, *etc*). The release of self-molecules is a sign of danger that is encountered by the PRRs only when there is a cellular damage. It has been observed in fish toxicology studies, that heat shock proteins are up-regulated and the expression of cytokines is affected when organisms are exposed to high levels of contaminants, indicating possible immuno-modulation of the fish through these pathways (Eder *et al.,* 2008). As stated by Wallin *et al.* (2002) and Dixon and Stet (2001), the detection of pathogen molecules or danger related self-molecules by PRRs possibly act in synchronicity (Figure 1.2), although such mechanisms already confirmed in mammals still need to be established in fish.

2.1.1.3 **Receptors of danger**

During the innate immune response, the molecules that recognise danger or non-self structures are mediators with specific tasks such as opsonisation, activation of complement, phagocytosis, *etc*. This arbitration work is performed by different PRR´s with affinities for a broad range of PAMP´s *e.g.* nucleic acids, proteins, carbohydrates, compound molecules (glycolipids, phospholipids, peptidoglucan, lipopolysaccharides, lipopeptides) (Lata & Raghava, 2008). Distinct classes of PRR's are present in teleosts and every family has a function more and less conserved through evolution (Aoki *et al.,* 2008) (Table 1.2). Toll Like Receptors (TLRs) (Purcell *et al.,* 2006), NOD Like Receptors (Laing *et al.*, 2008) (NLRs), RIG-1 (retinoic acid-inducible gene)-Like proteins (RLRs) (Zou *et al.,* 2009), C-type Lectin Receptors (CLRs), Mannan Binding Lectins (MBLs) (Russell & Lumsden, 2005), and complement receptors (Boshra & Sunyer, 2006) have all been described for teleosts (Table 1.2). In mammals, it is known that PAMPs are recognised by PPRs and these start a flow of events through different receptors that finally prime cells to release mediators (*e.g.* cytokines) (Kawai & Akira,

2009). Sepulcre *et al.* (2007) demonstrated that this could occur in teleosts by showing that macrophages and granulocytes, after being primed by PAMPs, released pro-inflamatory cytokines. The assemblage of the response, through these PRRs, provides the starting point of inflammation (Kawai & Akira, 2009).

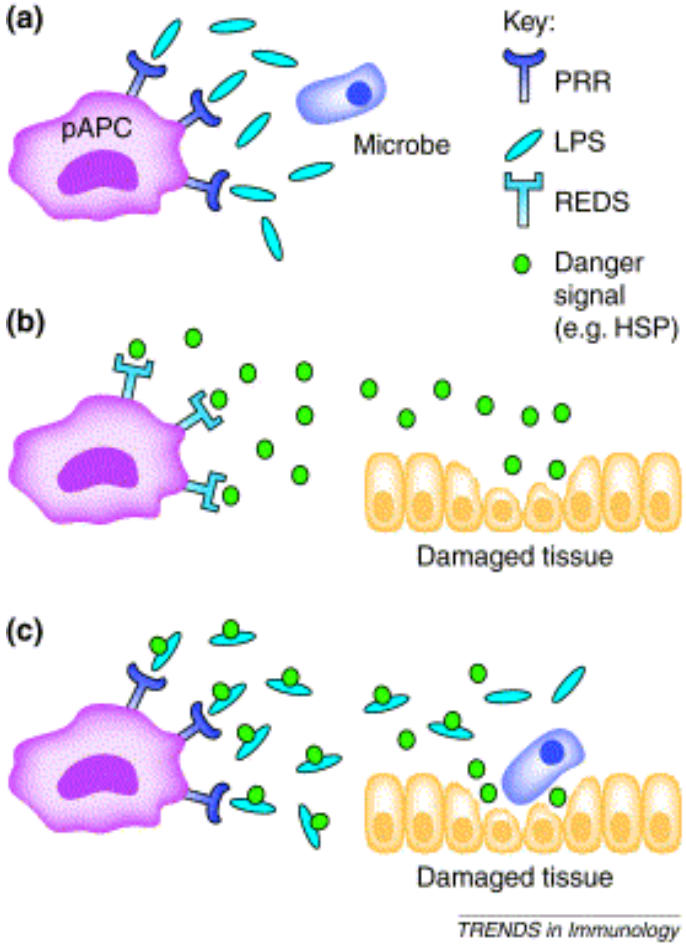


Figure 1.2 "Three models are shown that might explain how endogenous and/or microbial products activate professional antigen-presenting cells (pAPCs). (a) In the 'extended self, non-self theory' proposed by Janeway, microbial products are recognized by pAPCs expressing pattern-recognition receptors (PRRs). (b) In the 'danger theory' proposed by Matzinger, endogenous substances produced or released in response to tissue damage caused by microbes are recognized by pAPCs with receptors for endogenous danger signals (REDSs). (c) In a third model, there is the possibility of an overlap between these two models, such that stress-induced endogenous proteins form complexes with microbial products, and these complexes are recognized by pAPCs. Note that these models are not mutually exclusive. Abbreviations: HSP, heat-shock protein; LPS, lipopolysaccharide". From (Wallin *et al.,* 2002, page 133)

Table 1.2 Pattern-recognition receptors found in mammals and some identified in teleosts (from Aoki *et al.*, 2008; page 266).

Family	Function/Structure	Taxon	Member	Source
TLRs	Pathogen associated molecular patterns (PAMPs) recognition on the cellular membrane. Extarcellular leucin-rich repeats (LRRs) and intracellular Toll/IL-1 receptor (TIR) domain.	Mammals	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, LTR12, TLR13	Lee and Kim 2007; Roach *et al.* 2005 Jault C, *et al.*, 2004
		Teleost Fish	TLR1*, TLR2, TLR3, TLR4*(Z), TLR5, soluble TLR5, TLR7*, TLR8*, TLR9, TLR14 (P), TLR21*, TLR22, TLR23 (P)	
Lectins	Involved in the carbohydrates recognition. Secretion form and membrane form are exist, and generally function as multimeric structure.	Mammals	Mannose binding lectin (MBL), Dectin-1#, Intelectin, Pentraxins	Lee and Kim 2007; Russell and Lumsden
		Teleost Fish	MBL, Intelectin, Pentraxins	
Complement receptors (CRs)	Involved in the leukocytes activation and migration.	Mammals	CR3 and CR4 (integlins), C5aR	Lee and Kim 2007; Boshra H, *et al.* 2004 Qian Y *et al.* 1999
		Teleost Fish	CD18 (β chain for the CR3 and CR4), C5aR	
NLRs	Involved in the PAMPs recognition in the cytoplasm. The receptors posses the centeral nucleotide oligomerization domain (NOD), caspase activation and recruitment domain (CARD) and LLRs.	Mammals	NOD1, NOD2, NALPs, IPAF, NAIP5	Lee and Kim 2007; Stein *et al.* 2007
		Teleost Fish	NOD1, NOD2	
CARD helicases	Implicated in the cytoplasmic detection of viruses. The molecules possess the RNA helicase activity in its C-terminal portion and CARD.	Mammals	Retinoic acid-inducible gene I (RIG-I), melanoma-differentiation-associated gene 5 (MDA5)	Lee and Kim 2007
		Teleost Fish	Unknown	

*Isotypes were reported.
(Z) Reported from only the zebrafish.
(P) Reported from only the pufferfish.
Only dectin-1 is a lectin that be able to mediate the intracellular signaling directly after the ligand recognition.

2.1.1.4 **Coordinating the acute phase response**

The unspecific (or non-specific) components of the immune system that provide a rapid reaction to aggression can be humoral or cellular. These components directly attend the site of the insult, but also provide instructions to initiate the adaptive immune system (Kawai & Akira, 2009). Physiological and metabolic changes take place when aggression is incurred to the organism; foreign or self molecules present at the wound site can stimulate secretion of pro-inflamatory cytokines, chemokines, and lipid mediators that initiate a group of processes called the acute phase response (APR) (Bayne & Gerwick, 2001).

The initial phase during inflammation occurs by a rapid alteration of the homeostasis of the organisms. Humoral mediator molecules are secreted or released with the objective of modifying vascularisation, to facilitate the aggregation and activation of haematopoietic cells, eliminate the stimulus, clear away debris and restore normal structure and functions. Central nervous system, liver, and several populations of cells are stimulated to up-regulate or down-regulate secretion of proteins during APR. The secreted proteins that eventually increase (positive) or decrease (negative) with respect to normal plasmatic levels, are specific for the acute phase response and are called acute phase proteins (APP) (Bayne & Gerwick, 2001; Russell & Lumsden, 2005). Cellular participation during APR is promoted by the release of mediators such as cytokines and chemokines, and is required to remove foreign particles or microorganisms and dead cells through phagocytosis (Afonso *et al.,* 1998; Grayfer & Belosevic, 2009) (Figure 1.3).

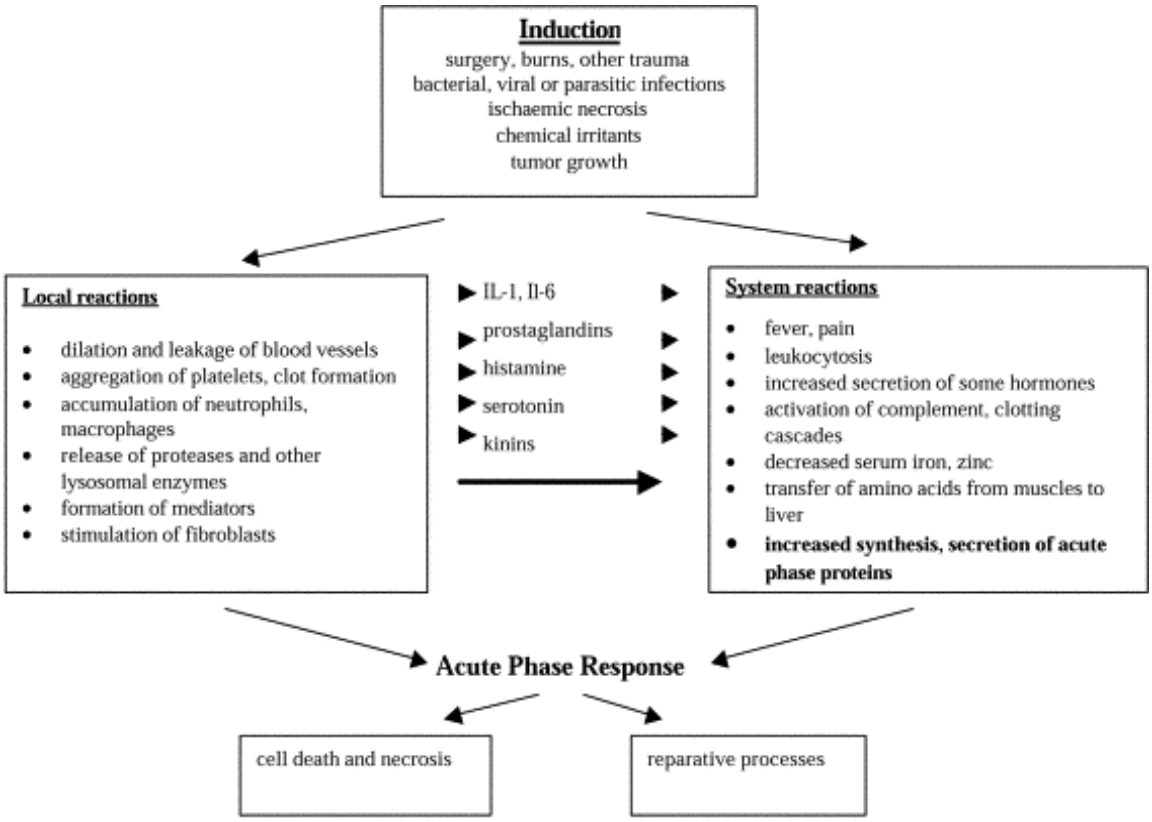


Figure 1.3 "Diagram illustrating phenomena that induce an acute phase response in mammals, their immediate effects**, and the mediators that are produced and that induce systemic reactions" (from Bayne & Gerwick, 2001 page 727).**

a) Soluble mediators

Soluble factors called cytokines are the mediators of cell communication that act in a paracrine or autocrine manner in both the innate and the specific immune responses (Secombes *et al.,* 1997). For humans, cytokines are a large family of proteins that can activate, inhibit or regulate immune function. Cytokines can be categorised into separate functions mainly during the innate response, the adaptive response or haematopoiesis, however it has been shown that there is no clear division in these categories (Kabelitz & Medzhitov, 2007). So far, the cytokines that intervene in the innate immune response of fish are similar to those described for humans. There are molecules described for interleukin (IL)-1beta, tumor necrosis factor alpha (TNF-

alpha, interleukin 18 (IL-18) and chemokines such as IL-8 (Secombes *et al.,* 2001; Zou *et al.,* 2002), and MacKenzie *et al.* (2004) for instance, have found that TNF-alpha plays a similar role in the fish's immune response to that seen in mammals. From the results of their experiments, they suggest that TNF-alpha is secreted by terminally differentiated macrophages, and that this cytokine has an important function as an activator of their phagocytic activity. However, while the activity of most cytokines is known in mammals, this information is still unclear for fish and dependent on the availability of recombinant peptides.

b) APPs in teleosts

The pentraxins, C-reactive protein-like (CRP), and serum amyloid P-like (SAP) proteins have been detected in several species of teleosts. Pentraxin-like proteins from fish have shown similar behaviour to those in mammals where an increase is detected during inflammation (positive APP). Just recently, genes from these pentraxin-like proteins were found to be up-regulated during infection by *Yersinia ruckeri* induced in *O. mykiss* (Raida & Buckman, 2009). These data, together with findings of agglutination and affinity to certain types of molecules from pathogens, suggest that CRP and SAP-like proteins are important mediators of the immune response of fish as agglutinins and secreted PRRs (Bayne & Gerwick, 2001; Cook *et al.,* 2003).

The non pentraxin serum amyloid A (SAA) is a common protein in APR for vertebrates. However, different responses have been found in fish making it difficult to relate the SAA to the APR (Raida & Buchman, 2009; Villarroel *et al.,* 2008).

Transferrin is a glycoprotein that binds iron and can sequester it when environment levels are low; where bacteria find it difficult to survive at these low iron

levels. The level of transferrin in APR decreases in mammals, however, in contrast it has been found that in rainbow trout and catfish, levels of transferrin may increase during APR (Bayne & Gerwick, 2001, Peatman *et al.,* 2007).

The plasma protein α2-macroglobulin modulates the immune response by inhibiting or inactivating proteases. Peatman *et al.* (2007) found this molecule amongst the up regulated genes in catfish during infection.

The complement system consists of 35 soluble and membrane bound proteins. Complement participates in the elimination of pathogens by creating pores in the membrane of the microbe or by facilitating phagocytosis through opsonisation of the microbe. Characteristically it produces an amplified response when triggered by a stimulus producing a cascade phenomenon. The complement cascade can be activated by three pathways: the classical pathway, which is stimulated by the binding of antibodies to the microbe membrane, acute phase proteins or directly by viruses, bacteria or infected cells. The alternative pathway is activated through contact with microorganisms independent of antibodies, and the lectin pathway is initiated by binding to a mannose-binding lectin. It is known that the main features of complement activation in fish occur in a similar manner to that of mammals, however, the mechanisms and molecules for some modes of activation are not clearly defined (Holland & Lambris, 2002; Boshra, Li & Sunyer, 2006). Some important components of the complement system have been identified as positive APP (Bayne & Gerwick, 2001, Peatman *et al.,* 2007).

Lysozymes are enzymes that occur not only in mucus secretions, but also in plasma, peritoneal macrophages and blood neutrophils. Bacterial cell walls are attacked by lysozymes by cleaving peptidoglucans present in the bacterial cell call (Bayne &

Gerwick, 2001). These molecules have been the centre of various studies with an increase in the plasma concentration detected during inflammation (Magnadottir *et al.,* 2005; Mackenzie *et al.,* 2008)

c) Phagocytic cells

The ability of granulocyte to phagocytose *in vitro* and *in vivo* has also been at the centre of many studies. Migration and phagocytic cellular activity have been observed during experimental inflammation in several fish species (Do Vale *et al.,* 2002; Pressley *et al.,* 2005; Serada *et al.*, 2005; Chaves-Pozo *et al.,* 2005; Cuesta *et al.,* 2007 & 2008). The identification of cells involved early during inflammation has been difficult due to a lack of specific cell markers; even so, populations of phagocytic cells have been identified such as monocytes, macrophages, granulocytes, and more recently B-cells (Li *et al.*, 2006; Overland *et al.,* 2010). For instance Sepulcre *et al.* (2007) demonstrated that acidophilic granulocytes and macrophages express PRRs and are stimulated during inflammation, showing active phagocytosis (neutrophils) and/or release of cytokines. Another interesting observation was the phagocytic behaviour seen for B-cells of salmon, cod and trout indicating the participation of this population during the innate immune response (Li *et al.*, 2006; Overland *et al.,* 2010). Phagocytes have an important role in the defence mechanism of fish. These are cells that engulf microbes and kill them through the production of potent bactericidal substances. These products include reactive oxygen species (ROS) such as superoxide anion, H_2O_2 and the hydroxyl free radicals (OH^+). In addition, macrophages can produce nitric oxide (NO) and subsequent peroxynitrites and hydroxyl ions (Ellis, 2001; Neuman *et al.,* 2001).

2.1.2 Specific immunity

The specific immune response provides strong protection against both extracellular and intracellular pathogens. These mechanisms are characterised by two important features: specificity and memory. The components of the specific or adaptive immune response include both humoral and cellular elements (Table 1.1). Specificity and memory are adaptations to the immune system that are obtained after the first encounter with the pathogen.

As in mammals, macrophages and monocytes have the ability to present antigens to specialized cells for the initial propagation of the specific immune response (Vallejo *et al*., 1992, Sugamata *et al.,* 2009). Specialised cells have been observed in fish, B cells responsible for antibody production (Manning & Nakanishi, 1997) and T cells that mediate cellular activity demonstrated both *in vivo* (Abelli *et al.,* 1999) through graft recognition and *in vitro* (Meloni *et al.,* 2006) by T cell proliferation during cytotoxity assays. Similarly molecules related with specific immunity have also been discovered in fish and these are mentioned later.

Antibodies in fish are immunoglobulins (Ig) that consists of heavy (H) and light (L) chains with a tetrameric structure; it is usually referred to as IgM. Like mammals, fish have the ability to develop immunological memory, following the first encounter with the antigen; the B memory cells retain the ability to be re-stimulated by the antigen. A secondary antibody response in fish is faster and results in higher Ig titres in the serum, than the primary response (Kaattari & Piganelli, 1997, Manning & Nakanishi, 1997). B cells participate in the immune defence process through the production of humoral antibodies and T cells directly participating by specifically recognising pathogen peptides processed by exogenous and endogenous pathways.

Thymus derived cells or T cells, secrete a membrane heterodimer Ig-like molecule called the T-cell receptor (TcR) that is linked to the major histocompatibility complex (MHC) and CD3 complexes, and mediates the immune response towards the specific cellular recognition of antigens. The immunocompetent cell subpopulations can be differentiated by CD4 and CD8 markers. Helper T-cells ($CD4^+$) participate by recognising exogenous antigens presented by the MHC II complex, releasing cytokines, and promoting the activation and maturation of cytotoxic T-cells and macrophages. Cytotoxic T-cells ($CD8^+$) participate directly by destroying infected cells when it is ligated to the peptide-MHC I complex, and releasing cytokines (Roitt, 1997). For teleost so far, genes of the polymorphic (MHC) molecules class I and II have been cloned and sequenced in a number of fish species as well as T cell receptor (TcR) homologues (Rast & Litman 1994; Koppang *et al.,* 2003; Dijikstra *et al.,* 2001; Nakanishi *et al.,* 2002; Dixon *et al.,* 2003). Added to that, detection of RAG (recombination activating genes) has demonstrated that recombination activity in receptors of mature B and T cells of teleosts occurs (Wienholds *et al.,* 2002; Huttenhuis *et al.,* 2005). Furthermore, molecules such as CD4 and CD8 markers from specific immunity have been described allowing differentiation of helper and cytotoxic T cells in teleost (Picchietti *et al.*, 2009; Shibasaki *et al.* 2010).

2.1.3 Cellular activation

In humans, cellular activation processes are initiated by signal to cell (*e.g.* cytokines-macrophage) or cell to cell interactions (*e.g.* TcR-APC) where particular membrane locations participate during cellular communication. At this leukocyte surface site where the interaction occurs a variety of receptors and ligands take part (*e.g.* TcR-MHC) depending on the identity of the participating cells (Moser 2003; Smith-

Garvin, Koretzky & Jordan, 2009). So far, there has research in the ways in which this interaction occurs, even so many gaps that clarify the links between signalling molecules and cellular outcomes still exist. Activation occurs in several pathways where the initial triggering stimulates up/downregulation of participating proteins expression. Such proteins may be involved in signal transduction to change cell function, mediation to definite this function, and cytoskeletal rearrangement of a signalling zone to maintain or improve the cellular function (Smith-Garvin, Koretzky & Jordan, 2009*,* 2009). For teleosts, cellular activation has been mostly studied in terms of the initial stimulus and final outcome of cell, but specific pathways and involved molecules are fairly well understood due the lack of tools for labelling cells (Burnet *et al.,* 2000; Verjan *et al.,* 2008).

2.1.4 Markers

In teleost immunology, mAbs recognising known surface molecules *e.g* the CD3 marker for the TcR, or other molecules distinguishing between cell phenotypes or the functional state of T-cell populations, do not exist to nearly the same extend as those available for mammalian immunology (Table 1.3). Consequently, the existence of teleost cell subpopulations, such as CD4 and CD8 cells, has been deduced from indirect results and evidence of their functional activity (Iwama & Nakanishi, 1997; Partula, 1999; Romano *et al.,* 2005). In 1999, Scapigliati *et al.* (page 14) stated that "few monoclonal antibodies (mAb) can be considered good T-cell markers", and more than ten years later the situation is still nearly the same, although some results based on molecular approaches to developing markers have been added to the list of mAbs (Fisher *et al.,* 2007). For human and murine cells, there are nearly 150 recognized membrane proteins to discriminate between cellular populations. Rombout *et al.* (1997)

found that from 469 clones obtained from one mouse immunised with membrane lysates (from carp *Cyprinus carpio*) only 18 detected thymocytes, and after the screening with flow cytometry just one (mAb WCL9) stained live thymocytes. However, this mAb developed against carp thymocytes reacted with a population of thymocytes (30-50 %) and did not recognise peripheral blood cells. In another attempt to produce mAbs against T-cells, Rombout *et al.* (1998) found that one mAb out of 230 (WCL38) showed an affinity for intra- epithelial cells (IEL, apparently rich with T-cells), the source of the antigens utilised for immunising the mouse. This mAb (WCL38) recognised 50-70 % of the IEL and gill lymphoid population, less than 6 % and 3 % of cells from thymus and spleen respectively, and only a few cells in peripheral blood leukocytes (PBLs).

Immunological studies to understand different immune processes in humans and mice have been greatly enhanced by the use of mAbs that recognise specific cell markers. The persistent lack of cell markers for teleosts has greatly hampered the development of more advanced immunological studies for teleosts; as these are especially important for understanding the dynamics of the fish immune system against infectious pathogens. This is quite a different situation from the knowledge acquired for human immunology, facilitated by over 150 markers used in the CD nomenclature to characterise epitopes of leukocytes (Fisher *et al.,* 2007).

Table 1.3 Monoclonal antibodies developed against teleost leukocytes.

Fish species	Immunisation antigen	Specificity	Screening	References
Carp	Thymocytes	Other leukocytes		Secombes *et al*., 1983
Cyprinus carpio	Thymocytes	Early thymocytes	ELISA, thymocytes, cytotoxic inhibition, FCM	Yamaguchi *et al.*, 1996
	Thymocyte plasma membranes	Majority of leukocytes	FCM, Leukocytes, , PHA enriched stimulated cells	Rombout *et al.,* 1997
	Intraepithelial leukocytes membranes	Mucosal T-cells	FCM	Rombout *et al.,* 1998
		Thrombocytes		Nakayasu *et al.,* 1998
		Macrophages		Romano *et al.,* 1997
	PBL	Phagocytes		Nakayasu *et al.*, 1997
Catfish	Thymocytes and Ig-negative PBL	35 kDa antigen		Miller *et al.,* 1987
Ictalurus punctatus	Thymocytes and peripheral T-cells	Not T cell-specific	FCM, mitogen stimulated lymphocytes	Passer *et al.*, 1996
	NK-like cells			Evans *et al.,* 1988
	Thrombocytes			Passer *et al.*, 1997
	Granulocytes			Bly *et al.,* 1990
				Ainsworth *et al.,* 1990
Sea bass *Dicentrarchus labrax*	Thymocytes	Fixed thymocytes, peripheral T-cells	FCM, IIF,	Scapigliati *et al.*, 1995
Yellowtail *Seriola quinqueradiata*	Thymocytes	Majority of leukocytes		Nishimura *et al.,* 1997
Rainbow trout	TcR DNA constructs	Cross-reaction, non-T	ELISA, FCM,	Timmusk *et al.,* 2003

Oncorhynchus mykiss	Synthetic peptides, TcR sequence	cells	leukocytes, recombinant proteins	Jansson *et al.,* 2003
	Thymocytes, enriched Ig-cells	Thrombocytes	ELISA, FCM, peptides, leukocytes	Slierendrecht *et al.,* 1995 Hill and Rowley 1998 Kollner *et al.,* 2004
		Granulocytes Macrophages		Slierendrecht *et al.,* 1995 Kuroda *et al.,* 2000 Koellner *et al.,* 2001
Atlantic salmon *Salmo salar*		Granulocytes		Pettersen *et al.,* 1995 Hamdani *et al.,* 1998
Hybrid catfish *Pseudoplatystoma corruscans x P. fasciatum*	Thymocytes and thymocyte cytoplasmic membranes	Lymphoid like cells	ELISA, FCM, cells, FITC differential,	Beelen *et al*., 2004
Fugu *Takifugu rubripes*	(polyclonal) cDNA fragment encoding fugu CD8α	Lymphocytes, thrombocytes, and monocytes/macrophages	Mitogen stimulated cells, FCM,	Araki *et al.,* 2008
Several species	B cells	IgM		Raison & Hidelman, 1984 Estevez *et al.,* 1994 van der Heijen *et al.,* 1995 Scapigliati *et al.,*1996, Uchida *et al.,* 2000 Stenvik *et al.,* 2001 Morrison *et al.,* 2002 Li *et al.,* 2007 Rathore *et al.,* 2008

Compared with bovine, equine or swine species, research in fish immunology has experienced significant achievements in a relatively short time, partly because of the increasing importance of aquaculture as a source of food. There are now several successful vaccines and immunostimulants available to boost the fish immune system and increase the immunological processes to combat invading pathogens. However, for the majority, the mechanisms by which these enhance the immunity of fish are not very well understood due to limited knowledge about the fish immune system (Gudding, Lillehaug & Evensen, 1999). One of the main areas of interest is the detection, functional differentiation and organization of the cellular populations involved in the host response to infectious pathogens. In human and murine immunology, specific immunocompetent subpopulations have been identified as collaborators or direct executors of pathogen elimination by exogenous and endogenous pathways. However, for teleosts this distinctive functional participation has not been probed given a lack of mAbs to mark cellular subpopulations (Somamoto, Nakanishi, & Okamoto, 2002). Significant efforts in teleost immunology research have focused on the development of these markers, although these are few currently available. Such mAbs against the cells involved in the immune process would permit their detection, isolation, and/or discrimination to better develop and comprehend functional studies *in vivo* and *in vitro*.

The present project, as a part of this effort to increase the number of fish cell markers, aimed to obtain a mAb to assist in the characterisation of thymus derived cell populations from rainbow trout (*Oncorhynchus mykiss*).

Aims of the study:

The aim of the present study was to identify surface molecules associated with the phenotype of leukocytes that contributes to the understanding of teleost cellular function. This was investigated by using T-cell mitogen activation to up regulate the molecules that assist such a phenotype or functional differentiation of teleost cells, and to search possible methods that allow evaluation of the participation of surface molecules in cellular activation.

Chapter 3 Development of Monoclonal Antibodies (mAbs) Against Thymic Cells of Rainbow Trout

3.1 Introduction

A great deal of effort has been made to generate mAbs against surface epitopes for differentiation between leukocyte populations of teleost immune cells. A variety of approaches have been used by researchers to develop mAbs against these cells. However, attempts to make these mAbs have been hampered by the high inter and intra-specific genetic heterogeneity between fish (Miller *et al.*, 2001). Over 28 publications have been produced focusing on the development of specific markers for immunocompetent cells and components of the immune response of fish (not including IgM´s from B-cells), based on traditional mAb technology (Table 1.3, Chapter 1). However, only a few of these have provided convincing evidence that specific populations of cells are recognised by these mAbs (Randelli *et al.*, 2008). The few reports of markers raised against teleost thymocytes include mAb DLT15 (Sea Bass, *Dicentrarchus labrax*) (Scapigliati *et al.,* 1995), CfT1 (channel catfish, *Ictalurus punctatus*) (Passer *et al.,* 1996), WCL9 (Carp, *Cyprinus carpio*) (Rombout *et al.,* 1997), and PST 33 (hybrid surubim catfish, *Pseudoplatystoma corruscans* x *P. fasciatum*) (Beelen *et al.*, 2004).

A number of points need to be considered regarding the methodology used to develop mAbs suitable for identifying cell markers in fish. Of particular note are the immunisation and screening protocols used to produce the mAbs. The type of antigens used for immunising mice varies greatly between studies from simple to complex preparations of cells. Whole thymic cells (Secombes *et al.,* 1983; Yamaguchi *et al.,* 1996;

Nishimura *et al.,* 1995), thymocyte plasma membranes (Rombout *et al.,* 1997), and thymocytes enriched with Ig^{-ve} cells isolated from peripheral blood leukocytes (PBLs) (Passer, 1996) have all been used to immunise mice. Effort has been made to maintain the antigenic repertoire of these cell preparations in their native form (*i.e.* by using unfixed cells). These preparations generally include the isolation, and enrichment of leukocytes, and lysing with mild detergents (*e.g.* Passer *et al.* 1996). Inactive cells, cell membranes and/or lysates are then mixed with adjuvants to enhance the immune response of the mouse to the antigens. Scapigliati *et al.* (1995), however, used a preparation of whole thymocytes fixed with 2 % para-formaldehyde, mixed with adjuvant, to immunise the mouse. The process of fixing the cells may have denatured immunogenic antigens on the cells, however these researchers did successfully develop a mAb from their fusion that was able to recognise a T-cell population in Sea Sass, using either live or fixed cells (*i.e.* mAb DLT15). More recently, advances in genetic technology have been employed to immunise mice. DNA constructs containing the gene for the α chain of the TcR have been used to vaccinate mice (Timmusk *et al.*, 2003), while synthetic peptides prepared for the α and β chain gene sequences of the TcR were used by Jansson *et al.*, (2003). The aim of their studies was to immunise the mice with specific antigens in the hope of producing antibodies against the TcR portion on the T-cell. However, given the divergence of the gene for TcR found in mammals (Jansson *et al.*, 2003), the antibodies produced in these studies were not specific for the TcR of teleost.

It has been shown through years of producing mAbs against mammalian leukocytes that the specificity and applicability of the mAbs produced depends greatly on the methodology used to screen and select the mAbs during their production

(Vollmers *et al.*, 1998). A diverse range of immunological assays have been used to screen the mAbs produced against fish thymocytes (Scapigliati, Romano & Abelli, 1999; Randelli, Buonocore & Scapigliati, 2008). For example, enzyme linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF), flow cytometry (FCM), immunohistochemistry (IHC) and mitogenic simulation have all been used in the selection of potential hybridomas and to help in their subsequent characterisation.

The use of ELISA for the initial screening of the fusion allows a large number of samples to be tested. Lysates, recombinant proteins and peptides, used to immunise the mice, have then been used to coat ELISA plates for screening the fusion (Yamaguchi *et al.*, 1996; Jansson *et al.*, 2003; Timmusk *et al.*, 2003). Other methods, such as IHC and IIF, are particularly useful for characterising mAbs of interest. The basis of these methods is staining of cells so they can be visualised by microscopy or FCM, and they are thus helpful in the identification and characterisation of mAbs which recognised specific cell population and thus potential cell markers. By using these methods, it is possible to examine the distribution and levels of positively stained cells within different tissues. These are particularly useful in immunologically rich tissues such as peripheral blood, thymus, spleen, gut, epithelia, head kidney, gill, or skin where there is a source of leukocytes. For instance, Scapigliati *et al.* (1995) selected mAb DLT15 as a T-cell marker because it was able to stain living cells in FCM and also in fixed tissue sections by IHC. The results obtained by FCM clearly showed that thymocytes were specifically stained by mAb DLT15. Further characterisation of this mAb confirmed it's specificity for sea bass T-cells (Abelli *et al.*, 1999; Scapigliati *et al.,* 2000).

After screening by FCM, Passer *et al.* (1996) selected mAb CfT1 from its immunostaining pattern with catfish cells stimulated with mitogens against T or B-cells. They found an increase in staining by mAb CfT1$^+$ with cells stimulated with the T-cell mitogen, Concavalin A (Con A). In contrast, a decrease in the numbers of positively stained cells stimulated with the B-cell stimulator Lipopolysaccharide (LPS). So far, it has been possible to differentiate between four different leukocyte populations for rainbow trout using mAbs, *i.e.* B cells (Sanchez, *et al.* 1995), thrombocytes (Slierendrecht *et al.*, 1995; Hill & Rowley, 1998; Koellner, 2004) granulocytes (Slierendrecht *et al.,* 1995), and macrophages (Kuroda, Okamoto & Fukuda, 2000; Koellner, 2001). Although convincing results have been obtained using these markers, the exact identity of the epitopes labelled with the mAbs is still unknown; except for Igs on B cells (see Chapter 1). Meloni *et al.* (2006) were able to examine the role of T-cells activated by non-histocompatible recognition during a mixed leukocyte reactions (MLR) for sea bass using one of the mAbs they developed against T-cells. Most of the studies developing mAbs against fish leukocytes have used antigens either present on cells (*i.e.* whole cell preparations) or as molecules purified from unstimulated cells (*i.e.* using cells under static cellular conditions). Given the fact that the molecules were isolated, synthesised, or present on unstimulated cells, they do not necessarily reflect dynamic cellular function. An alternative strategy for developing mAbs against teleost thymocytes is presented in the current chapter, which was to screen hybridoma supernatants with cells in a dynamic state *i.e.* responding to a stimulus. This approach was used in an attempt to produce mAbs against functional cell surface antigens. It was thought that an assay that promotes T-cell functional display by detection of foreign MHC could be used to detect mAbs which reacted with

T-cell populations by FCM. The MLR is a commonly used method to evaluate the allogenic activation of immunocompetent cells *in vitro* against incompatible MHC molecules in graft studies (see Figure 2.1) (Sprent, 1998; Mak & Saunders, 2006). During a MLR, TcR on $CD4^+$ cells respond with an intense activation that induces proliferation and liberation of mediators *via* cross-linking with non-self MHC-II. Additionally, $CD8^+$ cells respond and are activated by directly recognising MHC-I in foreign cells by means of the TcR (Roitt, 1997).

An assay in which mixed allogenic PBLs are used in order to promote a leukocyte reaction has been employed by researchers to hypothesise the existence of T-cell subpopulations. This type of assay has allowed researchers to establish that $CD8^+$ and $CD4^+$ -like functions are present in teleosts, where the cells are capable of being activated, and respond to non-self molecules and undergo cellular proliferation (Miller *et al.,* 1987; Abelli *et al*., 1999; Meloni *et al.,* 2006; Utke *et al.,* 2007; Araki *et al.,* 2008; Moulana *et al*., 2008). In the present study, a histocompatibility reaction (or activation via foreign histocompatibility complexes, HCA) was chosen as a suitable test to help select positive hybridomas by detecting activated T-cells. It is known that there is a high degree of variability between individual fish in the ability of their cells to respond *in vitro* against allogeneic molecules or unkwon major histocompatibility complexes (Meloni *et al.,* 2006). It was therefore thought that to ensure the presence of responsive cells from at least one fish in the reaction, the PBLs from more than two fish should be mixed together. It was considered that the response of fish to the leukocytes of more than one fish in this multiple reaction would improve the overall numbers of $CD4^+$ and $CD8^+$ T-cells obtained for screening the hybridoma supernatants.

A modification was made to this multiple way HCA (mHCA). Mitogens were added to the reaction to enhance the "basal" activation of T-cells during the mHCA. The mitogen Con A, a lectin that promotes activation and proliferation of human T-cells (Sharon & Lis, 2004; Stone *et al.*, 2009) was added to the mHCA to "enhance" the basal levels of activation of the T-cell population already reacting in the mHCA. It was thought that this would help in the selection of hybridomas able to detect differences in the activated cells reacting in the mHCA, stimulated with the mitogen.

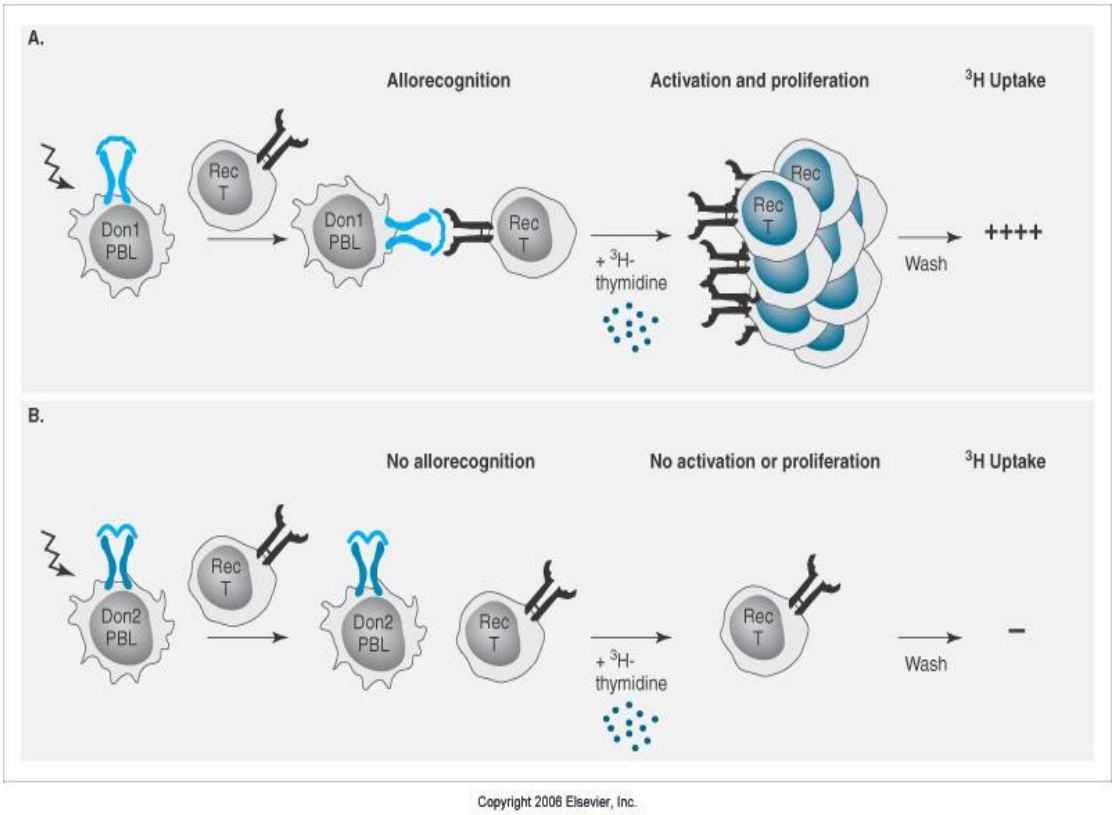


Figure 3.1 Mixed Lymphocyte Reaction (MLR). Example: screening of donor 1 and donor 2 for suitability as tissue in graft transplant. The donor "stimulator" PBLs are isolated and irradiated (jagged arrow), further the PBLs from the "responders"are mixed with recipients PBL. (A) T cells from the recipient are activated by allorecognition of MHC from donor 1 cells and incorporate [3 H]-thymidine into their DNA as they proliferate (high radioactivity). (B) The recipient T cells are not activated by donor 2 cells so there is minimal proliferation and incorporation of 3H-thymidine corresponding to a MHC matching. (From Mak & Saunders, 2006, page 893).

A method used to evaluate the histocompatibility is to assess the level of proliferation, detected by the incorporation of [^{3}H]-thymidine into the DNA of proliferating cells (Figure 2.1) However an alternative to this method can be FCM analysis to assess cell activation and proliferation, but relies on mAbs that react with cell markers for T-cells to do so. In an attempt to visualise differences on the detection ability of preliminary selected hybridomas after the cloning procedure, a final screening using FCM and stimulated cells was used.

Aim:

The aim of this Chapter was to generate antibodies against activated T-cell populations of rainbow trout obtained by immunising mice with whole thymocytes isolated from the thymus of rainbow trout. Positive hybridomas were initially selected by their ability to detect surface epitopes on thymic cells by Dot blot. A subsequent screening was performed by FCM to identify clones able to detect cells with lymphocyte characteristics in whole blood. Finally, the positive clones were assessed to determine their ability to show differences in labelled cells during mHCA assays using FCM.

3.2 Material and Methods

3.2.1 Isolation of leukocytes from rainbow trout

Rainbow trout, *O. mykiss,* obtained from Buckieburn Fish Farm, Stirlingshire, UK (56° 2'33"N 4° 0'25"O), were maintained in tanks at ambient temperature with flow-through water at the Aquatic Research Facility, University of Stirling, Stirling,

UK. Fish weighing between 0.2 - 0.4 kg were fed commercial dried trout pellets daily to satiation. The fish were killed with an overdose of benzocaine 10 % w/v in ethanol (Sigma Aldrich, Dorset, UK).

Leukocytes were isolated following the procedure of Scharsack *et al.* (2001) unless otherwise stated. Medium RPMI 1640 (Sigma Aldrich) used to isolate the cells was prepared by diluting with 10 % distilled water to adjust the osmolarity of the medium. Heparin was added to the medium at 50 units ml^{-1} (Sigma Aldrich) and the isolation procedure was performed either on ice or at 4°C.

3.2.1.1 **Peripheral blood leukocytes (PBLs)**

Five ml syringes were prefilled with 2 ml of RPMI medium, diluted as described above. Blood was withdrawn from the caudal vein into the prefilled syringe and centrifuged at 90 x *g* for 5 min then 400 x *g* for a further 10 min. The buffy coat of leukocytes was collected, layered onto 5 ml Ficoll Histopaque 1.077 (Sigma Aldrich) and centrifuged at 750 g during 30 min at 4°C. The PBL´s were recovered from the Histopaque layer and washed with phosphate buffered saline (PBS; 0.02 M phosphate, 0.15 M NaCl, pH 7.2 adjusted with HCl) at 1000 x *g* for 10 min.

3.2.1.2 **Tissue leukocytes**

Thymus and spleen were removed from fish, and teased through a cell strainer consisting of a 100 μm mesh (BD Biosciences, Oxford, UK), into a 5 ml Petri dish containing diluted RPMI medium. The final volume of the cell suspension was approximately 3 ml which was layered onto 5 ml of Ficoll Histopaque 1.077 and centrifuged at 4°C in a swing-out rotor 30 min at 800 x *g*. The thymic leukocytes (TL's) were recovered from the interface and washed twice with diluted medium by

centrifuging for 10 min at 1000 x *g* (Scharsack, 2001). The procedure was performed on ice whenever possible.

3.2.2 Thymic leukocyte activation

Culture medium was prepared according to Scharsack, (2001). For this, diluted RPMI 1640 was supplemented with 10^5 IU L^{-1} of penicillin, 100 mg L^{-1} of streptomycin, 4 mM L-glutamine, 15 mM HEPES buffer, 25 μM 2-mercaptoethanol (all from Sigma Aldrich) and 5 % rainbow trout plasma. The plasma used was a pool of plasma from six individual fish that had been heat inactivated at 56°C for 30 min. This was then filter sterilised using a 0.22 μm filter (BD Biosciences) and frozen until used.

The TL's were activated by culturing them at a concentration of 1 x10^7 cells ml^{-1} for 3 days in RPMI culture medium as described and supplemented with 30 μg ml^{-1} Concanavalin A (Con A, from *Canavalia ensiformis*, type IV-S) (Sigma Aldrich.) in sterile 6 well plates (Nunclon, Fisher Scientific, Leicestershire UK). Activated cells were recovered and washed with PBS by centrifuging for 10 min at 1000 x *g*.

3.2.3 Monoclonal antibody production

One BALB/c mouse was immunised with an intraperitoneal injection of 0.1 ml PBS containing 1 x10^7 non-activated thymocytes (M1) and another mouse was immunised with activated thymocytes (M1CA) emulsified 1:1 with Titermax Gold (Cyt Rx Corporation, Stratech, Cambridgeshire, UK). Mice were boosted 6 weeks later with non-activated or activated thymocytes as before. Four days before harvesting the spleen thymocytes for the fusion, mice were finally boosted intravenously with non-activated or activated thymocytes through the tail vein.

3.2.3.1 **Generation of monoclonal antibodies**

Mice were sacrificed by CO_2 asphyxiation. The spleen from mouse M1CA was removed and passed through a cell strainer. The cells were collected and washed with DMEM containing additives (Sigma Aldrich) at 85 x *g* for 7 min. The pellet was recovered and mixed 1:1 with SP2 myeloma cells (Sigma Aldrich) before fusing the cells using polyethylene glycol (Sigma Aldrich). The fused cells were seeded into 96 wells plates using mouse red blood cells as a feeder layer with DMEM medium (Sigma Aldrich) supplemented with 20 % foetal calf serum (FCS) (Sigma Aldrich), and 2 % hypoxanthine-aminopterine-thymidine (HAT). The hybridoma supernatants from the first, second and third cloning were tested for the presence of antibodies to thymus leukocytes by Dot blot analysis as described in Section 2.2.3.2. Once cloned by the limiting dilution method (Campbell, 1984), the positive clones were re-screened by flow cytometry (FCM). The positive hybridomas from the Dot blot were first probed to determine their ability to detect cells in the lymphocyte region of whole peripheral blood. Hybridoma supernatants detecting cells in the R1 region were evaluated by FCM with cells from the mHCA as is shown in Figure 2.2.

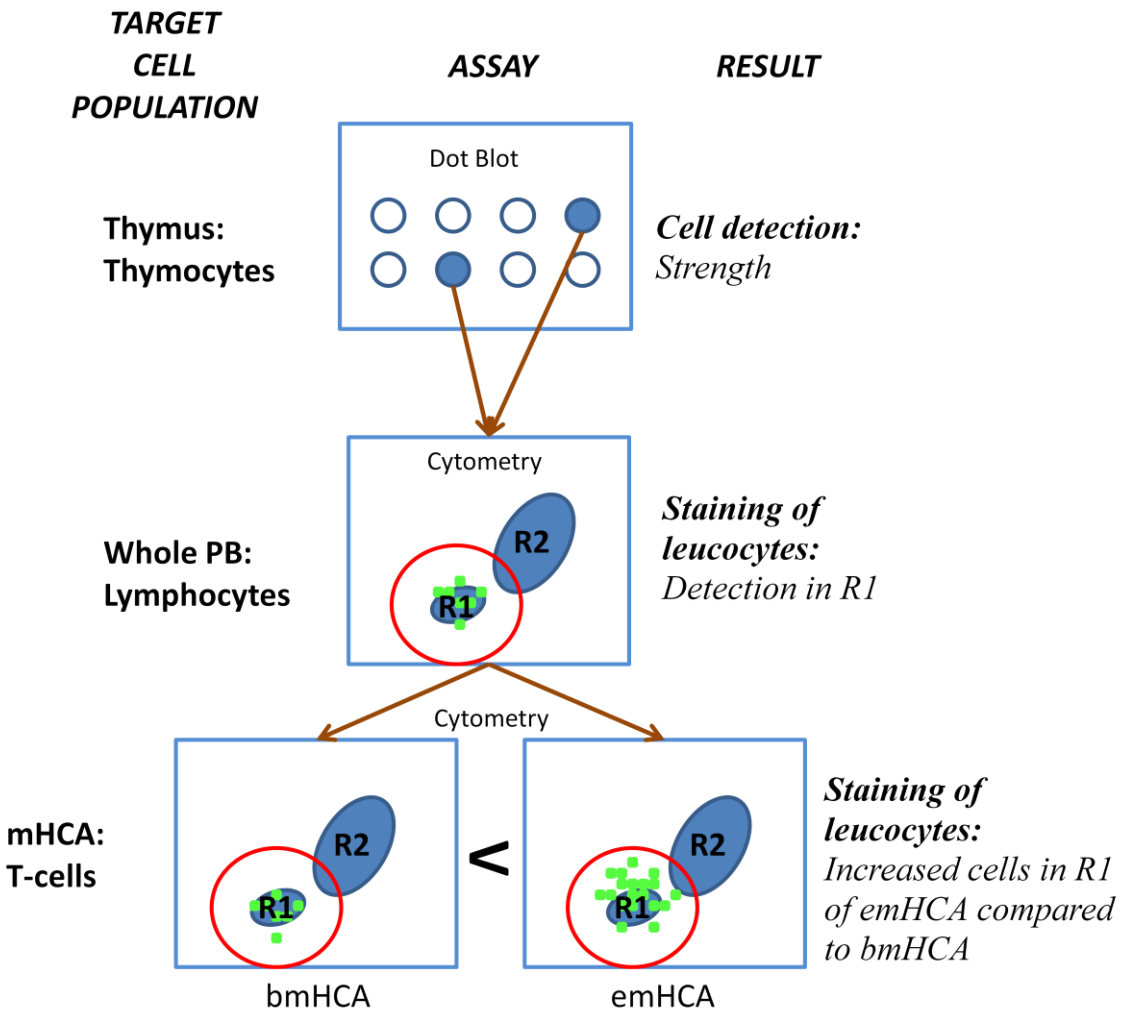


Figure 3.2 Diagram showing the process used for screening the hybridomas. PB, peripheral blood; bmHCA, basal multiple way histocompatibility activation; emHCA, enhanced multiple way histocompatibility activation.

3.2.3.2 Dot blot optimisation and selection of monoclonal antibodies

Hybond-ECL nitrocellulose membrane (GE Healthcare, Chalfont, UK) was pre-wet with Tris buffered saline (TBS: 20mM Tris-HCl, 500mM NaCl, pH 7.5) and placed in the Bio-Dot Microfiltration Apparatus (BioRad, CA, USA). Thymic and spleen cells were isolated as described in Section 2.2.1.2, and added to sample wells of the Dot blot at a concentration of 5 x10^4 or 1 x10^5 cells in 50 µl of Foetal Calf Serum solution (1% FCS in TTBS: TBS containing 0.05% Tween 20). The wells containing cells were drained and the cells were trapped onto the membrane, after which 200 µl of

blocking solution containing FCS (1% FCS in TBS) was added to each well for approximately 60 min before draining the well by gravity. The wells were then washed twice with 300 µl of TTBS by applying a vacuum to the membrane. Test bleeds from the two mice, one immunised with non activated cells (M1) and the other with activated cells (M1CA) were evaluated by diluting the serum 1:100 with antibody buffer, (TTBS with 1%BSA) and 100 µl of diluted serum or hybridoma supernatant was added to each sample well and allowed to filter into the membrane by gravity as before. For the negative control, HT culture medium was used instead of cells. Wells were washed three times with TTBS before adding 100 µl of the secondary antibody *i.e.* anti-mouse IgG-Horseradish peroxidase (HRP) (Sigma Aldrich) diluted 1:100 in antibody buffer and filtered though the membrane by gravity. The wells were washed twice with 300 µl of TTBS and twice with TBS to remove excess Tween. The assay was developed by adding 100 µl of 4 CN peroxidase substrate (KPL, USA) to each well. Once the colour development was complete, the reaction was stopped by washing the membrane with 200 µl of distilled water. The Dot blot colour development was evaluated visually.

3.2.4 Cytometry screening

3.2.4.1 **PBLs isolation**

In order to obtain sufficient numbers of cells for hybridomes screening with FCM, PBL´s were isolated. For the first screening by FCM, whole peripheral blood was collected from the caudal vein of an anesthetised fish, as previously described in Section 2.2.1, using a 5 ml heparinised syringe. Whole blood from individual fish was then prepared for FCM analysis. The hybridoma supernatants which reacted with cells

localized in the LL region, a zone expected to contain mainly lymphocytes and thrombocytes, were selected (Scharsack *et al.,* 2001).

When using cells from the HCA to screen clones from the second cloning by FCM, peripheral blood leukocytes were isolated according to Scharsack (2001) with modifications. Anesthetised fish were bled by caudal venipuncture with a 5 ml syringe containing 2 ml of diluted heparinised RPMI 1640 medium. Samples were maintained on ice whenever possible. Diluted heparinised blood was first centrifuged for 5 min at 90 x *g* and then subsequently 10 min at 400 x *g* in polystyrene tubes. The layer containing the leukocytes was separated from the erythrocytes and diluted with 2 ml of diluted RPMI medium. Subsequently, the suspension was layered onto 3 ml of Histopaque 1077, and centrifuged for 45 min at 900 x *g*. The PBL's were then recovered from the density gradient and washed with diluted medium for 10 min at 1000 x *g*. Pellets from five different fish were pooled in cell culture medium. Numbers of cells were counted using a haemocytometer, and adjusted to 4 $x10^7$ cells ml^{-1} with cell culture medium.

3.2.4.2 **Multiple way histocompatibility activation (mHCA)**

The PBL's from 5 fish were pooled using 1 $x10^7$ from each fish. The pooled cell suspension was plated into a 96 well flat bottom plate at a density of 1 $x10^6$ cells per well in 175 µl cell culture medium. Two different sets of wells containing the pooled leukocytes from the 5 fish were prepared in a mHCA assay by triplicate: one set of wells contained a "basal multiple way Histocompatibility Activation" (bmHCA) (*i.e.* control wells), to which was added 25 µl of cell culture medium <u>without</u> Con A to make the volume up to 200 µl per well. The other set of wells contained an "enhanced

multiple way Histocompatibility Activation" (emHCA) with Con A. This was prepared by adding 25 µl of culture medium containing Con A to obtain a final concentration of 30 µg Con A ml^{-1} per well (Figure 2.3). Plates were incubated for 6 days at 15°C in an atmosphere with 100 % relative humidity and 3 % CO_2. Following this, cells were recovered for analysis by FCM.

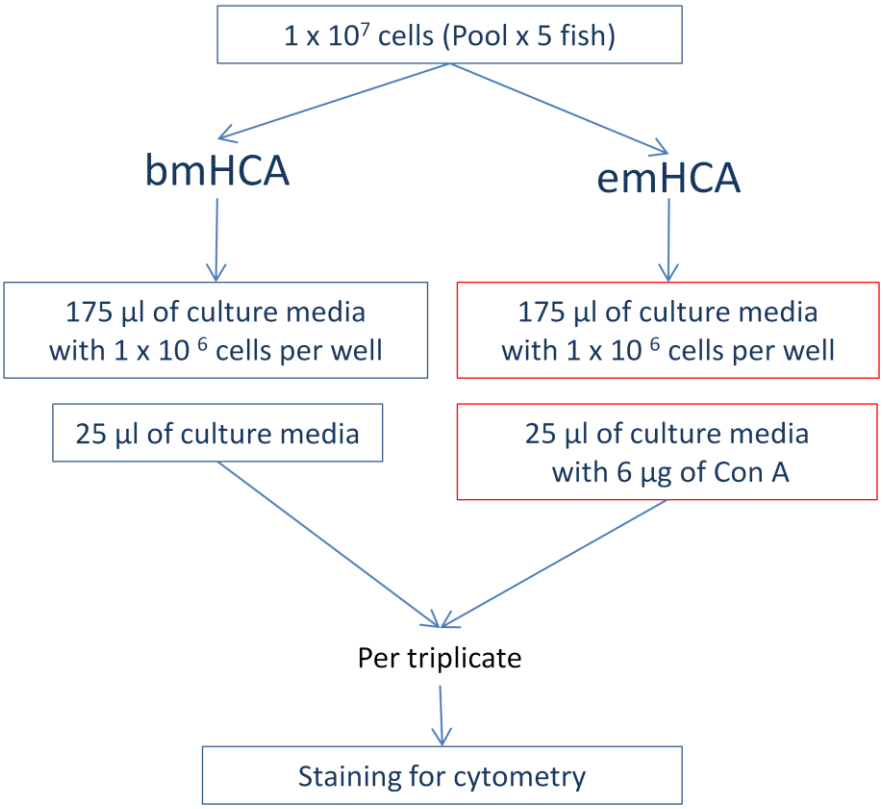


Figure 3.3 Diagram showing mHCA assay preparation. bmHCA, basal multiple way histocompatibility activation; emHCA, enhanced multiple way histocompatibility activation.

3.2.4.3 IIF staining for first screening by FCM

Fifty µl of complete peripheral blood were incubated with 180 µl of hybridoma supernatant for 30 min. Cells were kept on ice with frequent shaking. Two ml of PBS/BSA was added to each tube and washed as described above. The cells were

resuspended in 200 µl of secondary antibody (rabbit anti-mouse FITC conjugate diluted 1:50 with PBS with 1 % bovine serum albumin (BSA), Vector). Samples were kept on ice in the dark for 30 min with frequent shaking. The cells were washed with 2 ml PBS/BSA. Cell pellets were resuspended by vortexing with 1 ml of FACS buffer (BD Biosciences), stored on ice in the dark and analysed by FCM as soon as possible thereafter. Background controls were prepared by adding FITC conjugate secondary antibody to cells without the first antibody. For the negative control neither the first nor secondary antibodies were added. Positive controls to determine the intensity level of fluorescence were prepared by staining cells from mHCA with polyclonal antibodies (M1CA) as the first antibody then the FITC-conjugate as the secondary antibody.

3.2.4.4 **Multiple way histocompatibility activation (mHCA) screening by flow cytometry (FCM)**

After 5 days, cells from the HCA were resuspended using plastic tips with bored ends (cut with sterile scalpel) to avoid cell damage and transferred to polystyrene tubes (Falcon). For every positive hybridoma selected by Dot blot, two tubes containing cells from bmHCA and two with emHCA cells were prepared (the contents of one well tube^{-1}). The cells were washed with PBS containing 0.5 % BSA and 0.05 % sodium azide (PBS/BSA) for 10 min at 1000 x *g*. Afterwards, indirect immuno-fluorescence staining was performed by incubating the isolated cells in 200 µl of hybridoma supernatant for 30 min, then following the procedure described in Section 2.2.4.3.

3.2.4.5 **Flow cytometry (FCM)**

Forward scattered light (FSC), side scattered light (SSC) and fluorescence intensity were registered for 5,000 or 10,000 events without gating using a flow cytometer with an argon laser at 488 nm (FACScan®, Becton Dickinson, Germany).

Data were analysed with the WinMIDI program (version 2.9) which provided histograms for fluorescence illustrating intensity on a logarithmic scale. The data were acquired in a 1024 channel scale producing a dot plot that represented a nominal range of four decades of intensity (as presented in histograms) and adjusted by calibrating with Calibrite beads (BD Biosciences). The flow cytometer settings were adjusted and saved for every batch of experiments. During acquisition, to analyse the various sets of experiments, the saved settings were retrieved and maintained. Statistical means and the standard deviations were calculated for the positive hybridoma supernatants to compare the proportions of labelled cells between the sets of HCA´s, and a one way ANOVA analysis was performed.

3.3 Results

3.3.1 Dot blot optimisation and selection of monoclonal antibodies

Polyclonal sera from immunised mice (M1 and M1CA) were used to optimise the Dot blot assay (Figure 2.4). Although both polyclonal antibodies had a good response in the assay, only one mouse was actually used for the fusion due to the impracticality of managing large numbers of clones at the same time using Dot blot and IIF to perform the screening. Two different cell concentrations were evaluated for the Dot blot, *i.e.* 5 x10^4 and 1 x10^5cells. A strong reaction was obtained with the fewest number of cells (5 x 10^4) and this cell concentration was therefore employed for subsequent assays. This was so that there were sufficient numbers of TL's to perform the screening of the hybridomas. Differences in the level of staining obtained between thymocytes and splenocytes with the polyclonal antibody (pAb) were small, but more stain was present with the thymic cells than the spleen cells. The M1CA mouse was

used for the fusion and a concentration of 5 x10^4 of thymic leukocytes was used to screen the hybridomas, as shown in Figure 2.4.

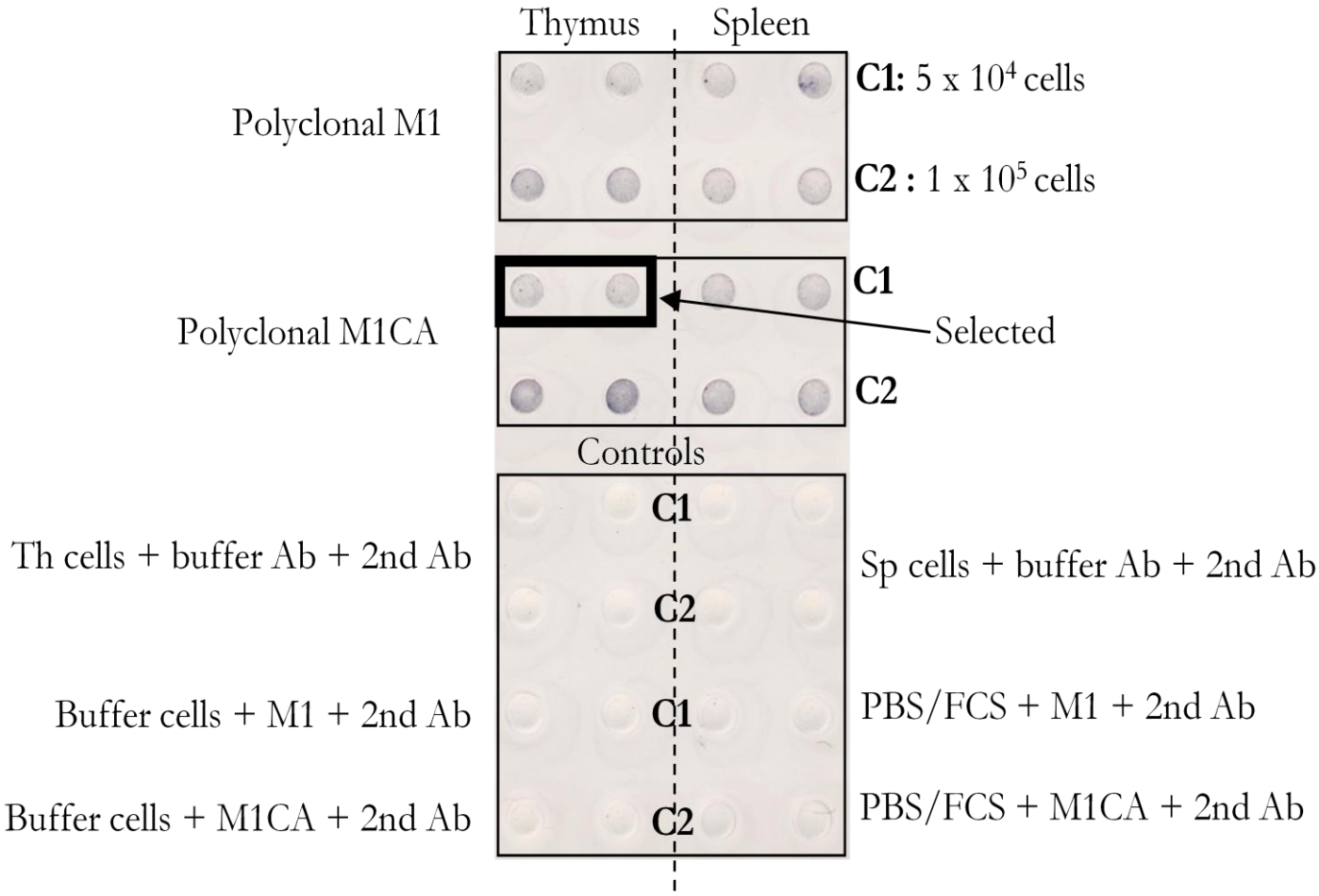


Figure 3.4 Optimisation of Dot blot screening. Samples were used in duplicate. Concentration 1, C1 and Concentration 2, C2.

3.3.2 Screening the fusion by Dot blot

A total of 564 hybridomas were screened from the fusion, 41 were selected for the first cloning, based on the strength of staining obtained in the Dot blot against TL's (Figure 2.5). By the end of the 3rd cloning 24 clones were selected for further screening by FCM.

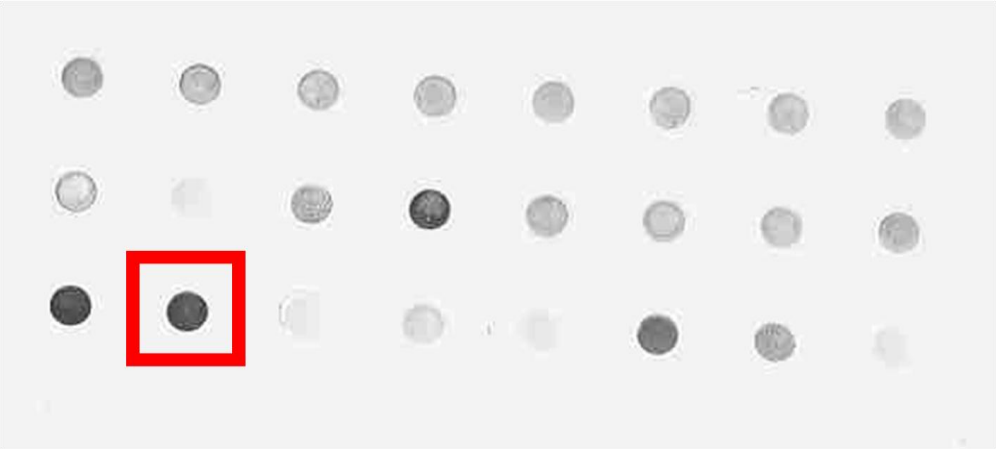


Figure 3.5 Screening hybridomas by Dot blot against thymus leukocytes. **Square indicates colour intensity of positive clones.**

3.3.3 Screening by flow cytometry (FCM)

Twenty four clones were screened by FCM using whole peripheral blood. Eighteen of these gave insufficient staining within the lymphocyte region and no further characterization of these was carried out. The four remaining hybridomas, TcOm14, TcOm15, TcOm34 and TcOm36, were further evaluated due to their ability to detect cells in the lymphoid region of the FCM dot plot (Figures 2.6- 2.11). The immunoglobulin isotyping indicated that the four hybridomes remaining belonged to the subclass IgG1.

These 4 clones were screened by FCM to evaluate their ability to detect differences in numbers of cells within the R1 population using cells from an emHCA

and a bmHCA. The mAb TcOm15 was considered a potential marker since reacted with cells gated in the R1 region, the population considered as lymphocytes. This mAb also gave more defined staining of medium size cells with low cytoplasmic internal complexity mainly within region R1. Forward side scatter or the relative size of the cells was from 200 to 450 while SSC or relative granularity was from 40 to 125 (Figure 2.7). From 3 replicate samples, mAb TcOm15 detected an increase of about 1.68 times of labelled cells in emHCA population compared with cells from the bmHCA (Table 2.1 & Figure 2.7 c). It also detected a significantly greater percentage of total labelled cells in the enhanced reaction compared with the basal mHCA (p value ANOVA= 0.024). When proportions of fluorescing cells in the R1 region were compared with respect to the total population, a twofold increased in the proportion of labelled cells was observed (Table 2.1 & Figure 2.7 d). There was a significant increase in the number of cells detected by mAb TcOm15 within the gated region of R1 in the enhanced mHCA compared with the basal mHCA (p value ANOVA= 0.004). This mAb was potentially positive for cells apparently undergoing activation.

From the remaining three clones evaluated by FCM, mAbs TcOm14 and TcOm36 were discarded as possible cell markers because they showed a high level of staining of cells outside R1, (Figure 2.8 & Figure 2.9). MAb TcOm34 detected an increase number of cells in the emHCA cell population compared to the bmHCA cells (Figure 2.10). However, the staining with this mAb was considered non-specific because of the irregular pattern of detection of cells outside the R1 region.

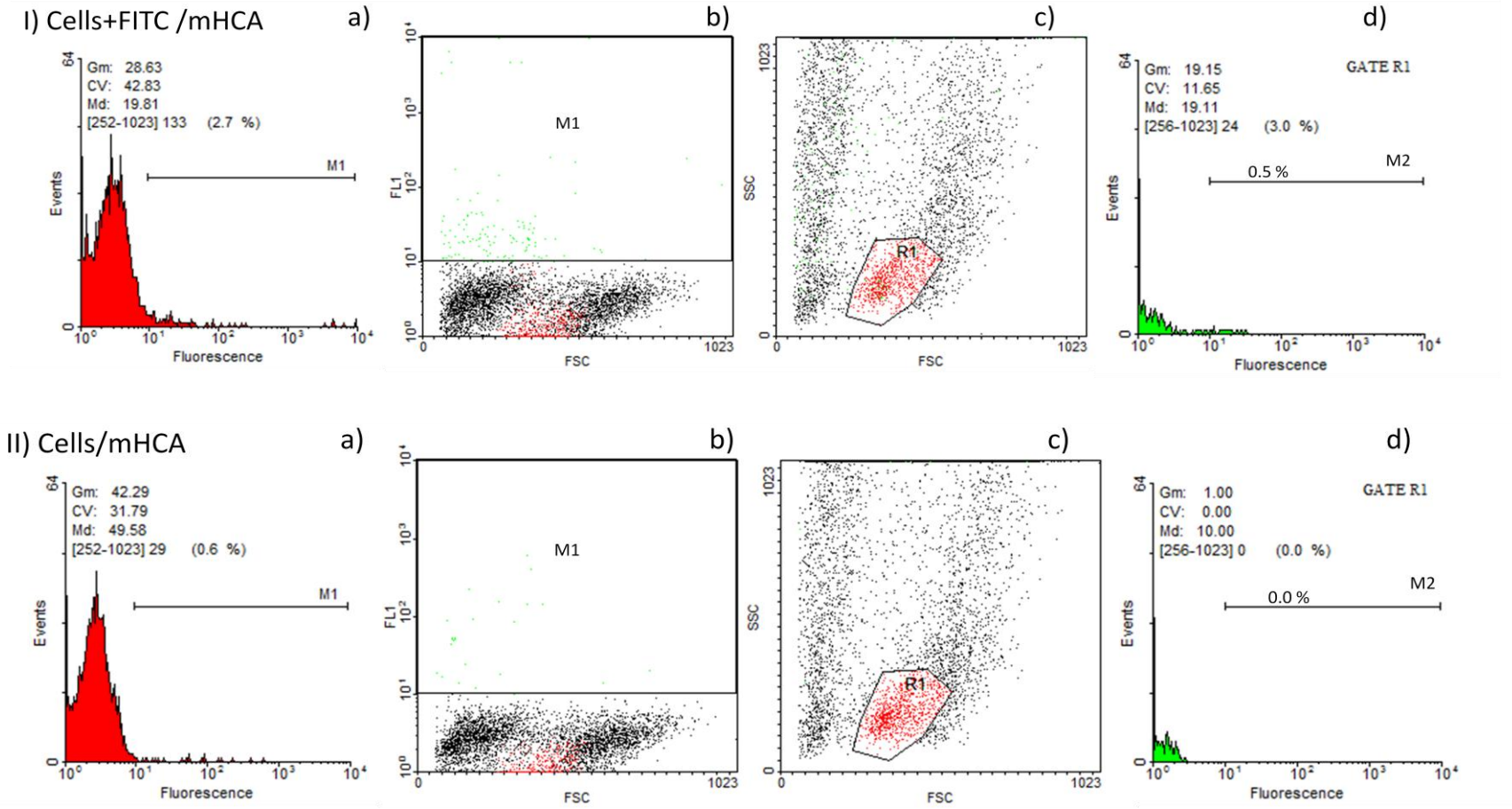


Figure 3.6 Pattern of cells detected in FCM from mHCA assays with no antibody staining. I) FSC/SSC dot plots and FSC/FL1 histograms of cells prepared for background control. II) Cells prepared for negative control. (a) percentage of fluorescing cells from total events; mark 1 (M1), indicates percentage of cells in the positive fluorescence intensity value (b) FSC/fluorescence of labelled cells from the total counted cell; (c) Gate R1; and (d) percentage of fluorescing cells from gate R1 in mark 2 (M2). Green dots, fluorescing cells.

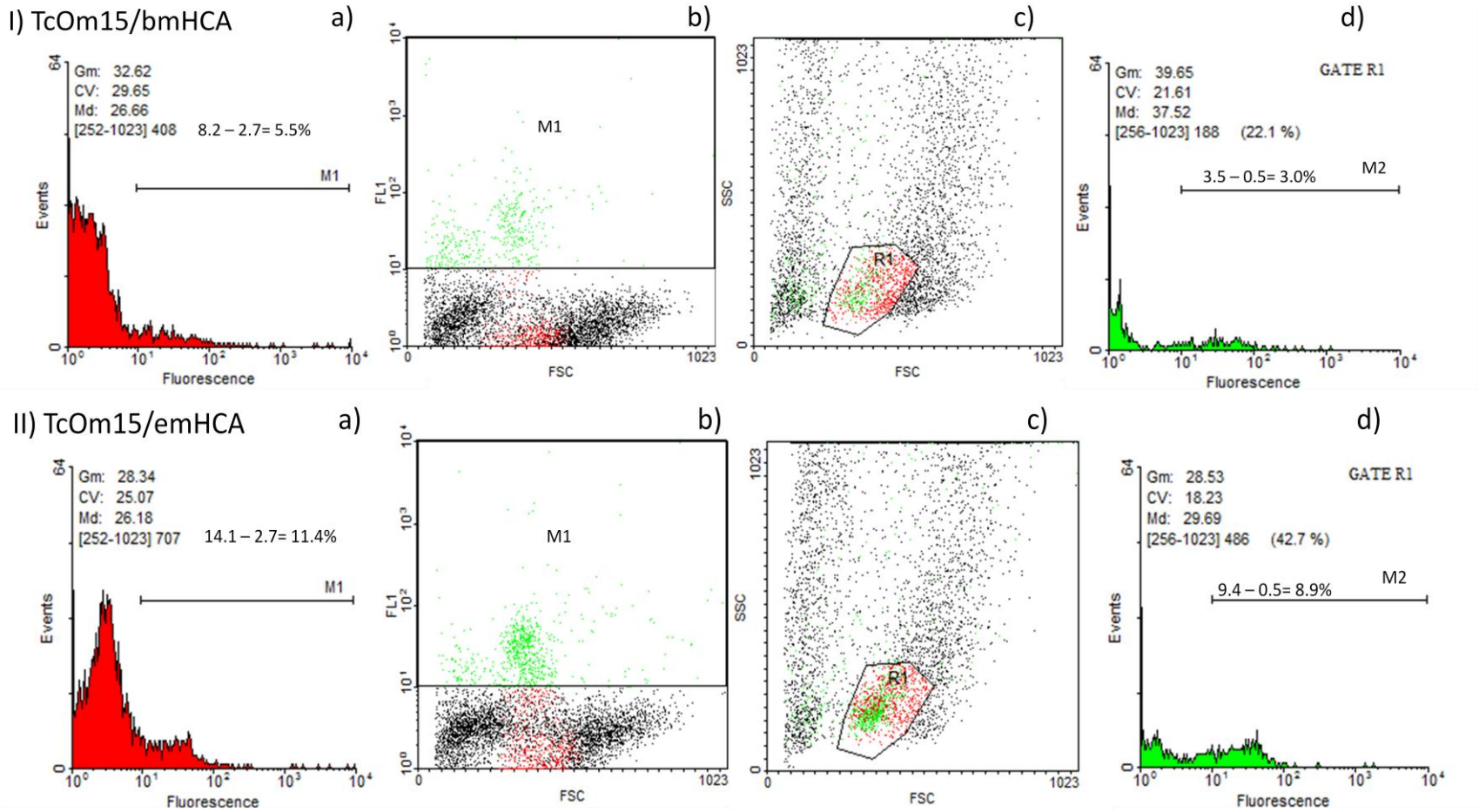


Figure 3.7 Pattern of cells detected by FCM stained with mAb TcOm15. I) FSC/SSC dot plots and FSC/FL1 histograms of cells from the basal mHCA. II) Cells from the enhanced mHCA. (a) percentage of fluorescing cells from total events; mark 1 (M1), indicates percentage of cells in the positive fluorescence intensity value (b) FSC/fluorescence of labelled cells from the total counted cell; (c) Gate R1; and (d) percentage of fluorescing cells from gate R1 in mark 2 (M2). Green dots, fluorescing cells. Minus background, a) and d).

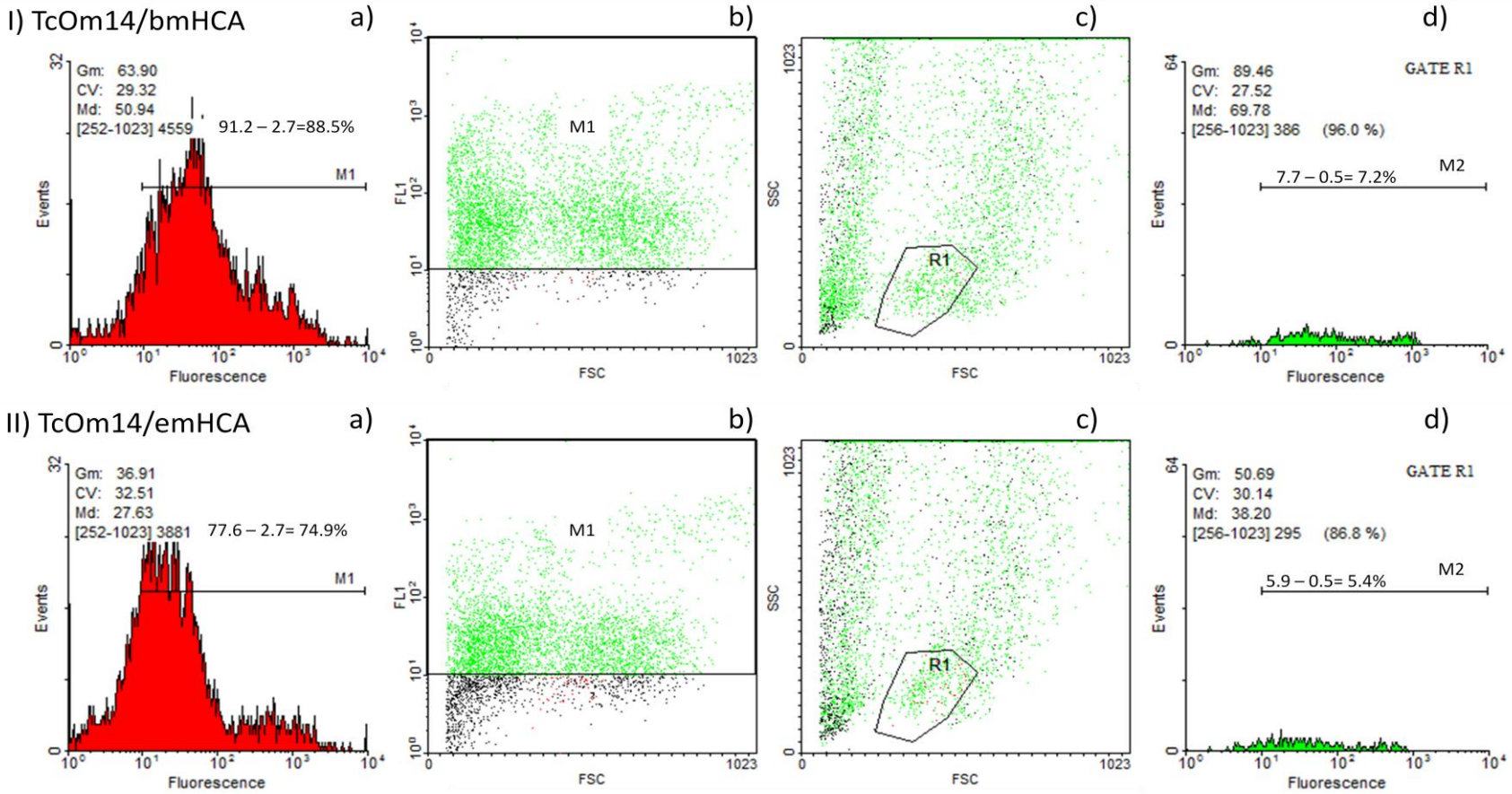


Figure 3.8 Pattern of cells detected by FCM stained with mAb TcOm14. I) FSC/SSC dot plots and FSC/FL1 histograms of cells from the basal mHCA. II) Cells from the enhanced mHCA. (a) percentage of fluorescing cells from total events; mark 1 (M1), indicates percentage of cells in the positive fluorescence intensity value (b) FSC/fluorescence of labelled cells from the total counted cell; (c) Gate R1; and (d) percentage of fluorescing cells from gate R1 in mark 2 (M2). Green dots, fluorescing cells. Minus background, a) and d).

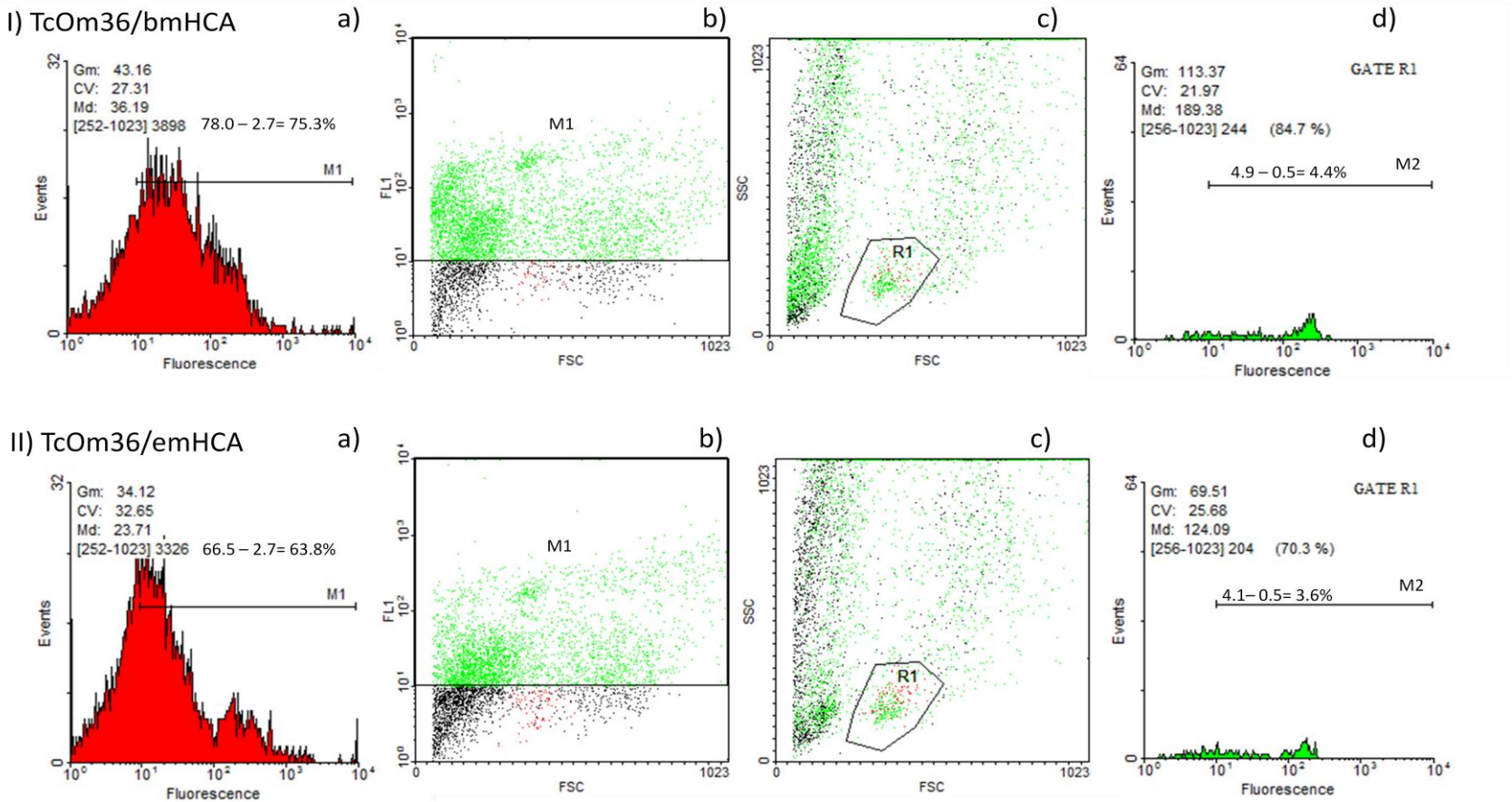


Figure 3.9 Pattern of cells detected by FCM stained with mAb TcOm36. I) FSC/SSC dot plots and FSC/FL1 histograms of cells from the basal mHCA. II) Cells from the enhanced mHCA. (a) percentage of fluorescing cells from total events; mark 1 (M1), indicates percentage of cells in the positive fluorescence intensity value (b) FSC/fluorescence of labelled cells from the total counted cell; (c) Gate R1; and (d) percentage of fluorescing cells from gate R1 in mark 2 (M2). Green dots, fluorescing cells. Minus background, a) and d).

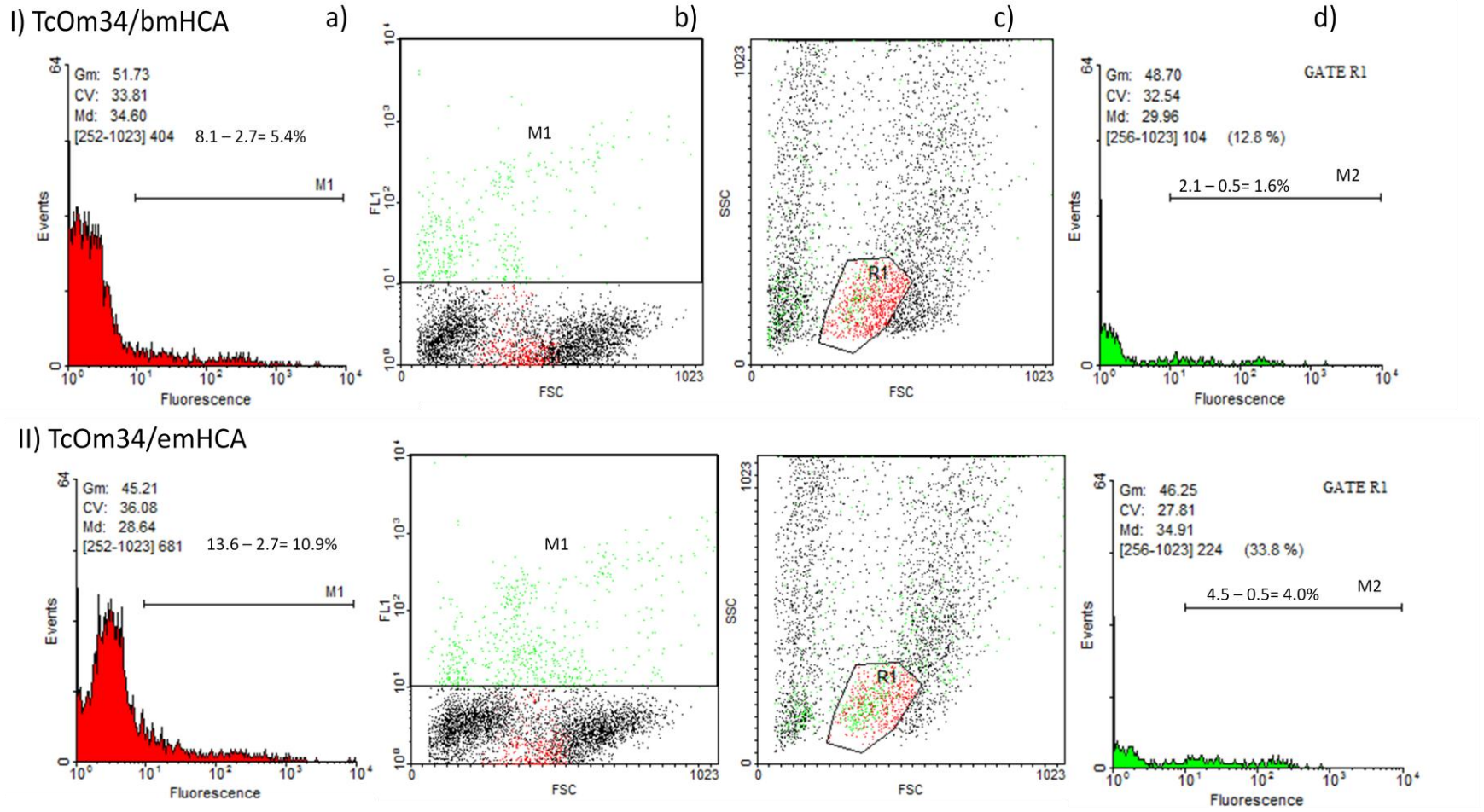


Figure 3.10 Pattern of cells detected by FCM stained with mAb TcOm34. I) FSC/SSC dot plots and FSC/FL1 histograms of cells from the basal mHCA. II) Cells from the enhanced mHCA. (a) percentage of fluorescing cells from total events; mark 1 (M1), indicates percentage of cells in the positive fluorescence intensity value (b) FSC/fluorescence of labelled cells from the total counted cell; (c) Gate R1; and (d) percentage of fluorescing cells from gate R1 in mark 2 (M2). Green dots, fluorescing cells. Minus background, a) and d).

Table 3.1 Proportions of rainbow trout blood leukocytes from mHCA assays labelled with mAb TcOm15 by IIF evaluated by flow cytometry. Background was subtracted.

Replicate ID	# Events	Culture days	% TOTAL fluorescing cells	Detected increase E/B=	% GATED fluorescing cells in R1	Detected increase E/B=
			Basal Reaction			
15bmHCAc	10000	5	5.5		2.7	
15bmHCAd	10000	5	7.2		5.0	
15bmHCA2	5000	7	8.2		3.5	
		$\overline{X}$ ± s.d.	7.0 ± 1.2		3.7 ± 1.2	
			Enhanced Reaction			
15emHCAa	10000	5	11.4		7.8	
15emHCAb	10000	5	10.2		8.3	
15emHCA1	5000	7	14.1		9.4	
		$\overline{X}$ ± s.d.	11.9 ± 2.0*	1.7	8.5 ± 0.8*	2.2

B=basal mHCA, E=enhanced mHCA
*P <0.024 for Total B *vs* E
*P <0.004 for Gated R1 B *vs* E

3.4 Discussion

Immunization of a mouse with Con A-stimulated thymic cells (M1CA) resulted in the production of pAbs that reacted with whole thymic cells. The underlying principle of using the M1CA mouse for the fusion was based on the possibility that the lectin promoted the activation of the target T-cell population (Manning & Nakanishi, 1997). In humans, this mitogen is capable of binding to membrane glycoproteins from different clones of T-cells initiating transduction signals, activation, expression of molecules, and finally proliferation (Roitt, 1997; Jason & Inge, 2000). It was expected therefore that pAbs produced by the M1CA mouse would have a higher reaction against thymic cells than that of the M1 mouse (immunised with non-stimulated thymocytes). However, when pAbs from the M1 and M1CA mice were compared for immunorectivity against whole thymic and spleenic cells no obvious difference was seen between their reactivity. This apparently similar antibody response was based on the results of the Dot blot and does not mean that the epitopes recognised by the antibodies were the same. Also the titres of the sera from these two mice were not measured, so possibly there may have been differences in the levels of reacting Ab between the two sera. The strength of staining obtained with the pAb was related to the amount of cells used in the Dot blot assay, implying a proportional reduction or increase of recognised epitopes. As the pAbs recognised surface epitopes on both thymocytes and splenocytes, suggesting the presence of Abs that possibly recognised epitopes on T cells and B cells, the hybridomas were screened using only TL's in the Dot blot, relying on the intensity of staining with these to select positive hybridomas. It was assumed that a strong Dot blot reaction was indicative of a high detection of

TL's. However, it is possible to find abundant immunogenic moieties in leukocytes (De Luca, Wilson & Warr, 1983; Greenlee & Ristow, 1993); hence it is possible that in the evaluated pAb, there existed Ab's that were reacting to common antigens on the leukocytes surface.

The success in producing cell markers that are able to differentiate T-cell subpopulations in fish has been very poor. Authors attempting to develop these have found that just one clone may have the potential to be a useful marker out of hundreds of clones that are produced during the fusion (Scapigliati *et al.,* 1995; Passer *et al.*, 1996; Rombout *et al.*, 1997 &, 1998). In the present work, of the 564 clones produced, only 24 were shown to react with cells from the thymus in the Dot blot, and from the FCM, only one of these 24 clones appeared to be able to specifically label lymphocyte-like cells.

The twenty four clones selected by Dot blot gave a strong reaction against thymic cells. However, when hybridoma supernatants were screened by IIF staining using peripheral blood cells, the proportion of cells detected by FCM was low and appeared non-specific. Due to the fact that the antigens used to immunise the mouse were thymic cells, it was expected to obtain a higher reaction with thymocytes rather than PBLs. The reaction of supernatants from the 24 clones with cells in whole peripheral blood was very low and these stained cells outside the R1 region. During the development of the mAb DLT15 for sea bass, one of the two mAbs available against T-cells of teleosts, was selected from 349 clones, chosen because of its ability to stain living and fixed thymocytes (Scapigliati *et al.*, 1995). By FACS these authors obtained 2.2 % staining of whole blood cells (Scapigliati *et al.,* 1995). Similarly, Passer *et al.* in 1996 immunise mice with whole thymic cells and developed a T-cell specific mAb

(CfT1) that stained 10 % of PBL´s (not whole blood) when assessed by FCM. In the present work, the use of whole peripheral blood for the first screening by FCM, permitted the selection of a mAb detecting cells with FSC and SSC similar to that obtained for lymphocytes, however very low levels (1.4%) of recognition were obtained.

The second screening by FCM was designed as an attempt to enrich the T-cell population by using a multiple way activation by recognisement of foreign histocompatibility complex (mHCA) and Con A stimulation, and hopefully leaving granulocytes, thrombocytes, and monocytes unstained. However, due to the incipient experience on FCM during evaluation, the selection of appropriate controls that facilitated the analysis was lacking (*i.e.* positive and no-immunised mouse serum). However, taking the previous into account during analysis, the mAb TcOm15 was considered a potential positive due to the reaction against a cluster of cells that several authors consider as mainly a lymphocyte population, and out of the precursors and debris area (Scharsack, 2001; Kfoury *et al.,* 1999, Chilmonczyk, 1999). Additionally, the mAb TcOm15 detected an increase by 1.7 to 2 times in number of labelled cells treated with mitogen Con A, localised by FSC/SSC similarly to the cells detected in the mHCA without Con A. Passer *et al.* (1996) clearly demonstrated the ability of the cell marker CfT1 to detect Con A stimulated cells which increased ~5 times when compared with a control and stimulus with a B-cell activator (LPS). Due to the modifications made to the stimulation assay (mHCA and Con A) for the screening of the mAb´s in this investigation, it was possible that there was activation for a subpopulation of cells but suppression of another population. However, due to the detection of differences in numbers of cells in the emHCA when compared with

bmHCA, it was assumed that mAb TcOm15 labelled cells with lymphocyte characteristics that showed up-regulation of surface molecules related with the allogeneic stimulation in the presence of Con A. The identity of the cells, and the antigen on the cells, recognised by this mAb, and the dynamics of its staining were investigated further in Chapters 3 and 4.

Chapter 4 Identification of the Antigen Recognised by mAb TcOm15

4.1 Introduction

Monoclonal antibodies (mAbs) are highly specific for the antigenic molecules they have been raised against. This specificity can be used to identify particular components such as receptors, molecules associated with cell activation, and adhesion markers within complex systems such as cellular membranes (Goldrath *et al.,* 1995). The detection of surface marker molecules on immunological cells can help to identify and elucidate cellular components of the immune response. The expression of these molecules on a particular leukocyte population depends on the morphological and/or functional differentiation of the leukocyte lineage that the leukocytes belong to (Scapigliati, Romano & Abelli, 1999; Moulana, *et al.*, 2008; LeBien & Tedder, 2009). Thanks to the availability of cell markers, studies on human immunology have demonstrated that surface molecules on leukocytes have important functions, such as receptors that bind antigens, ligands involved in cell to cell signaling or in initiating immune response activity (LeBien & Tedder, 2009). Not only have mAbs been used to discriminate cells during different stages of cell development, but have assisted in understanding the dynamics of the molecule's expression during functional processes. Such molecules detected on human leukocytes have been named human leukocyte differentiation antigens or HLDA. In order to group the antibodies that detect similar molecules and have similar patterns of reaction, human immunologists have organised the mAbs using the nomenclature

scheme, Clusters of Differentiation (CD). So far, there are about 350 leukocytes surface molecules detected by mAbs with a CD nomenclature for human leukocytes (www.hcdm.org). Workshops are organised annually to discuss how new antibodies fit into this nomenclature scheme.

A greater understanding about the types of mAbs available for swine immunology came from the animal sections held at the HDLA workshop, leading to studies in which the lineages and subsets of cells involved in the immune response of swine were examined (Haverson *et al.*, 2001). According to Piriou-Guzylack & Salmon (2008), the progress made in swine immunology in recent years has been achieved through the classification assignment of CD nomenclature to mAbs against swine leukocytes. The criteria used to establish an official swine CD nomenclature are based on: (1) "the binding to the cluster of two or more mAb resulted in a pattern of reactivity that is typical of the same CD marker seen on human cells", (2) "the molecular weight (MW) of the antigen recognised by the mAb is similar to that of the human antigen" and (3) "the reactivity with the gene product or functional studies of the identified molecule" (Piriou-Guzylack & Salmon, 2008 page 3). Monoclonal antibodies used to identify surface molecules on pig leukocytes are able to differentiate cell types from lymphoid and myeloid lineages into T-cells, natural killer cells, B-cells, monocytes, macrophages and dendritic cells. It is also possible to examine different functions as activation, adhesion and attachment as well as identifying functional molecules during the immune response of pigs (see Figure 3.1).

However, as yet no similar nomenclature exists for teleosts (Fischer & Koellner, 2007). In fish immunology there is a great need to produce mAbs that are able to differentiate the various components of the fish immune system and to help understand the behaviour of the fish immune response.

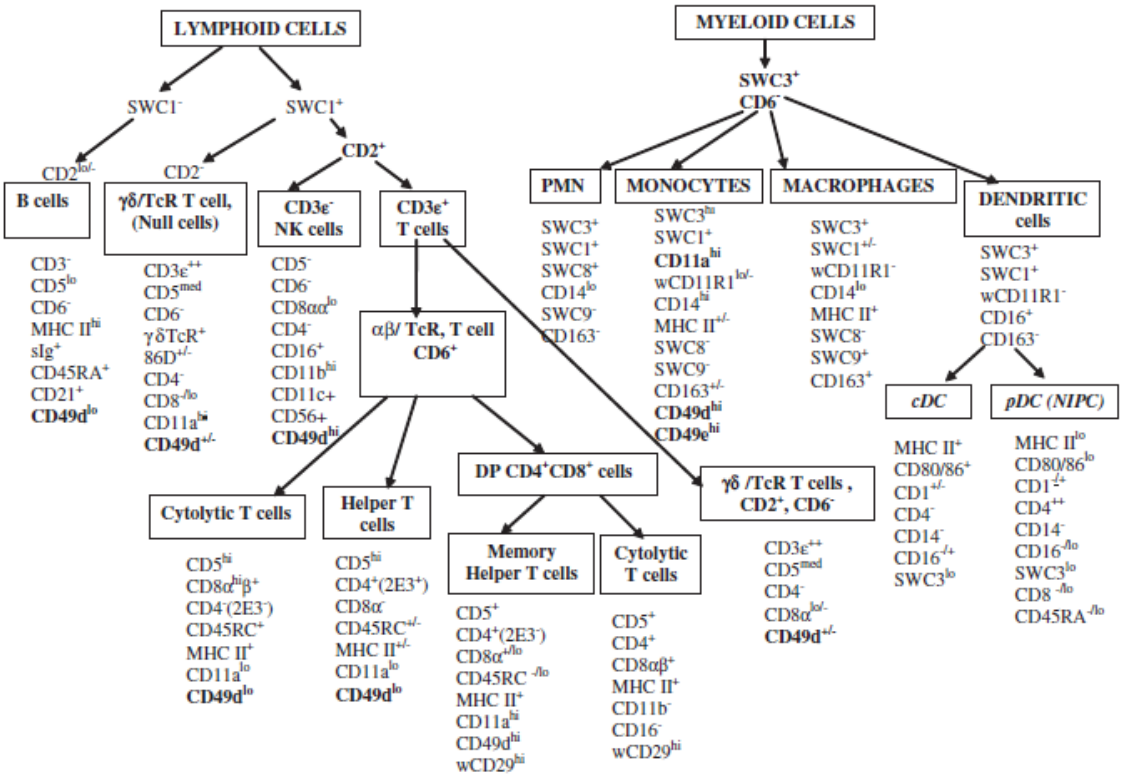


Figure 4.1 Example of clusters of discrimination used to phenotype blood swine leukocytes, macrophages and dendritic cells using CD mAbs (from Piriou-Guzylack & Salmon, 2008, page 16).

It is still not possible to describe the role of the different membrane molecules of leukocytes during recognition of antigens and the physiological communication between immune cells of fish. As mentioned earlier in Chapters 1 and 2, there are very few mAb cell markers available for fish. In an effort to identify mAbs which might be useful for teleosts, 382 mAbs commercially available for

humans were recently tested against rainbow trout and carp cells (Fisher & Koellner, 2007). From this long list, only 4 mAbs showed any reaction with leukocytes from trout (mAbs anti-human CD14, CD20cy, CD45RO and Xg molecules) and 2 against carp cells (mAbs anti-human CD20cy, and CD45RO molecules) by flow cytometry. However, only one of these showed any reaction in immunohistochemistry (IHC), and none were reactive in Western blot analysis or immunoprecipitation (Fisher & Koellner, 2007). Even though a reaction was obtained with the human mAbs, the possibility that these mAbs were able to identify identical CD markers in fish could only be demonstrated through functional assays. Several mAbs have now been developed for fish, as shown in Table 1.3 in Chapter 1. However, it is necessary to look at the reactivity of all these mAbs in a teleost workshop with the purpose of identifying possible clusters where these can be assigned and to initiate a nomenclature system for fish.

Aim:

Given the findings in Chapter 2, the aim of the present chapter was to determine which types of cells that were recognised by mAb TcOm15 and to identify the antigen being detected by the mAb. Cells and tissues stained with the mAb were analysed by microscopy, proteins from leukocytes were extracted and immunodetection was performed with the mAb in an attempt to identify the antigen and possibly establish its identity by Mass Spectrometry (MALDI-TOF) analysis.

4.2 Material and methods

4.2.1 Isolation of leukocytes

Gills, thymus, spleen, liver, head kidney, and intestine were removed and leukocytes were isolated and washed with PBS at 1000 x *g* for 10 min at 4°C. The cell concentration was determined (Section 2.2.1) and cells were used as required for each of the following protocols.

4.2.2 Flow cytometry (FCM)

Immunolabelling of cells from different organs was carried out to determine the proportions of leukocytes labelled with mAb TcOm15 from the different tissues. The methodology was performed as described in Section 2.2.4.

4.2.3 Microscopy

4.2.3.1 **Immunohistochemistry (IHC)**

Samples were processed according to Adams and Marin de Mateo (1994). Tissues from the various organs (thymus, spleen, gill, liver, intestine, and head kidney) were fixed with 10 % neutral buffered formalin. Tissue was embedded in paraffin, and 5 μm sections were cut and placed on microscope slides. Tissue sections were dewaxed and rehydrated in xylene (2 times for 5 min), in 100 % ethanol for 5 min, in 70 % ethanol for 3 min. Endogenous peroxidase activity was blocked by incubating the slides for 10 min at 21°C with H_2O_2 in methanol and washed three times with PBS. Non-specific binding sites were blocked with normal goat serum diluted 1/10 in PBS for 20 min at 21°C. Tissue sections were circled with a wax pen and then covered with hybridoma supernatant containing mAb

TcOm15 and incubated for 30 min at 21°C in a humid chamber before washing three times with PBS. Goat anti-mouse IgG HRP conjugate (Vector, Peterborough UK) diluted 1/200 in PBS was added to the slides for 30 min. Finally, the reaction was visualised by incubating the slides for 10 min with substrate using a Vector VIP Peroxidase Substrate Kit following the manufactures instructions. The tissue sections were counter-stained with methyl green (Vector) for 5 min following the manufactures instructions and mounted for microscopy observation.

4.2.3.2 **Indirect immunofluorescence (IIF)**

The IIF protocol was performed using the tissue sections and the IHC protocol described in Section 3.2.3.1 with modifications. The HRP conjugate was substituted with a rabbit anti-mouse FITC conjugate (Sigma Aldrich), diluted 1:50 with PBS containing 1 % BSA, incubating with this for 30 min. Slides were washed with PBS and mounted using Vector mounting shield containing DAPI (which stains DNA). The slides were maintained in the dark until observed under a Leica TCS SP2 AOBS Confocal scanning laser microscope. Images were captured using Leica Confocal software and lasers lines at 405nm, 488nm and 594nm.

Indirect immunofluorescence was also performed on PBLs (1 x 10^8 cells/ml) that had been allowed to adhere to µ-VI flat Ibidi slides (IBIDI, Munich, Germany) coated with poly-L-lysine for 1 h. Wells were washed with PBS containing 0.5% BSA and incubated with hybridoma supernatant containing mAb TcOm15 for 30 min. The secondary antibody used was an anti-mouse IgG conjugated with Texas Red (1.5 µg/ml) (Vector), incubating with this for 30 min. Wells were then washed three times with PBS. Cells were fixed with 2 % paraformaldehyde containing 3 %

sucrose in PBS for 20 min. Finally, cells were permeabilized with PBS plus 0.5 % Triton X-100 (Sigma Aldrich) for 10 min, and 2.5 % of methanolic solution of Alexa Fluor 488 Phalloidin (Invitrogen) in PBS (pH 7.4) was added for 20 min to stain F-actin. Vector mounting shield containing DAPI was added to the slides, and the cells covered with a coverslip then maintained in the dark until observed under a confocal microscope.

4.2.4 Characterisation of antigen recognised by mAb TcOm15 by gel electrophoresis

4.2.4.1 **Cell lysates and sample preparation**

Cells were isolated, as described previously in Chapter 2, and a sample from the mucus of skin was also obtained by scraping the fish with a scalpel to obtain approximately 0.5 ml of mucus. Proteins were extracted from the cells and mucus using six different detergents. A basic lysis buffer was prepared according to Camacho-Carvajal *et al.* (2004) containing 50 mM Tris-HCl, pH 7.5, 25 mM of NaCl and 1 mM of EDTA. One of the 6 different detergents was added to this basic solution, these being either 0.1 % of Saponin (Sigma Aldrich), 0.1 % of Triton X-100 (Sigma Aldrich), 0.1 % of Tween 20 (Sigma Aldrich), 0.1 % of Nonidet P-40 (Sigma Aldrich), 0.1 % of Tween 80 (Sigma Aldrich), or 1 % of CHAPS (Fisher Scientific, Loughborough, UK) and using 1 mM of phenylmethylsulfonyl fluoride (PMSF) (Promega, Southampton, UK) in each preparation as protease inhibitor. Cells (1×10^6 - 2×10^7) were incubated with 250 μl of each lysate buffer for 30 min on ice. Once cells were lysed the preparations were centrifugated at 5,000 g for 20

min at 4°C. Cell supernatants were retained and kept refrigerated or frozen until used.

4.2.4.2 **1D Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE)**

For SDS-PAGE, supernatants were prepared with sample buffer containing 100 mM Tris-HCL (pH6.8), 4 % SDS, 2mM DTT, and 0.02 % bromophenol blue (Laemmli 1970). Equal volumes of protein sample from Section 3.2.4.1 were mixed with a 2x concentration of sample buffer and boiled for 90 sec. Electrophoresis was performed according to Laemmli (1970). Twenty μg of the protein lysate from each organ was added to one of the lanes. Full range rainbow markers were added as a reference in order to compare bands from the Western blot analysis Section (3.2.4.4). Electrophoresis was carried out at 130 V and 90 mA until the bromophenol blue dye reached the bottom of the gel. Gels were stained with Comassie Brilliant Blue (Sigma) and destained with 40 % methanol/10 % acetic acid. A silver stain kit (Proteo Silver, Sigma Aldrich) was used to stained samples which gave weak staining with Coomassie Blue.

4.2.4.3 **2D Blue native polyacrylamide gels (BN-PAGE)**

In order to investigate membrane-bound complexes native gels were prepared with modifications according to Camacho-Carvajal *et al.* (2004), Lehner, Niehof & Borlak (2003), Nijtmans *et al.* (2002), and Schagger *et al.* (1991).

a) Protein supernatants/whole leukocytes lysates

Protein samples described in Section 3.2.4.1 were eluted through a filter column in order to eliminate small molecules and help improve the resolution of the

electrophoresis run under native conditions. Supernatants were centrifugated in a filter column (Microcon YM10; Millipore) where 375 µl of BN buffer (500 mM ε-amino caproic acid, 20 mM Bis-Tris, pH 7.0, 2 mM EDTA, 12 mM NaCl, 10 % glycerol, detergent and 1 mM phenylmethylsulfonyl fluoride) was added to the column. After centrifugation for 45 min at 15,000 x *g* at 4 °C, the flow-through from the column was discarded and the concentrated protein solution used for gel electrophoresis. The procedure was repeated two times with 450 µl of cold BN buffer (Camacho-Carvajal *et al.*, 2004).

b) 1D BN-PAGE

BN-PAGE electrophoresis was performed by preparing 5 %-13 % gradient native polyacrylamide gels (0.75 mm thick) according to Table 3.1 & Figure 3.2 A. Buffers used for the BN-PAGE were prepared as follows: **Blue Cathode Buffer** (50 mM Tricine, 15 mM Bis-Tris and 0.02 % of Coomassie Blue G250 (Sigma)), **Anode Buffer** (50 mM Bis-Tris) and **3x Buffer** (200 mM ε-Amino Caproic acid, 150 mM Bis-Tris). The cathode and 3x buffers were adjusted to pH 7 with HCl (4^{o}C).

The mini-Protean II electrophoresis system (BioRad labs Ltd.) was prepared according to the manufacturer's manual. Anode Buffer was first added to the reservoir in the lower chamber, and then the Blue Cathode Buffer was added to the upper chamber taking care to not mix the Cathode and Anode buffers. Samples (20 µl) were added to the sample wells, and ferritin (10mg/ml) and BSA (10mg/ml) in BN buffer were added to an additional well as MW markers.

Table 4.1 Volumes of reagents used to prepare gradient gels (modified from Nijtmans *et al.,* 2002).

Solution	***A*** ***5 %***	***B*** ***13 %***	***Stacking*** ***4 %***
Acryl-Bis mix (48:1)	1.01 ml	2.60 ml	0.40 ml
3x buffer	3.33 ml	3.33 ml	1.67 ml
water	5.39 ml	1.98 ml	2.87 ml
glycerol	0	2.00 ml	0
10 % APS	60.0 µl	30.0 µl	55.0 µl
TEMED	6.0 µl	3.0 µl	5.5 µl
Total volume	10 ml	10 ml	5 ml

During the BN electrophoresis, the proteins in the samples acquire their negative charge from the Coomassie Blue. The samples were first subjected to 80 V until the samples were observed to penetrate the gradient gel, and once in the gradient gel (separating gel) the voltage was increased to 200 V. The electrophoresis apparatus was connected to a water cooler (LEEC Incubator) to maintain the temperature of the gel at 4^{o}C during the electrophoresis procedure. After having reached the middle part of the gel, the Blue Cathode buffer was exchanged for a buffer prepared as described for the Blue Cathode buffer but without the Coomassie Blue (*i.e.* it was colourless), so this buffer destained the gel during the rest of the run. Once the electrophoresis run was completed, the gels were stained as described for SDS-PAGE in Section 3.2.4.2.

c) 2D SDS-PAGE

Once the BN-PAGE was complete, a lane was sliced from the gel as shown in Figure 3.2 and equilibrated for 15 min in Laemmli loading buffer (2x SDS). The

strip of the gel was placed horizontally onto a 10 % SDS gel with a 1.5 mm thickness, taking care to not introduce any bubbles between the interfaces of the two gels (Laemmli, 1970) as shown in Figure 3.2 B. A gap was introduced to the left of the 1D gel (BN) so that 20 µl of Full Range Rainbow marker (Amersham) could be added to the gel. The SDS-gel was run at 200 V until the bromophenol blue dye reached the bottom of the gel. Subsequent staining procedures were as described for the SDS-PAGE outlined in Section 3.2.4.2.

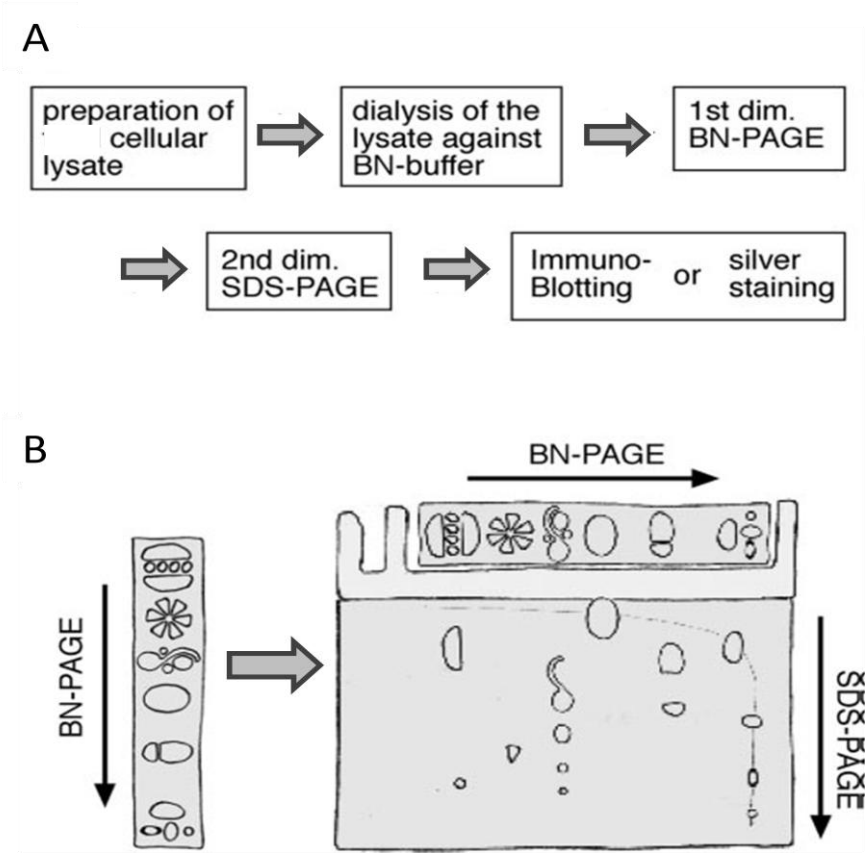


Figure 4.2. 2D Blue Native polyacrylamide gel electrophoresis. (A) Flow diagram of BN-PAGE procedure, *i.e.* extraction of protein, elution to remove small (< 10 kDa), running of 1D gradients gels, running of cut sample lane from 1D on SDS-PAGE gels for 2D analysis, and staining to visualise separated proteins; (B) Principle of 2DBN/SDS-PAGE. Cut lane from 1D BN-PAGE contain protein complex separated according to their size by the gradient gel, bigger at top and smaller complexes at the bottom of the lane. After SDS-PAGE run of the cut lane, the components of every complex are separated according size (image from Camacho-Carvajal *et al.,* 2004, page 177).

4.2.4.4 **Western Blotting (WB)**

In order to determine which proteins were being recognized by mAb TcOm15, the gels from SDS-PAGE and BN-PAGE were immunoblotted with the mAb. Firstly, proteins separated by electrophoresis were transferred onto a nitrocellulose membrane according to Wiens (1990). The nitrocellulose membrane was then incubated in 1 % BSA in Tris-buffered saline (TBS) for at least 60 min at 21^{o}C to block non-specific binding sites on the membrane. The nitrocellulose paper was then washed 3 times with TTBS (Tris buffered saline plus 0.1 % of Tween 20) for 5 min on each wash. Next, the membrane was incubated with hybridoma supernatant containing mAb TcOm15 for a minimum of 1 h and again washed as described above. An anti-mouse IgG biotinylated second antibody (Sigma Aldrich) diluted 1:200 in TBS was added for 60 min and the blot was again washed as described above. The blot was incubated for a further 60 min with streptavidin-HRP (Vector) diluted 1:200 in TBS and washed as described giving a fourth wash with TBS without Tween 20. The blot was finally developed with a KPL 4CN substrate kit (KPL, Maryland, USA) according to manufacturer's instructions.

4.2.5 Immunoprecipitation (IP)

4.2.5.1 **Sequential extraction of proteins**

Sequential extraction of proteins was performed according to Lehner *et al.* (2003). Cytosol proteins were extracted in buffer containing 40 mM Tris and 150 units ml^{-1} Benzonase (Sigma Aldrich) using sonication to mechanically disrupt the cells (Lehner *et al.,* 2003). Samples were vortexed for 5 min, and sonicated for 10

min, repeating this process twice. The membrane proteins were then extracted using CHAPS 1 % as described in Section 3.2.4.1.

In order to reduce the amount of contaminating proteins in the sample lysate a pre-clearing step was performed by incubating 450 µl of cell lysates with 50 µl prepared Protein G slurry (Sigma) for 45 min on ice according to eBiosciences protocol (www.ebioscience.com/ebioscience/appls/IP.htm). After incubation, the sample was centrifugated at 10,000 g for 10 min to remove the Protein G slurry. The supernatant was then recovered and used for immunoprecipitation (IP).

4.2.5.2 **Immunoprecipitation (IP)**

The supernatant collected in Section 3.2.5.1 was incubated overnight at 4°C with 200 µl of hybridoma supernatant containing mAb TcOm15. Protein G slurry was prepared in cold Lysis Buffer and 50 µl of this was added to the ag-ab complex. After 1 h at 4°C, the sample was centrifugated at 10,000 g for 30 sec and the protein G pellet collected. The Protein G beads containing the ag-ab complexes were extensively washed with 500 µl of cold lysis buffer and after centrifuging again the supernatant was removed from the pellet. Protein G beads were finally vortexed and heated for 10 min at 100°C with 1x Laemmli Sample Buffer (www.ebioscience.com/ ebioscience/appls/IP.htm). To recover the supernatant, the sample was centrifugated at 10,000 g for 30 sec and the supernatant used for SDS-PAGE and WB analysis according to Sections 3.2.4.2 and 3.2.4.4.

4.2.6 Protein identification

The antigen bound to mAb TcOm15 in the WB analysis were selected for identification by mass spectrometry. Protein samples were prepared from cells using lysis buffer (50mM Tris-HCl pH7.5, 25 mM NaCl, 1 mM EDTA, and 0.5 % NP40), and this was then sent for analysis by the FingerPrints Proteomics Facility at University of Dundee, UK. To increase the yield of proteins detected by Commassie Blue in SDS-PAGE, samples were concentrated using centrifugation in a filter column (Microcon YM30; Millipore). At the Proteomics Facility, samples were separated by electrophoresis, stained with colloidal Coomassie Blue and reductively alkylated in-gel. Bands of interest were selected and digested in 0.1 % n-Octyl Glucoside /20mM Ammonium Bicarbonate containing 12.5 μg/ml trypsin. Then, 20 μl of acetonitrile (ACN) were added to 20 μl of sample extract and 2.0 μl of the samples were spotted for peptide mass fingerprinting by MALDI-TOF or ESI MS/MS.

4.3 Results

4.3.1 Flow cytometry (FCM)

Levels of leukocytes in different tissues (PBL, thymus, spleen, and head kidney) were determined by staining the cells with mAb TcOm15 and analysed by FCM (Figure 3.3). The percentage of total positive leukocytes was highest in peripheral blood (39.4 % ± 3.2), moderate in spleen and head kidney (20.6 % ± 0.9 and 10.8 % ± 0.9), and very low in the thymus (7.1 % ± 0.8), as shown in Table 3.2. For all four organs examined, the highest number of labelled cells was observed in

the lymphocyte region (RLymph) of the dot plot in contrast to the granulocyte region (RGran), where stained cells were scarce. Leukocytes from peripheral blood were stained in a proportion of 27.1 % ±1.4 in the RLymph region and 9.3 % ±0.6 in the RGran region, while 13.8 % ±0.4 of spleen leukocytes were stained in the RLymph region and 4.5 % ±0.1 in RGran region when labelled with the mAb, and for head kidney 5.6 % ±0.6 of cells in the RLymph region and 4.5 % ±0.1 in the RGran region were stained. The exception was in the thymus, where a slightly higher number of reactive cells were seen in the RGran region of the dot plot *i.e.* 2.5 % ±0.3 compared to the proportion seen in the RLymph region, *i.e.* 2.0 % ±0.2 (Table 3.2 & Figure 3.4). A small percentage of staining was seen in the region marked as R1, for all organs where debris tends to be located (see Figure 3.4 and Table 3.2).

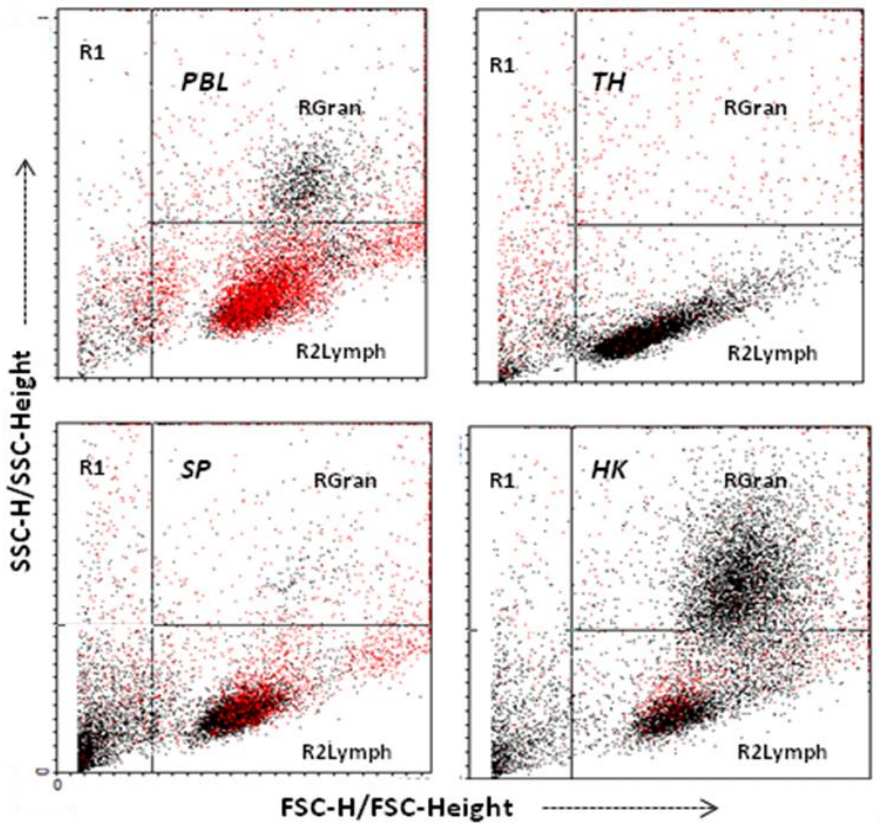


Figure 4.3 Example dot plots showing labelled cells in red for gate R1 (debris cells), R2Lymph (Lymphocytes), RGran (Granulocytes). PBL, peripheral blood leukocytes; TH, thymus; SP, spleen; HK, head kidney.

Positively labelled leukocytes in the peripheral blood and the spleen had a high measure of FSC and SSC in the region where lymphocytes are located and fewer cells were measured in the region where granulocytes can be found. Conversely, the proportion of positive cells, labelled with the mAb, seen in the head kidney and thymus populations of cells were similar for both the granulocyte region and the lymphocyte region (Table 3.2). Different percentages of TcOm15^{+} cells were obtained in the different organs examined for the three different fish analysed, however the pattern of labelling in the various organs was very similar between the three fish *i.e.* a high level of labelling with the PBLs, with a lower level of staining

with cells in the spleen, and head kidney and a small number of stained cells in the thymus (Table 3.2).

Table 4.2. Total percentages of leukocytes labelled with mAb TcOm15 in peripheral blood, thymus, spleen, and head kidney.

		Percentage ± s. d.
PBL	R1	3.0 ± 1.3
	Rgran	9.3 ± 0.6
	RLymph	27.1 ±1.4
	TOTAL	39.4 ± 3.2
TH	R1	2.6 ± 0.3
	Rgran	2.5 ± 0.3
	RLymph	2.0 ± 0.2
	TOTAL	7.1 ± 0.8
SP	R1	2.3 ± 0.4
	Rgran	4.5 ± 0.1
	RLymph	13.8 ± 0.4
	TOTAL	20.6 ± 0.9
HK	R1	0.7 ± 0.2
	Rgran	4.5 ± 0.1
	RLymph	5.6 ± 0.6
	TOTAL	10.8 ± 0.9

The results are presented as the mean ±s.d. for four organs from three fish with three replicate from each fish minus the background control value. PBL, peripheral blood leukocytes; TH, thymus; SP, spleen; HK, head kidney; R1 (primordial cells), R2Lymph (Lymphocytes), RGran (Granulocytes).

4.3.2 Microscope observation

4.3.2.1 **Immunohistochemistry (IHC)**

Positive staining was observed in all the tissues examined by IHC *i.e.* gills, thymus, spleen, head kidney, liver, and intestine.

Gills: tissue was strongly stained with mAb TcOm15 with preferential staining seen in the epithelial region of the tissue (Figure 3.4). Individual cells were stained in the primary lamellae with mAb TcOm15 (Figure 3.4 c & d).

Thymus: TcOm15^{+} cells seen in this tissue were restricted to the cortex of the thymus next to the epithelial cells of the branquial cavity. Groups of labelled cells were detected near to the cortex (see Figure 3.5 c & d). In contrast, cells from the medulla did not show any reaction with the mAb (see Figure 3.5 e). All thymus tissue appeared stained with the pAb serum collected from the fusion mouse when used as a positive control. No reaction was seen in the thymus tissue with the negative control (*i.e.* PBS).

Spleen: Positive staining was seen in the spleen with mAb TcOm15 (Figure 3.6). Stained cells were seen scattered throughout the tissue and the cells stained appeared "active" as it was possible to observe filopodia on cells (see Figure 3.6). Cells in spleen tissue did not show any preferential distribution of staining with the mAb.

Liver: The pAb gave a patchy pattern of staining in the lobules of liver tissue when used to stain this tissue (Figure 3.7 b and c). When liver was stained with the mAb numerous cells were labelled, and these were seen scattered throughout the liver tissue (Figure 3.7 d, e and f). Filopodia and pseudopodia, resembling interacting cells, were observed on many pairs or groups of labelled cells (Figure 3.7 e & f).

Head kidney: The pattern of cells stained with the mAb in the head kidney tissue, as shown Figure 3.8, was similar to that observed in the spleen with stained cells seen scattered throughout the tissue.

Intestine: The intestine displayed distinct staining with mAb TcOm15, reacting strongly with the contents of some goblet cells and secreted mucus (Figure

3.9). However, there were differences in the pattern of staining between the anterior and posterior intestine with the end of the intestinal tube being more reactive (Figure 3.9 e and f). Few stained cells were observed in lamina propia of intestine by IHC.

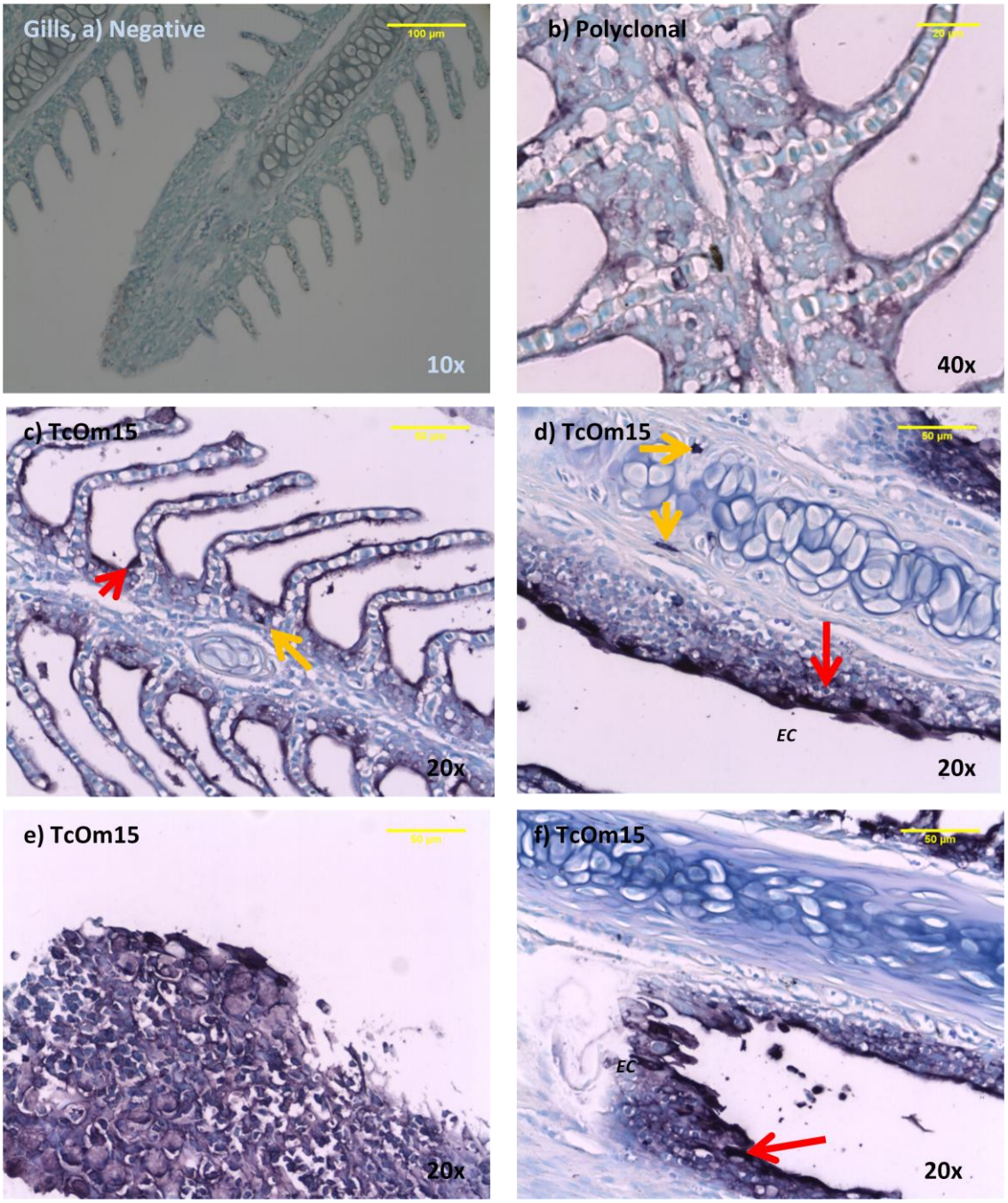


Figure 4.4 Immunohistochemistry of gills from rainbow trout. a) gill filament negative (i.e. PBS added in place of primary antibody), b) primary lamellae stained with polyclonal from M1CA, c), d), e) and f) stained with mAb TcOm15. e) tip of the primary lamellae showing strong staining of epithelia, and f) base of gill filament. Orange arrows, positive cells. Red arrow, strong epithelial staining. EC, epithelial cells.

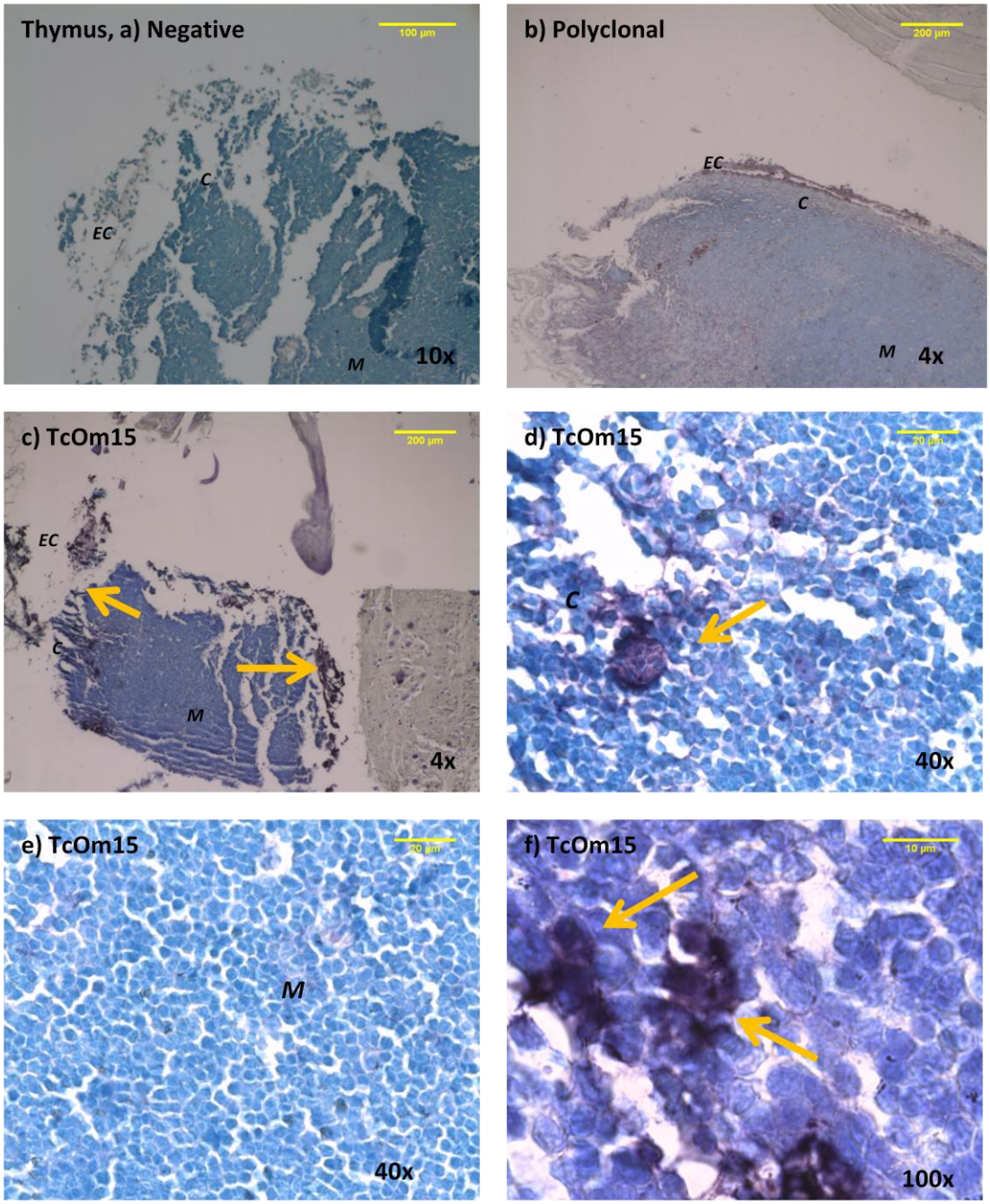


Figure 4.5 Immunohistochemistry of thymus from rainbow trout. a) tissue prepared as negative (*i.e.* PBS added in place of primary antibody), b) tissue stained with polyclonal from M1CA, c), d), e) and f) stained with mAb TcOm15. d) mAb TcOm15s showing labelled cells in groups (orange arrows), e) tissue from medulla (M) stained with mAb TcOm15 showing no reaction, f) stained cells from the cortex border (C) (orange arrows). EC, epithelial cortex.

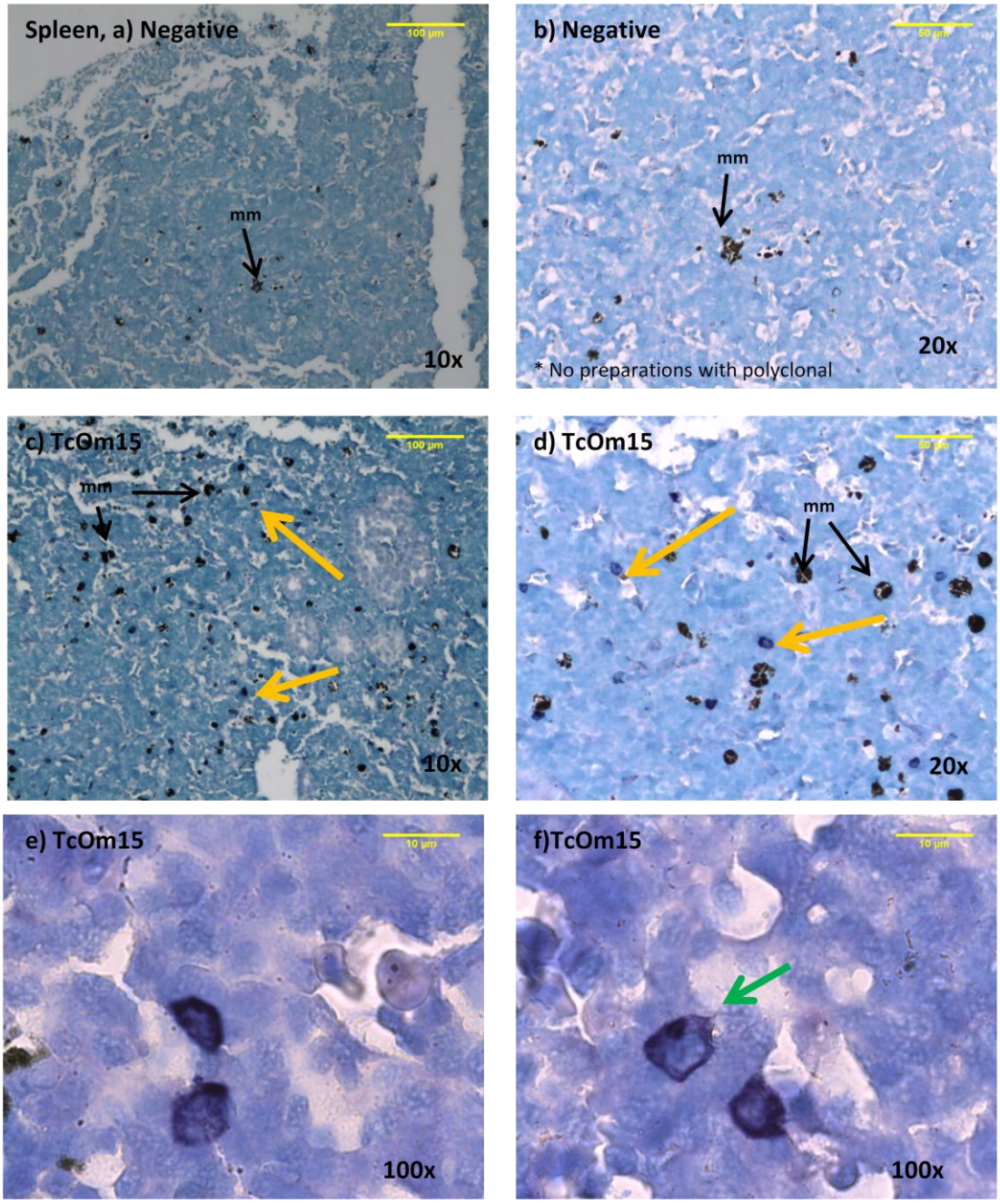


Figure 4.6 Immunohistochemistry of spleen from rainbow trout. a) and b) tissue prepared as negative (*i.e.* PBS added in place of primary antibody), c), d) e) and f) tissue stained with mAb TcOm15 (orange arrows show positive staining of cells), and filopodia can be observed in stained cells with mAb Tcom15 in f) indicated by green arrows. mm, melanomacrophages. Note: No polyclonal staining image was obtained.

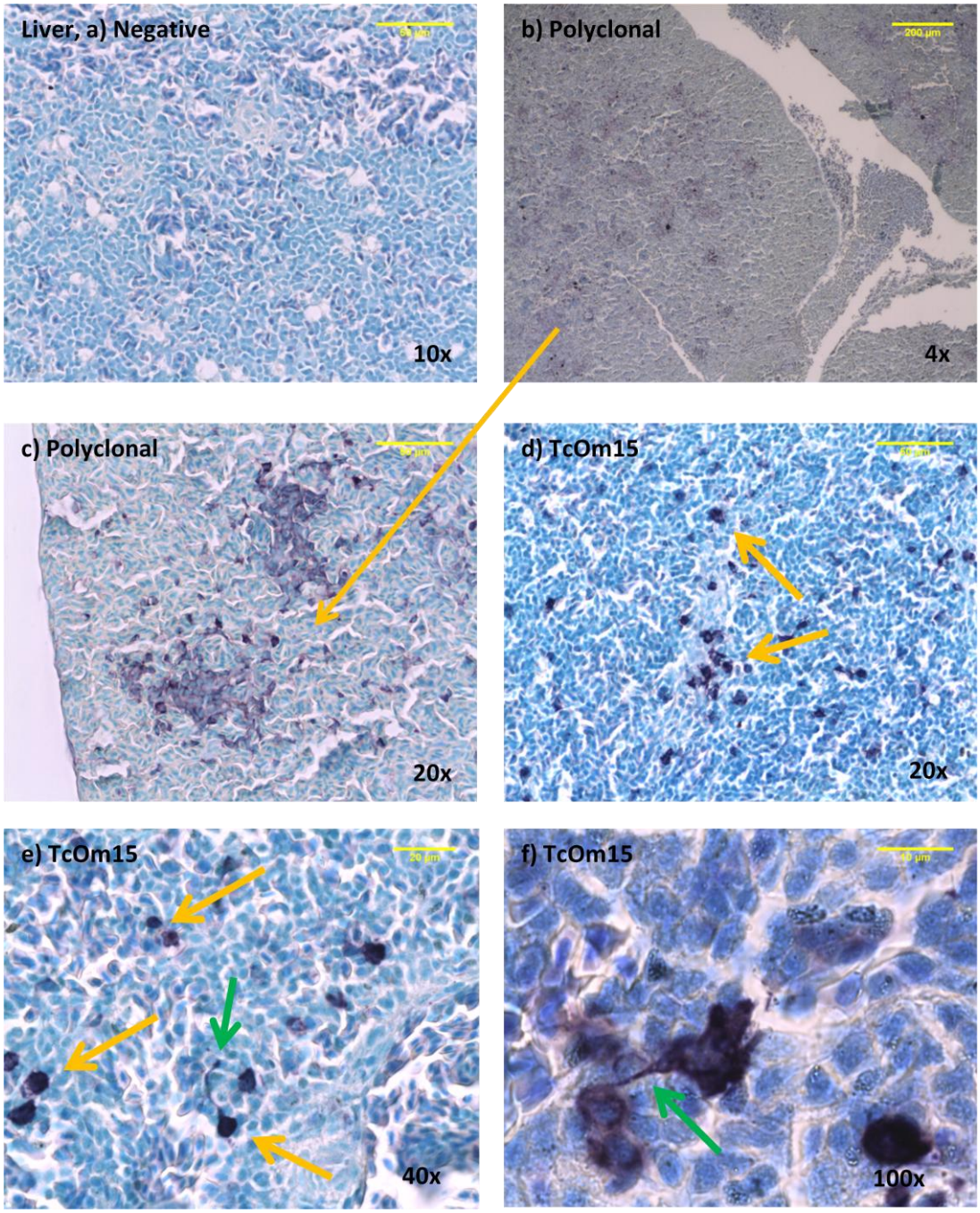


Figure 4.7 Immunohistochemistry of liver from rainbow trout. a) tissue prepared as negative (i.e. PBS added in place of primary antibody), b) and c) tissue stained with polyclonal from M1CA showing patched pattern of staining, d), e) and f) stained with mAb TcOm15. d) and e) tissue stained with mAb TcOm15 (orange arrows show positive staining of cells), and filopodia can be observed in stained cells with mAb TcOm15 in e) and f) indicated by green arrows.

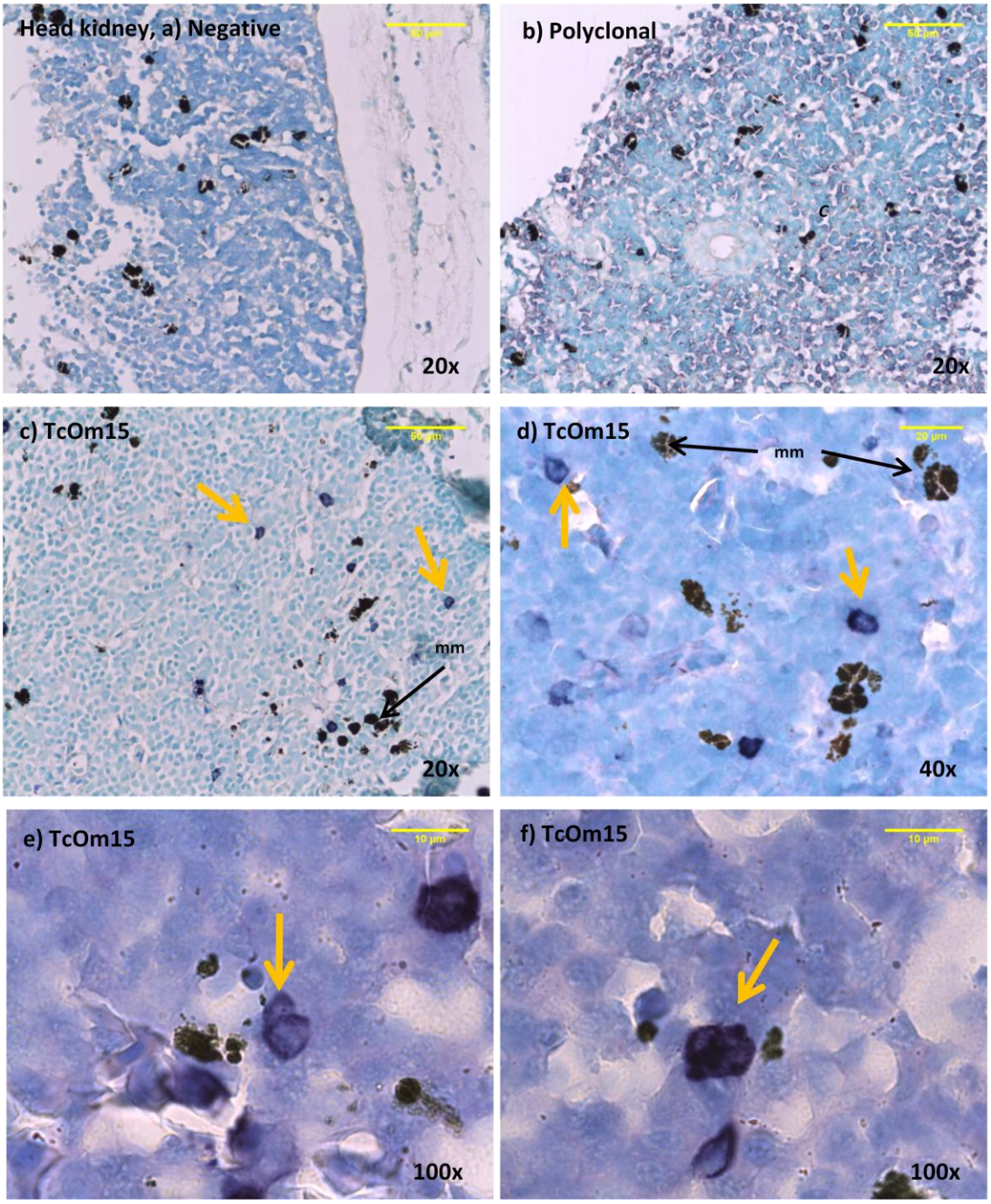


Figure 4.8 Immunohistochemistry of head kidney from rainbow trout. a) tissue prepared as negative (*i.e.* PBS added in place of primary antibody), b) tissue stained with polyclonal from M1CA, c), d), e) and f) stained with mAb TcOm15. Orange arrows show positive staining of cells. mm, melanomacrophages.

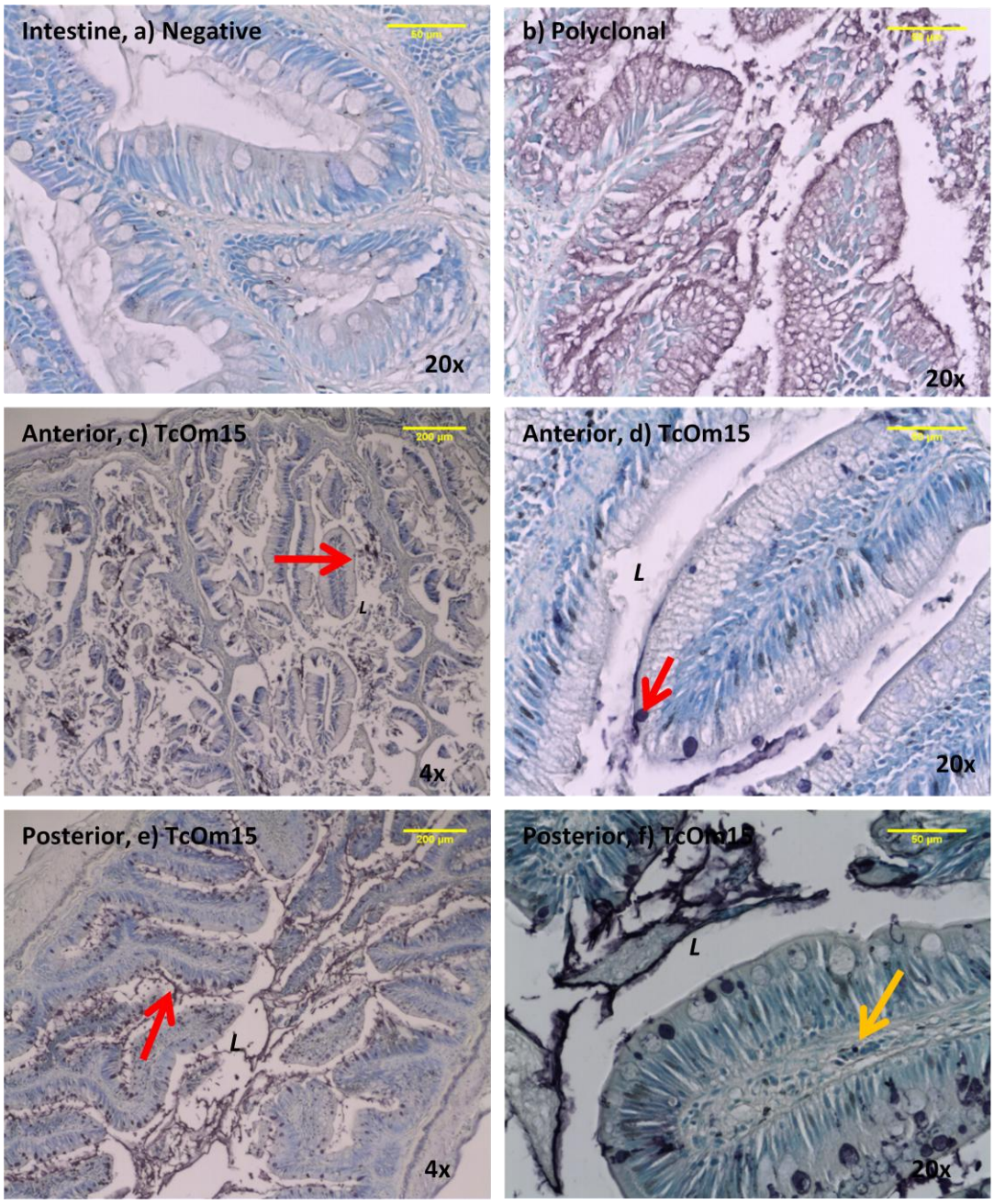


Figure 4.9 Immunohistochemistry of intestine from rainbow trout. a) tissue prepared as negative (*i.e.* PBS added in place of primary antibody), b) tissue stained with polyclonal from M1CA, c), d), e) and f) stained with mAb TcOm15. c) and d) IHC of anterior intestine, e) and f) IHC from posterior intestine. Red arrows show staining of mucus and some goblet cells. L, lumen.

4.3.2.2 **Confocal microscopy**

Peripheral blood cells were stained with mAb TcOm15 then viewed under a confocal microscope. Detailed observations indicated that certain cells did not stain with mAb TcOm15 (Figure 3.10 a). Stained cells that appeared to be granulocytes or lymphocytes showed polarisation of red staining that co-localised (white) with F-actin in green (Figure 3.10 b & c). The mAb react with lymphocyte-like cells, which were shown to have condensed cytoplasm and rounded nuclei (Figure 3.11). The cells had diffuse staining with the mAb over the whole membrane, as indicated by the red staining from the texas red fluorescent label used to identify binding with the mAb. Colocalisation of F-actin (indicated by green phalloidin), and the mAb TcOm15 reactive molecule (red) was observed (Figure 3.11). Monocyte-like cells with ruffling membranes were detected showing an irregular spotted staining with the mAb but not colocalization was detected (Figure 3.12). In Figure 3.13, it is possible to observe a lymphocyte-like cell and a monocyte-like cell where an apparent polarized staining was observed. Neither thrombocytes nor erythrocytes appeared to stain with the mAb TcOm15.

Due to the strong reaction observed with the mucus and secretions from goblets cells, some tissue sections were also prepared for confocal microscopy. When intestine was examined under the confocal microscope it was possible to visualise cells that reacted with mAb TcOm15 as shown in Figure 3.14. The strong reaction seen in IHC coincided with the staining seen under confocal microscopy, *i.e.* labelling of mucus and the secretions of some goblet cells in the posterior part of intestine, and negative controls were not stained observed (images not processed).

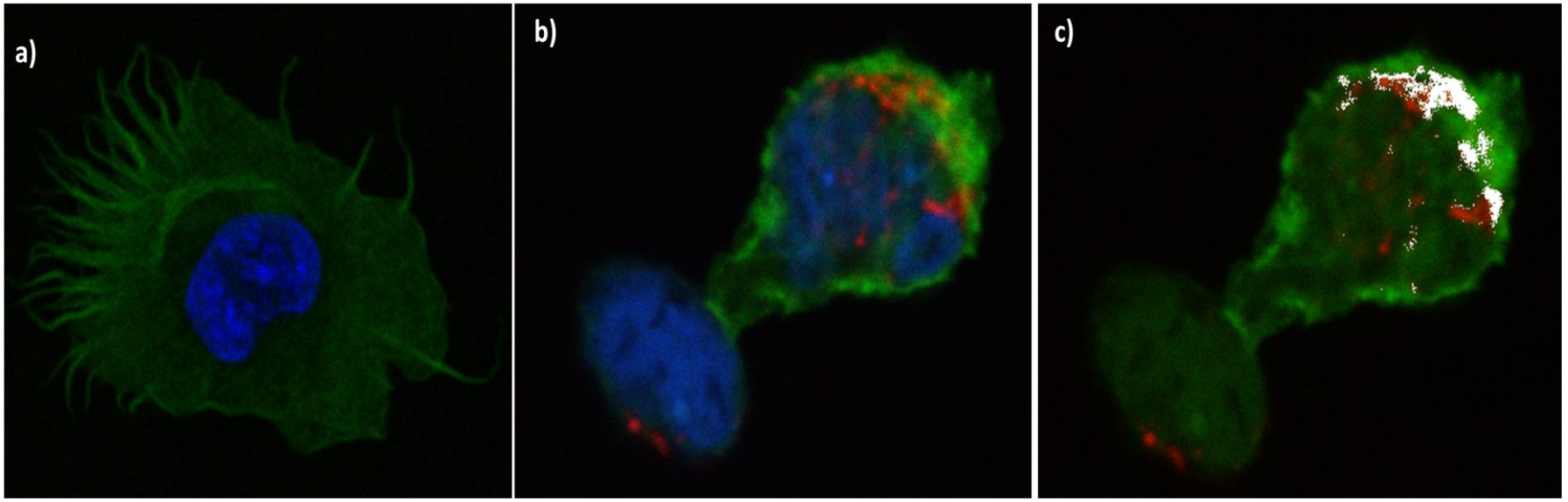


Figure 4.10 Confocal image of peripheral blood leukocytes from rainbow trout stained with mAb TcOm15 and anti-mouse Texas Red conjugate. a) image of monocyte-like cell showing no stain, b) granulocyte-like and lymphocyte-like cells with merged channels, and c) granulocyte-like and lymphocyte-like cells showing colocalization in white. Blue, nuclei stained with DAPI; green, actin stained with phalloidin; and red, TcOm15^{+} molecule labelled with Texas Red.

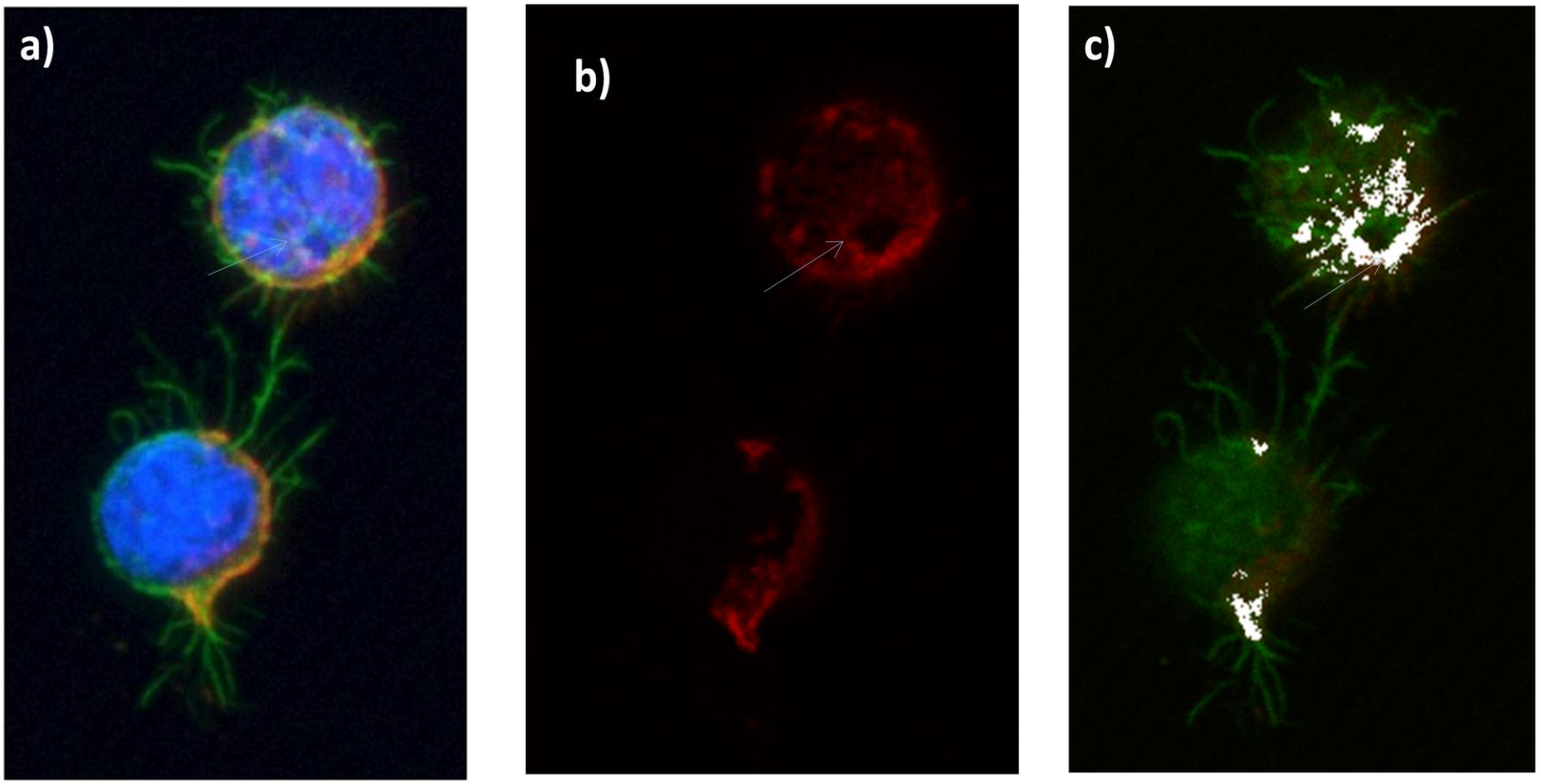


Figure 4.11 Confocal image of peripheral blood leukocytes from rainbow trout stained with mAb TcOm15 and anti-mouse Texas Red conjugate. a) image of lymphocyte-like cells with merged channels, b) separated channel for mAb TcOm15 in the Red channel, and c) image showing colocalization in white. Blue, nuclei stained with DAPI; green, actin stained with phalloidin; and red, TcOm15^{+} molecule labelled with Texas Red. Blue arrow shows a ring formation in the cytoskeleton.

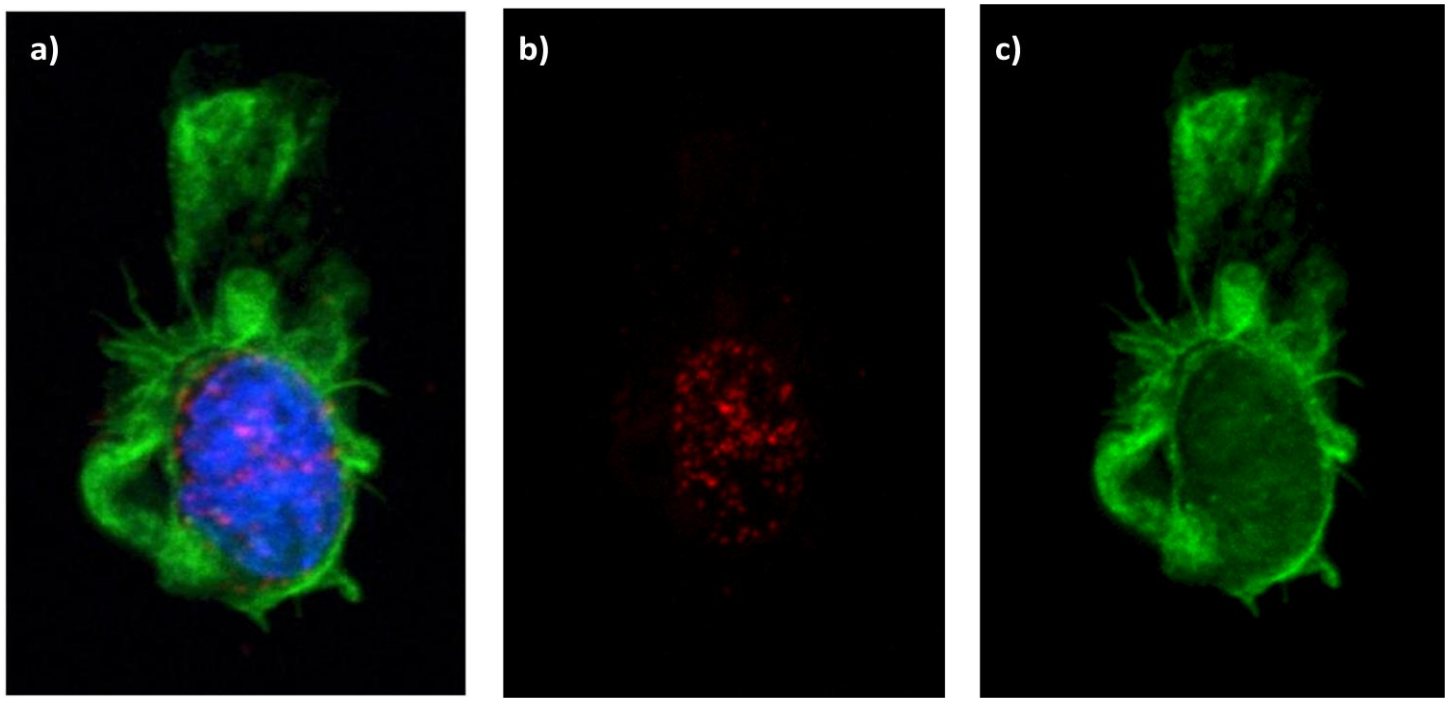


Figure 4.12 Confocal image of peripheral blood leukocytes from rainbow trout stained with mAb TcOm15 and anti-mouse Texas Red conjugate. a) Image of monocyte-like cell using merged channels, b) separated channel for mAb TcOm15 Red, and c) F-actin in the green channel. Blue, nuclei stained with DAPI; green, actin stained with phalloidin; and red, TcOm15^{+} molecule labelled with Texas Red.

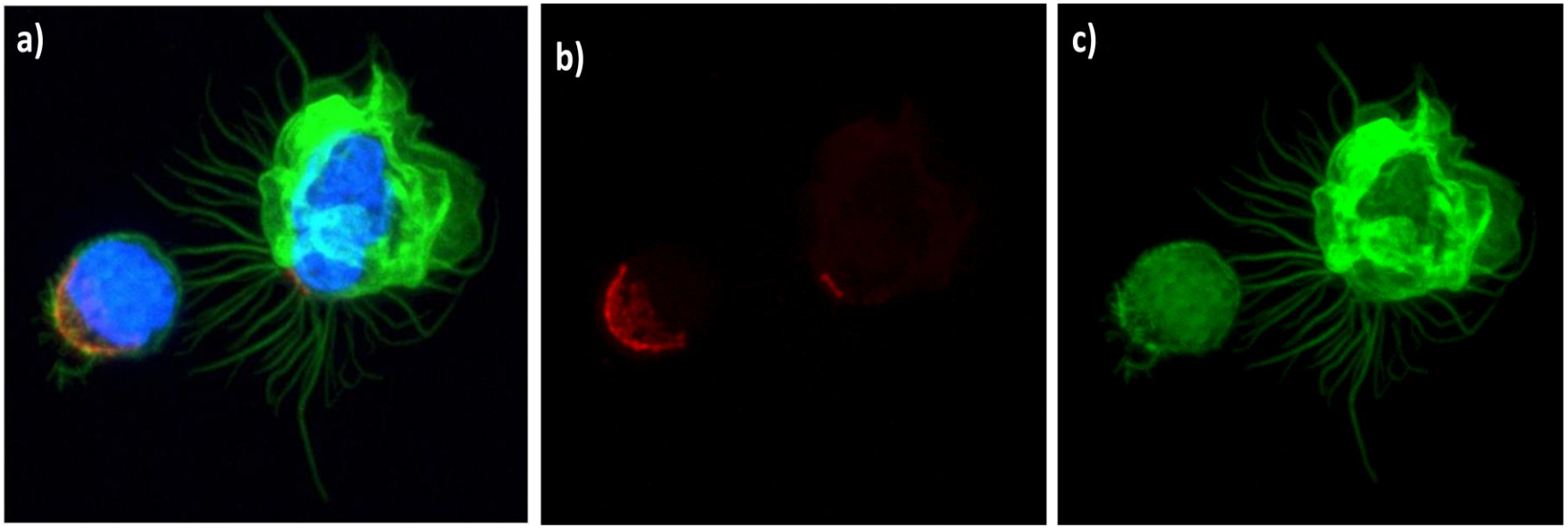


Figure 4.13 Confocal image of peripheral blood leukocytes from rainbow trout stained with mAb TcOm15 and anti-mouse Texas Red conjugate. a) image of a lymphocyte-like cell and a monocyte-like cell using merged channels, b) separated channel for TcOm15 in Red, and c) F-actin in the green channel. Blue, nuclei stained with DAPI; green, actin stained with phalloidin; and red, TcOm15^{+} molecule labelled with Texas Red.

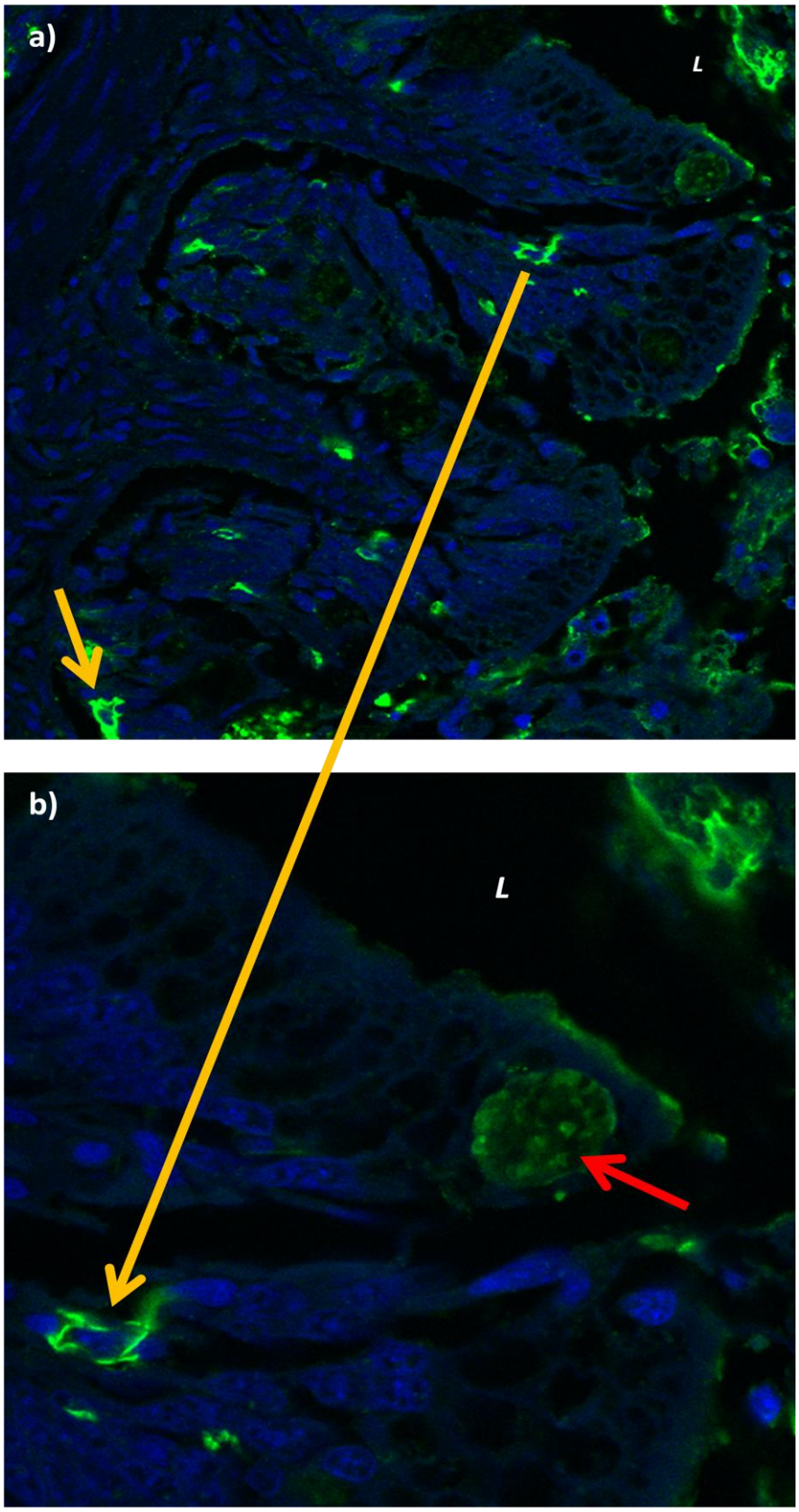


Figure 4.14 Confocal image of intestine from rainbow trout. a) and b) tissue stained with mAb TcOm15 and anti-mouse FITC conjugate (Green). Red arrows show staining of mucus and goblet cells. Orange arrow, stained cells. L, lumen.

4.3.3 Antigen identification

4.3.3.1 **1D SDS-PAGE and WB**

Polyacrylamide gel electrophoresis was used in order to determine the molecular weight of the antigen recognised by mAb TcOm15. As an initial approach, several detergents were tested in order to select the detergent that gave the better resolution of cell profiles in gel electrophoresis. Samples of PBL were lysed as described in Section 3.2.4.1. Nonidet NP40 was the detergent finally selected for lysis of cells as this detergent enabled optimal resolution of the protein bands (Figure 3.15 lane 4).

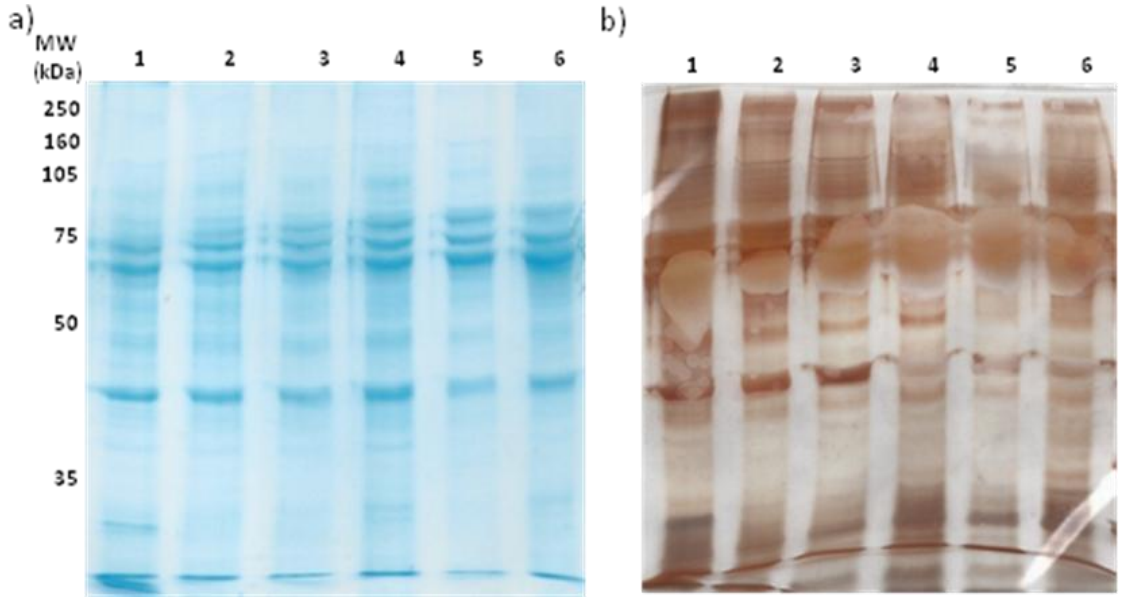


Figure 4.15 SDS-PAGE of cell lysates from rainbow trout PBLs extracted with 6 different detergents and stained with a) Coomassie Blue and b) silver stain. Lanes: (1) Saponin; (2) Triton x-100; (3) Tween 20; (4) NP40; (5) Tween 80; (6) CHAPS.

Several bands were detected by mAb TcOm15 in the solubilised leukocyte protein extracts prepared from different tissues treated with detergent NP40. Smeared staining was observed when gills, thymus, intestine, and mucus when

stained with mAb TcOm15 in Western blot analysis (Figure 3.16 & Figure 3.17). Intestine lysates had the highest number of proteins detected, but strong smearing was noted with this tissue (Figure 3.16). For proteins extracted from skin mucus an additional protein at 90 kDa was detected (Figure 3.16), and in gill and thymus a double band at 60 kDa was observed (Figure 3.17). Three proteins bands were detected at 280 kDa, at 160 kDa and 105 kDa in Western blot in spleen and head kidney tissue leukocytes (Figure 3.16 & Figure 3.17). When avidin amplification was used, faint bands at 280, 160 and 105 kDa were observed in PBLs and thymus (Figure 3.17). Two more bands at 70 and 130 kDa were detected due to a cross-reaction of the amplification (Figure 3.17). Protein bands detected by mAb TcOm15 in Western blot analysis, common between the PBL, thymus, spleen and head kidney tissue leukocytes were prepared for MS identification (*i.e.* 280, 160 and 105 kDa) (Figure 3.16 & Figure 3.17).

4.3.3.2 **2D Blue native-PAGE and Western blotting (WB)**

Further analysis of the antigen recognised by mAb TcOm15 was performed using two dimension gels under native conditions (BN-PAGE) to establish if the bands detected in WB were part of a complex of proteins.

Proteins from PBL were extracted with buffers containing one of the six detergents mentioned above. Solubilised proteins extracted with 1 % of CHAPS gave the best resolution after electrophoresis as observed in Figure 3.18. Previous attempts to run electrophoresis showed smearing when proteins were extracted with Tween 80 (0.1 %), a problem that was resolved by using the detergent CHAPS (1 %).

Solubilised proteins from leukocytes isolated from different tissues were separated on linear 5-13 % gradient polyacrylamide gels. Several bands were obtained showing successful separation of the proteins. However, when attempts were made to perform Western blot on the 1D BN gels (nature conditions), it was not possible to successfully transfer the proteins onto the nitrocellulose membrane. Due to the higher resolution and greater band definition obtained using denaturing conditions (Figure 3.18) and non-denaturing conditions for proteins from spleen leukocytes (Figure 3.19), these cells were used as the samples for the 2D gels. Initially WB analysis of 2D gels with mAb TcOm15 gave a reaction with a band between 80 and 250 kDa in a 10 % gel (Figure 3.20 indicated by the red boxes). However, it was not possible to observe this reaction again when the assay was repeated.

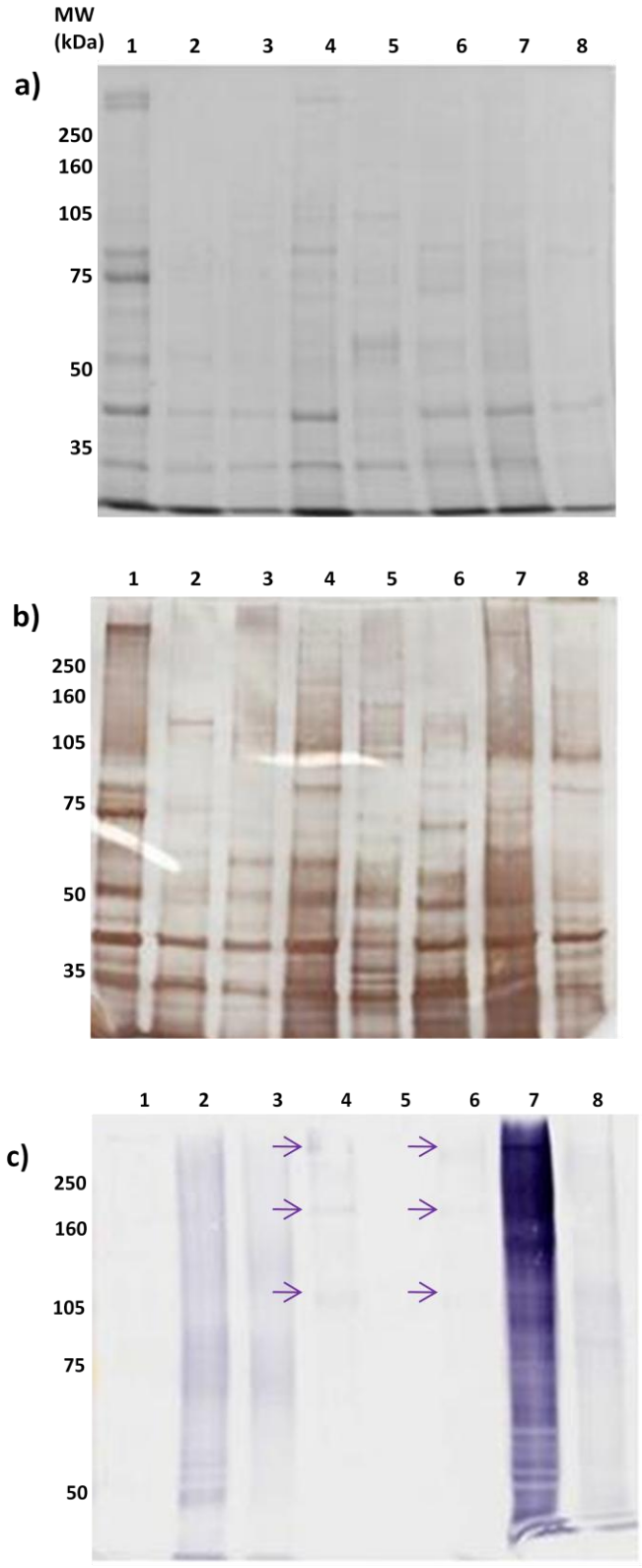


Figure 4.16 Gel electrophoresis and Western blotting analysis of eight different tissues from rainbow trout. a) SDS-PAGE stained with Coomassie Blue, b) Silver stained, and c) Western blot with mAb TcOm15, arrows indicate common proteins bands found in spleen and head kidney leukocytes. 7 % gels under reducing conditions. (1) PBL, (2) gills, (3) thymus, (4) spleen, (5) liver, (6) head kidney, (7) intestine, (8) mucus.

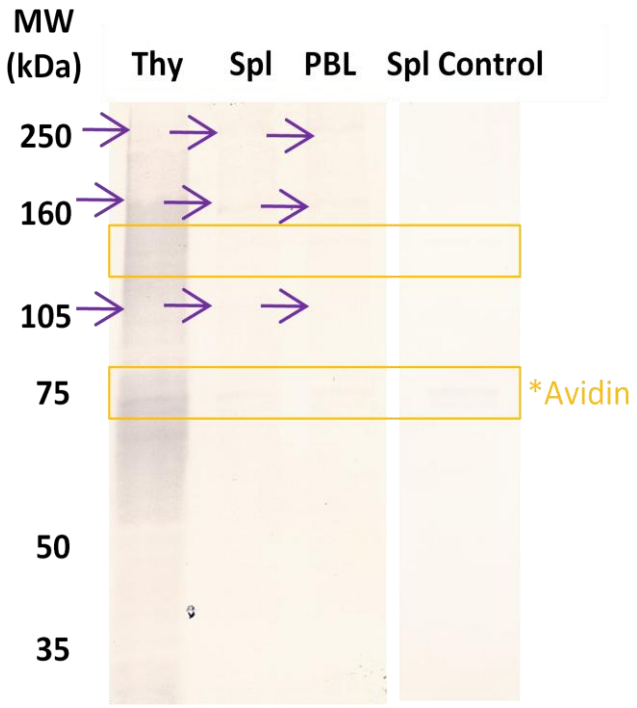


Figure 4.17 Western blot analysis with mAb TcOm15 and marked with a box the proteins that cross-reacted with avidin. 7 % gel under denatured conditions. Protein leukocytes from Thy, thymus; Spl, spleen; PBL, peripheral blood; and Spl control, control for avidin-biotin. (Reaction amplified with avidin-biotin).

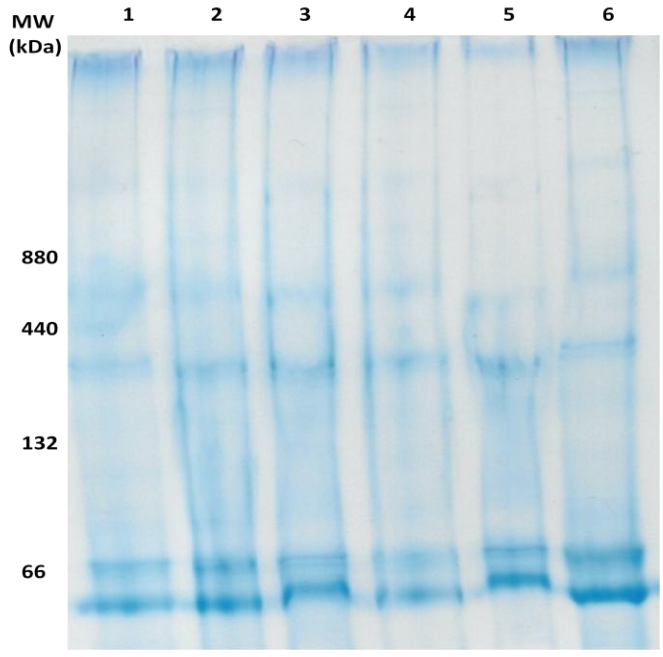


Figure 4.18 One dimensional blue native polyacrylamide gel showing solubilised proteins from PBL extracted with 6 different detergents and stained with Comassie Blue. (1) Saponin, (2) Triton X-100, (3) Tween 20, (4) NP40, (5) Tween 80, (6) CHAPS.

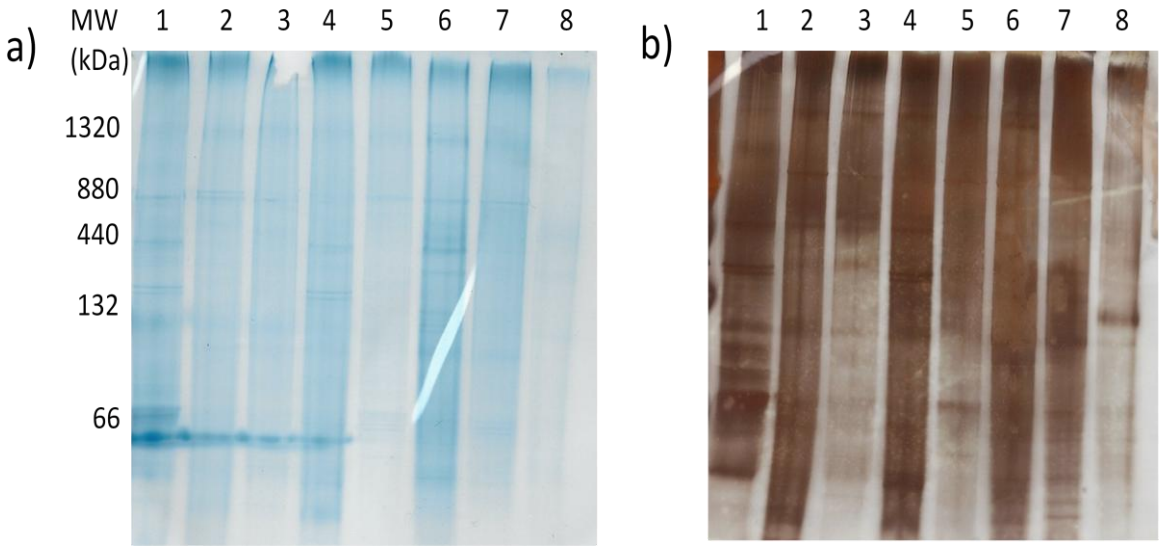


Figure 4.19 Gel electrophoresis and Western blotting analysis of eight different tissue sources of leukocytes stained with a) Coomassie Blue and b) Silver stain. Gradients gels from 5-13 % under nature conditions. (1) PBL, (2) gills, (3) thymus, (4) spleen, (5) liver, (6) head kidney, (7) intestine, (8) mucus.

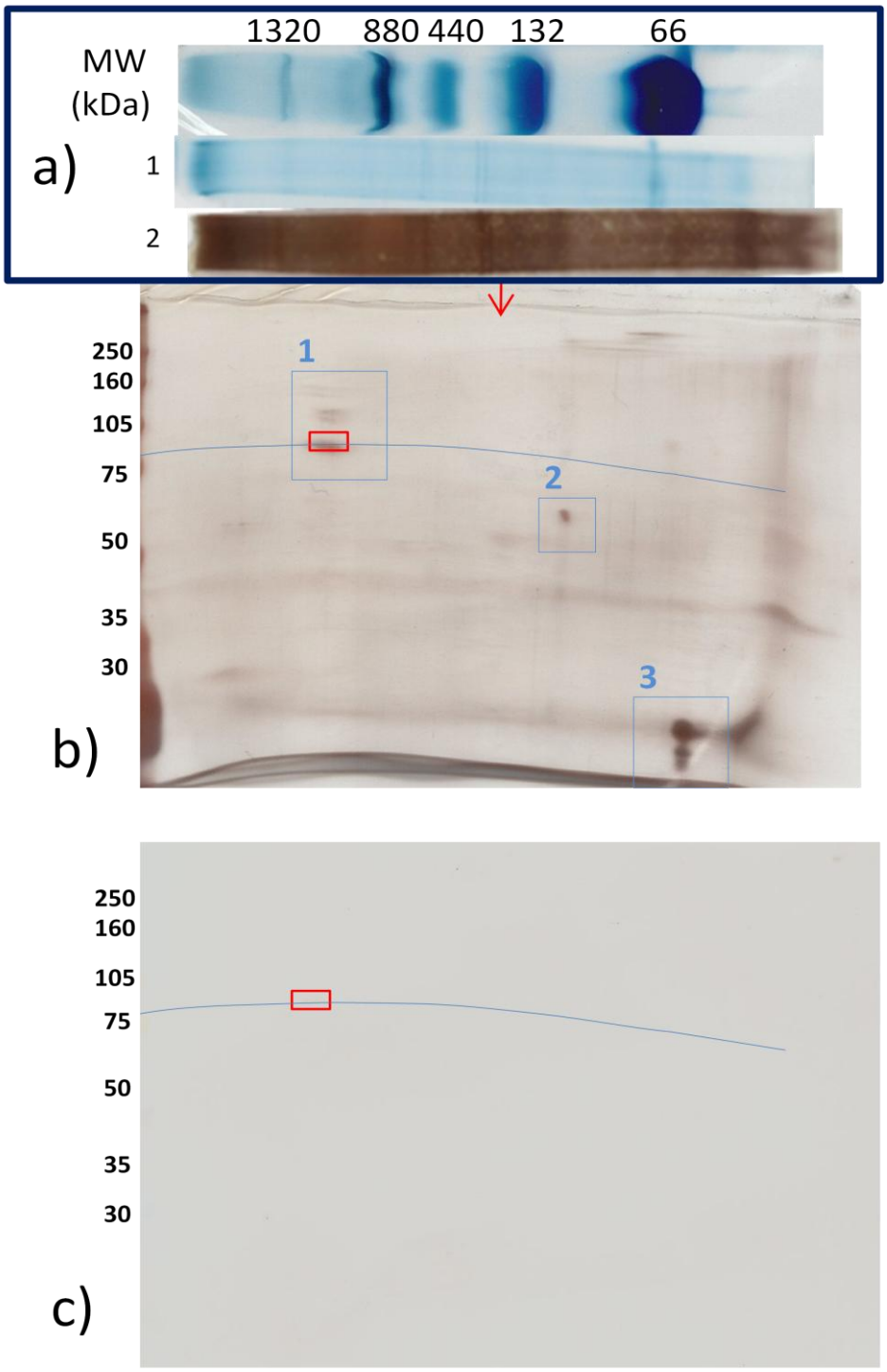


Figure 4.20 One dimensional blue native polyacrylamide gel of solubilised proteins from spleen cells a) Lanes, (MW) weight markers (ferritin and BSA), (1) Coomassie Blue, and (2) Silver stain. b) SDS-PAGE 8 % gel stained with silver showing resolved proteins, probably in a complex (blue box 1 and 3), and a single protein (blue box 2). c) Western blot gel showing a spot very faintly stained with mAb TcOm15 and indicated in b) and c) with a red box. Blue boxes showing proteins resolved in two repetitions.

4.3.4 Immunoprecipitation (IP)

Samples of spleen leukocytes were lysed for protein extraction. Proteins binding to mAb TcOm15 were immunoprecipitated according to the method described in Section 3.2.5. It was possible to isolate three proteins by IP (Figure 3.21) that had already been detected by mAb TcOm15 in lysates during Western blot analysis (Figure 3.15). The IP proteins from the cytosol lysates were detected at 105, 160 and 280 kDa, in an equivalent position to the bands previous detected in WB analysis. For membrane lysates, a faint band was observed at 105 kDa and another band at 160 kDa (Figure 3.21). Results comparable to the IP were obtained when solubilised proteins from cytosol and membranes that had not being immunoprecipitated were immunoblotted (Figure 3.21).

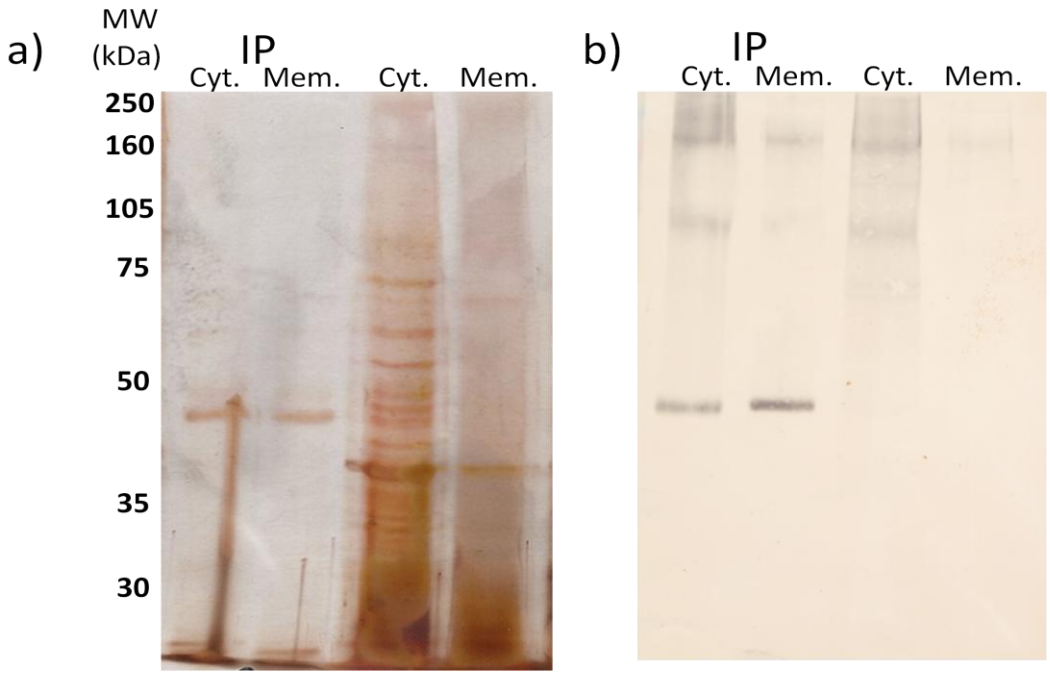


Figure 4.21 a) Silver stained SDS-PAGE gel of lysates from cytosol (Cyt.) and membranes (Mem.) from spleen leukocytes immunoprecipitated (IP) by mAb TcOm15 and non immunoprecipitated, and b) Western Blot labelled with TcOm15.

4.3.5 Protein identification

Attempts were made to identify the protein bands detected by the mAb TcOm15 in WB. Samples of leukocytes were prepared and sent to the Proteomics facility at the University of Dundee for analysis. The bands of interest were removed from the SDS-PAGE gel at the facility and the amino acid content of these was subsequently determined. The protein at 280 kDa was identified as Myosin (heavy polypeptide 10, non-muscle) with a molecular weight, according to the marker used by the Dundee facility, of 200 kDa. This protein showed the highest significant (>67) score of 127 from 5 different non-mucle myosins showed by the "hit" list during blasting with Mascot program.

From the band at 160 kDa, a long "hit" list (100 proteins) was obtained from the database search from which three results were significant. The results showed first a protein "Similar to spectrin αII chain (a non-erythroid α chain Spectrin)" with the highest score of 186 (>67). Whereas another two identities were immunoglobulins identified as Immunoglobulin lambda light chain (*Bos Taurus*) with a score of 131, and another "Similar to immunoglobulin lambda-like polypeptide precursor" with a score of 131 was registered.

The third protein at 105 kDa was identified as an α Actinin-4 (Figure 3.22) with a score of 376 from a short "hit" list where another 3 α Actinin-4 from three different species was obtained.

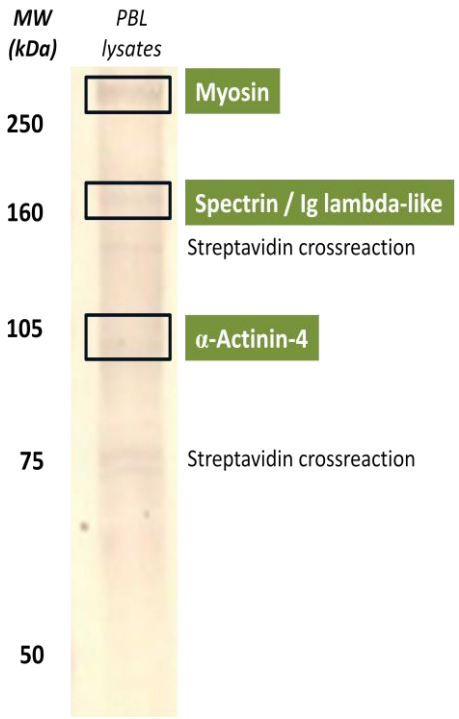


Figure 4.22 Western blot analysis of solubilised PBLs from rainbow trout with mAb TcOm15. Bands could be seen at 280, 160 and 105 kDa. Arrows indicate bands which also appeared in the Western blot negative control due to streptavidin amplification.

It was expected that proteins could be identified by IP, however it was difficult to obtain bands distinct enough and at a high enough concentration that could be selected for the mass fingerprinting technique. In an effort to obtain enough proteins, cells from PB, thymus, and spleen were cultured for 6 days with Con A and then IP with mAb TcOm15. This time it was possible to observe a band from the IP protein in the area of interest, *i.e.* a band at 160 kDa. From this preparation, the protein which bound to mAb TcOm15 was identified as a protein "similar to membrane-bound Immunoglobulin gamma-1 chain C region from *Bos Taurus*" (Figure 3.23). This identification was the only one score (292) significant from a list of 10 proteins showed.

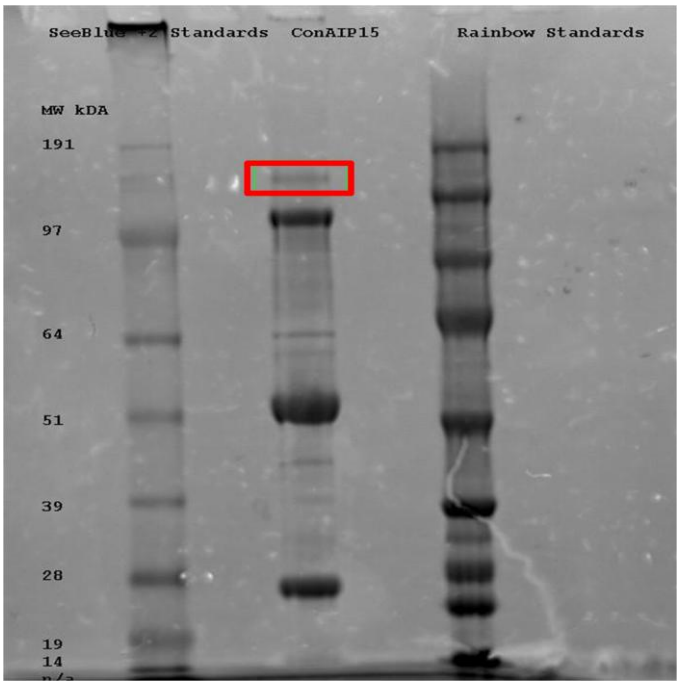


Figure 4.23 Electrophoresis gels performed at University of Dundee where a 160kDa band was isolated with immunoprecipitation of solubilised proteins from PB, thymus and spleen. Leukocytes were pooled after culturing for 6 days with Con A. Protein from band in red was identified as protein similar to membrane bound IgG1 C region from *Bos taurus*.

4.4 Discussion

Analysis by FCM, microscopy and WB showed that mAb TcOm15 not only reacted with thymic cells but also with cells from other tissues. From FCM analysis, the lymphocyte population was one of the main groups of cells recognised by this mAb (Figure 3.4 b). This region (RLymph) has been confirmed by several authors as a region containing populations of lymphocytes and thrombocytes (Romano *et al.,* 1997 & 1998; Esteban, Muñoz & Messeguer, 2000; Koellner *et al.,* 2001; Jansson *et al.,* 2003; Ronneseth, Wegerland & Pettersen, 2007). It is expected that the T-cell population is localised in this region, however, the proportions reported for fish T-cells in the thymus do not correspond to the ones analysed here by FCM. Of the two markers currently available against T-cells from fish, one recognised 73 % of labelled cells in the

thymus (Passer *et al.,* 1996) and the other gave a similar value of 74.8 % (Scapigliati *et al.,* 1995). The percentage of cells stained by mAb TcOm15 was very low *i.e.* 7 %. The results from IHC on the thymus show cells labelled with the mAb, distributed in the cortex of thymus with patches of cells near the thymic cortex. These findings thus indicate that mAb TcOm15 detects certain cells in the thymic population. It is interesting to note that Romano *et al.* (2007) found compartmentalisation in thymus *i.e.* cells expressing TcRβ localised in the periphery. This compartmentalisation was explained by a migration process where immature cells move from the subcapsular zone to the cortex and become mature. It is possible that, due to the fact that the cells used to immunise the mouse were activated, that those detected by mAb TcOm15 in the cortex are associated with cellular maturation, a presumption that is necessary to investigate.

The cells being labelled by mAb TcOm15 could possibly correspond to a B-cell population, however, when labelled cells were observed in IHC or confocal microscopy it was possible to observe cells other than lymphocytes being stained with the label. In the thymus 7 % of cells were labelled with mAb TcOm15 which was higher than labelling seen for Ig markers in the thymus of trout (1 % by 2A1 and 2H9 mAbs, Sanchez *et al.,* 1995) or 3 % DLIg3 for sea bass B-cells (Romano *et al.,* 1997). A considerably higher number of cells were stained in the thymus with mAb TcOm15 in IHC analysis compared to the 10 cells per section reported by Scapigliati *et al.,* (1995) detected with their DLIg3 mAb against B-cells IgM. Since the stained cells were detected in the lymphocyte region where thrombocytes have been reported, it is necessary to point out that cells stained for microscopy with mAb TcOm15 did not have the classical spindle shape described for thrombocytes (Koellner *et al.,* 2004).

From the results of FCM, another population that may be involved in the expression of the molecule recognised by mAb TcOm15 is granulocytes. However, no clear recognition was seen in the granulocyte region in FCM; instead disperse labelling of cells could be seen (Figure 3.3). Because of the inability to detect higher numbers of cells in the head kidney (an important tissue for granulocytes, Mulero *et al.,* 2007), it was presumed that mAb TcOm15 does not detect a specific population of granulocytes.

It was therefore concluded that mAb TcOm15 does not detect an antigen that can differentiate phenotypic differences between populations of cells, but rather is detecting molecules related to a functional state. Staining of PBL, when observed by confocal microscopy appeared to have diffuse staining in lymphocyte-like cells, possibly associated with the cytoskeleton, due to colocalization of F-actin and the TcOm15+ molecule (Figure 3.11, 3.12 & 3.13, F-actin and TcOm15) (Lodish *et al.,* 2001). It is important to notice that the staining preparation was aimed to detect surface molecules and therefore no permeabilization was performed on cells, where in such conditions mAb TcOm15 would have reach cytoskeletal molecules on cells. The staining in monocyte-like cells was not co-localised with F-actin, and showed a dotted pattern of the antigen localisation (Figure 3.14), suggesting no involvement of cytoskeletal molecules on those cells. More detailed research is needed in this respect.

Electrophoresis and Western blotting were performed to determine which specific proteins were detected by the mAb. Results of the Western blot using electrophoresed proteins under denaturing conditions with leukocytes from several different tissues showed that the mAb was not detecting a single protein. At least three proteins appeared to be identified by the mAb at 105, 160 and 200 kDa. The proteins

were identified as α actinin-4, spectrin αII chain or Ig-like protein and myosin (MYH10) by MALDI-TOF analysis and these are molecules that are compositional elements for the cytoskeleton of different types of cells (Black *et al.*, 1988; DeMatteis & Morrow, 2000, Dustin & Cooper, 2000; Xu *et al.,* 2003). As for spectrin it has been suggested that this molecule has, in humans, an important property as a migration mediator, and shares a role with actin and myosin, in mechanisms that are involved in lymphocyte activation such as cellular polarization and uropodia (Black *et al.*, 1988; Gregorio *et al.*, 1992; Evans *et al.,* 1993; Wang, Ostberg & Repasky, 1999; Bialkowska *et al.,* 2000). In non-erythroid human cells, spectrin functions as a cross-linker with actin filaments to give shape to cells, as a participant in protein sorting, vesicle trafficking or endocytosis (Bialkowska *et al.,* 2000). Even when it is ubiquitous in metazoan cells, there exist seven encoding genes for spectrin that express seven variants in specific cellular patterns, where αII spectrin is common for nucleated cells (Baines, 2010). Spectrin however, contains a common domain called SH3, which can be found in different proteins that participate in intramolecular interaction, subcellular localisation of signals, and mediate multiprotein complexes assemblies (Mayer, 2001). Further, spectrin repeats are found in cytoskeletal related proteins (actinin, dystrophin, calyrin, *etc.*) that serve as multivalent binding sites where structural proteins, cell membrane receptors, zinc-finger proteins, protein kinases, and other adaptor proteins can interact (Mislow *et al.,* 2002). On the other hand, cytoplasmic domains of transmembrane proteins directly interacting with spectrin repeats has been registered for integrins, ICAMs, L-selectin, and other receptor subunits (Djinovic-Carugo *et al.,* 2002). Therefore, the identification of these proteins by mAb TcOm15 suggests an ability of this mAb to detect an epitope appearing during a specific function, possibly related

with the synchronicity of expression or interaction for the three proteins identified. This hypothesis proposed by the lack of an extensive detection of common cytoskeletal proteins during microscopy studies, again indicates a possible recognition of the mAb that occur under certain circumstances. Another possibility is a strong carrier role during multiprotein complex formation for the interacting proteins. Such a carrier role has been reported for instance in α-actinin studies that demonstrate a relationship of the cytoskeletal protein with a transmembrane protein (L-selectin) evidenced initially by coimmunoprecipitation (Pavalko *et al.,* 2002). The results from the Western blot for cytosolic and membrane solubilised proteins, draws attention to the likelihood that the main protein being detected is Ig-like, because this molecule was detected more strongly in the membrane fraction. Another result confirms the involvement of Ig's due to the identification of the protein from the band at 160 kDa from immunoprecipitated proteins as a membrane-bound IgG-1 chain C region from *Bos Taurus*. On first impressions, it is possible to think that this finding is logical during immunoprecipitation due to the fact a mAb was used for the immunoprecipitation process. However, the MASCOT search to identify the protein at 160 kDa should have been able to give enough data for an IgG from mouse to be detected; instead it BLASTED the protein with a bovine IgG from membrane and not a secreted one from mouse. It is important to mention at this point that the identification from the protein at 160 kDa was difficult given the very low yield of protein for this band. Even then, the monoclonal with or without biotinylation always detected this band. Membrane bound IgGs are found in B-cells and have been the centre of many studies examining their role as membrane receptors (LeBien & Tedder, 2008). Initial studies on spectrin in humans aimed to explain the capping behaviour of membrane molecules

during activation or immunomodulation by mitogens (Turner, Newton & Shotton, 1988; Wang, Ostberg & Repasky, 1999). The rearrangement of molecules and the capping of antigen are thought to be a property of lymphocytes to allow membrane components to respond to stimulus during activation or function. With advances in human immunology, Masso-Welch *et al.,* (1999) found that the co-polarization of spectrin, ankyrin and protein kinase C at the site where membrane immunoglobulin from B cells capped was possibly involved in interactions of the antigen with T-cells. Taking into account the stimulation of the cells used for the immunoprecipitation of proteins (*i.e.* pooling cells stimulated with Con A) from which membrane bound IgG was identified, it is possible that this stimulation led to the promotion of some unknown signalling functions. Unfortunately, attempts to elucidate the interaction of the proteins involved, perhaps as a complex protein, by BN-PAGE were unsuccessful, possibly because the method was not completely optimized or the proteins involved were not bound together in a complex of molecules, even when they could interact with each other. However, it is a useful technique that has so far never been applied in fish studies. The possibility of using 2D BN-PAGE to separate proteins has advantages over isoelectrofocusing (IEF) during proteomics analysis given the possibility for having a higher representation of native membrane proteins. Added to that is the possibly of having information about the protein complexes themselves (separated in 1D gels) and about protein subunits that interact in the complex (in the 2D gels) (Reisinger & Eichacker, 2007). Results achieved with this technique for the separation of proteins in one dimension were successful, and indicate the possibility to using this method to help understand how the membrane proteins from fish leukocytes interact.

Based on results from the present chapter, it appears that mAb TcOm15 not only recognises part of a population of thymic cells, but also recognises other populations of leukocytes. However, even though the mAb does not help to differentiate cell populations it appears to be a potentially useful marker for examining the cytoskeleton activity of cells. This is proposed by the fact that mAb TcOm15 detected proteins involved in the organisation of the cytoskeleton and also detected cells undertaking certain functions; this needs to be further investigated.

Chapter 5 Antigen Expression on Activated Cells

5.1 Introduction

The induction of lymphocyte activation by mitogens *in vitro* is a technique widely used to study the ability of lymphocytes to be stimulated non-specifically. These assays have been applied to fish lymphocyte populations to evaluate the ability of the mitogens to promote mitosis (Kaattari & Yui, 1987; Reitan & Thuvander, 1991; Wang *et al.*, 1997; Siegl *et al.,* 1998; Scharsack *et al.*, 2000; Espelid, Steiro & Johansen, 2003; Gauthier *et al.*, 2003, Morrison *et al.*, 2004), to elucidate the genetic lineage of responsive populations (Araki *et al.*, 2008), to promote the expression of specific genes (Pelegrin *et al.*, 2001; Savan & Sakai, 2002; Blohm, Siegl & Kollner, 2003; MacKenzie, Planas & Goetz, 2003), and to understand mechanisms of lymphocyte activation (Ferriere *et al.*, 1999). A significant finding from these studies was the ability of $sIgM^{+ve}$ and $sIgM^{-ve}$ lymphocytes from fish to activate in response to stimulation with B-cell and T-cell mitogens, respectively (Kehrer, Hannan & Raison, 1998; Blohm, Siegl & Koellner, 2003, Araki *et al.*, 2008).

The mixed leukocyte reaction (MLR) is another important assay that has been used to examine the participation of specific subpopulations of T-cells during non-self recognition of allogeneic histocompatibility complexes. (Caspi & Avtalion, 1984, Miller, Deuter & Clem, 1986; Meloni *et al.,* 2006; Majji *et al.*, 2009).

Cellular activation is triggered by a variety of signal molecules from different biological and chemical origins such as proteins, carbohydrates, lipids and nucleic acids. Receptors on the surface of cells detect the signals of danger and initiate the mechanisms that promote the expression of molecules involved in cell activation. An

example of proliferation of B cells after successful activation of the immunocompetent cell by a thymus-dependent antigen is illustrated in Figure 4.1.

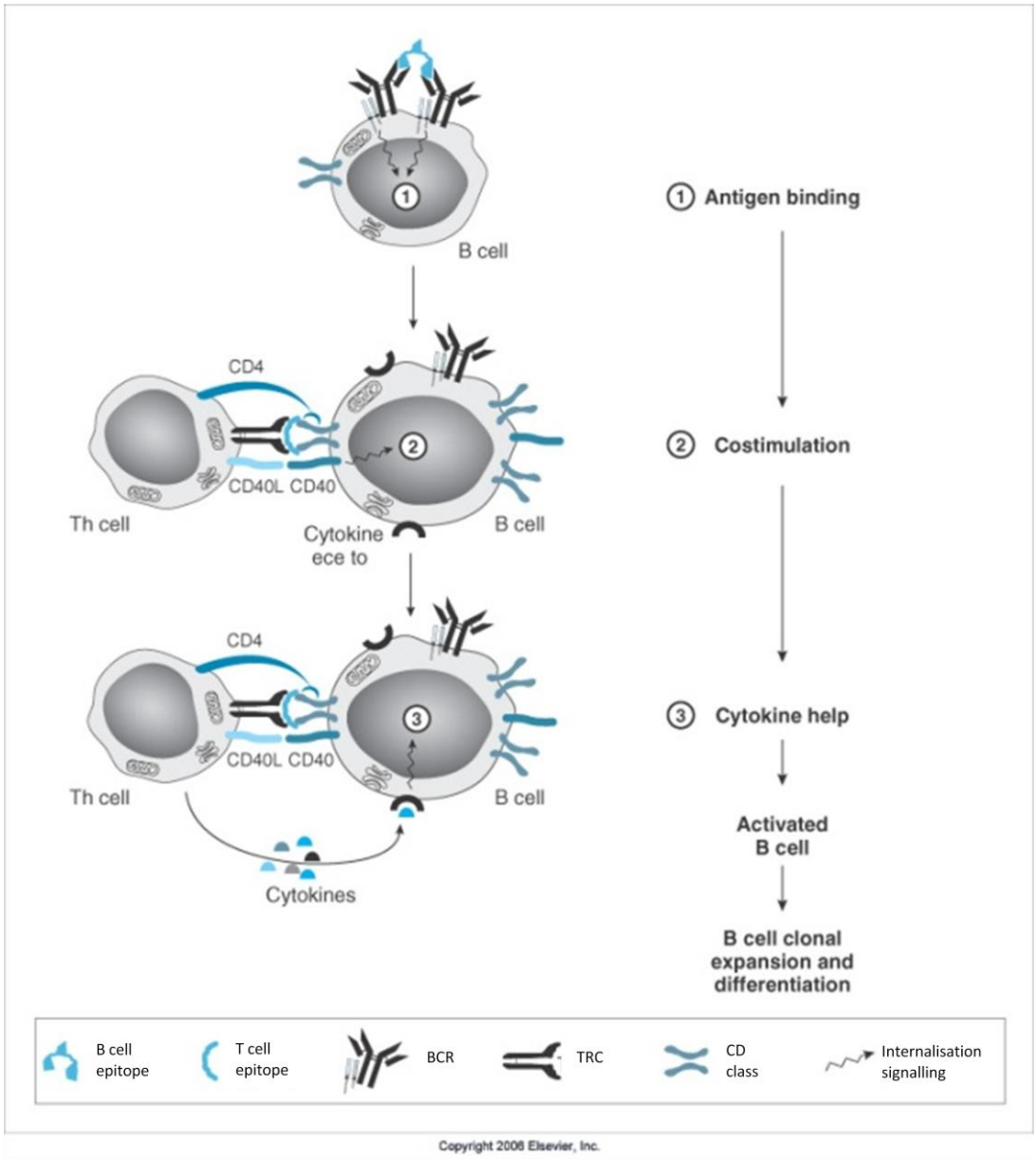


Figure 5.1 Diagram of the process of activation of B-cells by a thymic dependent antigen (from Mak & Saunders, 2006, page 219).

This activation process involves a number of complex phenotypic modifications that occur sequentially. The ability to detect human activated cells by means of membrane markers (for examples see Figure 4.2) has been used to evaluate the efficacy

of vaccines against important diseases such as measles and HIV (Schnorr *et al.*, 2001, Gurunathan *et al.*, 2009).

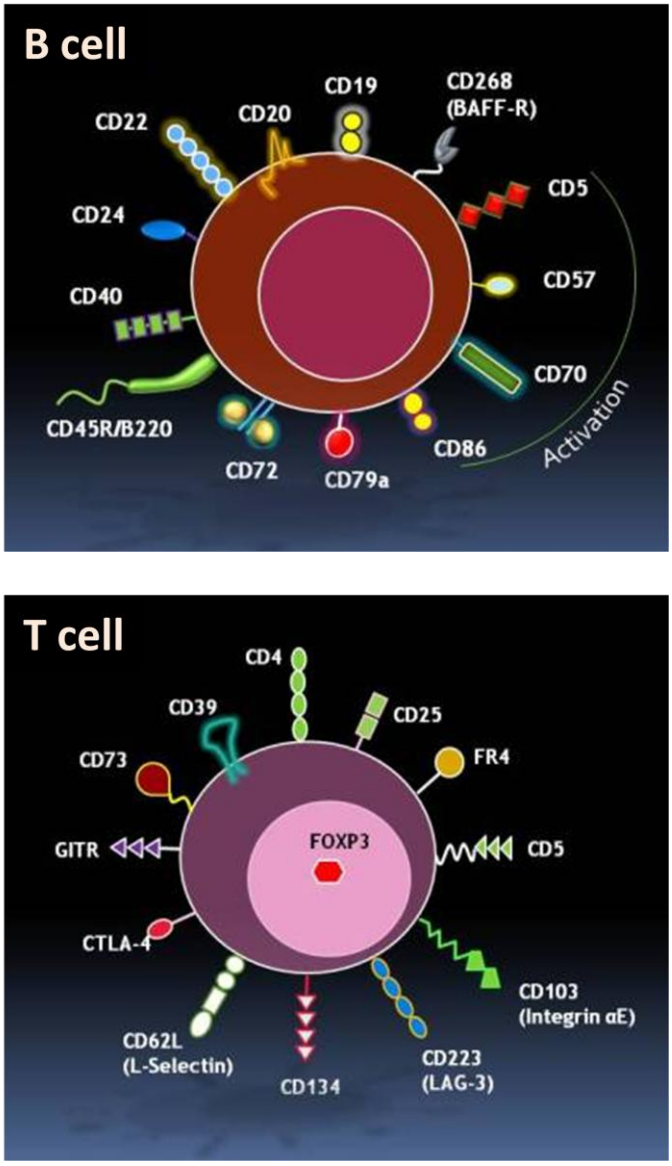


Figure 5.2 Example of markers for B-cells and T-regulatory-cells from human (images from www.biolegend.com; Nov, 2009).

Markers on the surface of leukocytes change both qualitatively and quantitatively according the different stages of differentiation or stimulation (Mak & Saunders, 2006). It has been found in mice, for example, that the number and type of

molecules expressed correlate with specific states of differentiation, as shown in Figure 4.3. The mechanisms of lymphocyte activation and expression of all markers during this activation are more and less understood for mammals, however, this is a relatively new field of research for teleost fish and the number of mAbs against membrane markers is still very limited.

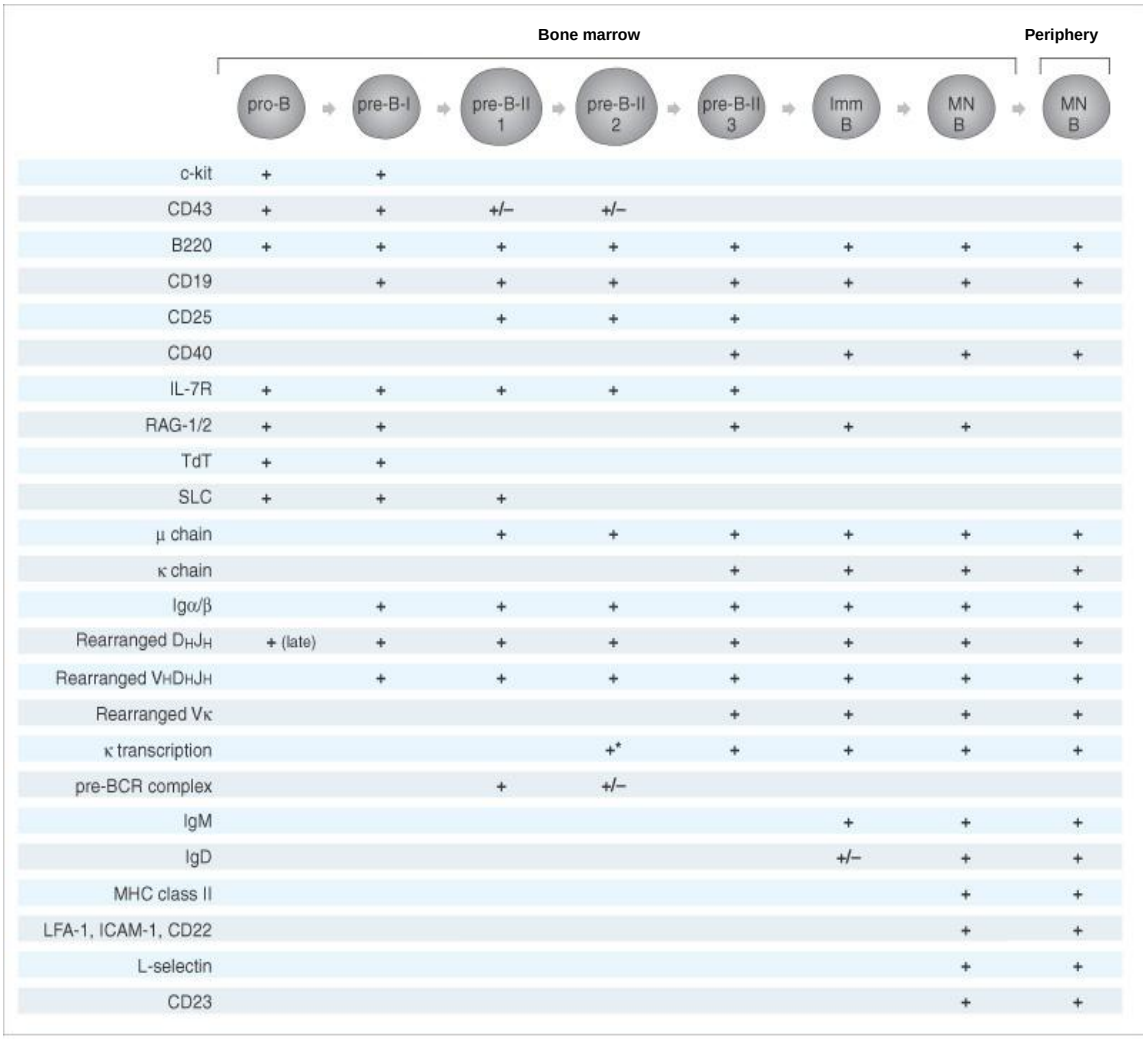


Figure 5.3 Markers for B-cells in mammals expressed through the maturation process (from Mak & Saunders, 2006, page 212).

Mitogens are compounds that have either been synthesized or extracted from plants or bacteria, and they can non-specifically activate cells in a variety of different ways. Lectins are mitogens from plants that interact with particular carbohydrate

moieties on membrane glycoproteins. Concanavalin-A (from *Canavalia ensiformis*), for example, is a mitogen for T-cells that has a mannose structure with an affinity for monosaccharides such as glucose and mannose. The phytohaemagglutinin from *Phaseolus vulgaris* (PHA) promotes the activation and proliferation of T-cells and binds to oligosaccharides such as galectins, while pokeweed mitogen (PWM), a lectin produced from *Phytolaca americana* induces activation and proliferation of B and T-cells, however the binding affinity has not been defined (Sharon & Lis, 2004).

Other types of compounds with mitogenic properties are the phorbol esters (*e.g.* PMA). These are toxic compounds derived from plants that interact with protein kinase C (PKC) and they affect the activity of a number of enzymes, biosynthesis of proteins, DNA, and polyamides, and also affect cell differentiation processes and gene expression. Hyper-activation of PKC by phorbol has been used to trigger activation and proliferation of lymphocytes (B and T) in stimulation assays (Goel *et al*., 2007) as well as stimulation of fish macrophages (Nagelkerke *et al.,* 1990). Polyriboinosinic polyribocytidylic acid (Poly I:C) is a synthetic double-stranded RNA that resembles double-stranded RNA present during viral infections, and is also used to stimulate macrophages. Lipolysaccharide (LPS) is a compound found on the outer membrane of Gram negative bacteria and it is able to initiate the activation and proliferation of B-cells (Han *et al*., 2003).

A different approach has been the use of various synthetic or naturally derived products from plants, animals, or bacteria to promote activation of the immune system *in vivo*. These substances have been used as immunostimulants to promote an immune response against infectious diseases in several fish species (Mulero *et al.,* 1998; Sakai 1999; Sealey & Gatlin III 2001, Cuesta *et al.,* 2003 & 2007, Bricknell & Dalmo, 2005).

Some studies have demonstrated that the administration of these substances during feeding can improve the production of specific components of the immune response, for example antibody production (Thompson *et al.* 1993), chemotaxis (Galindo *et al.,* 2004), migration and phagocytosis (Mulero *et al.,* 1998), or an increase in leukocyte numbers (Ogier *et al.,* 1996).

Activation of cellular immune components through the use of mitogens in the laboratory or immunostimulats *in vivo* could be useful in helping to understand which, how and when the molecules associated with activation and differentiation are expressed on the surface of fish cells.

Aim:

The aim of the present chapter was to examine the effects of different stimulators of leukocytes (*in vitro* and *in vivo)* on the expression of the TcOm15 antigen, and to evaluate the potential of this antigen as a cell marker.

5.2 Material and methods

The basic methodology for isolation and culture of leukocytes was previously described in Chapter 2. In the present chapter, modifications were made to these methods with respect to mitogen stimulation and evaluation. Mitogen stimulation was promoted using different doses selected to obtain the optimum response in assays and selection was based on related literature available (Caspi *et al.,* 1985, Lopresto, Kendall, & Burnett, 1994; Passer *et al.,* 1996; Siegl *et al.,* 1998; Espelid, Steiro & Johansen, 2003). The experiments will be described indicating the methodology employed. Data for

flow cytometry (FCM) were analysed with the WinMDI 2.9 programme from Joe Trotter, Scripps Research Institute, La Jolla, CA.

5.2.1 Detection of TcOm15+ cells on multiple way HCA assays by FCM

5.2.1.1 **Evaluation of one dose effects of Con A/LPS on mHCA assays**

Isolated peripheral blood leukocytes from three fish were prepared, as described in Section 2.3.1, and pooled together and prepared for culture according to the method in Section 2.3.2. Two different mitogens were used in separate cultures to enhance the mHCA. Con A from *Canavalia ensiformis*, type IV-S (Sigma-Aldrich) was added at a final concentration of 30 μgml^{-1} and LPS from *E. coli* (055:B5) (Sigma-Aldrich) at a final concentration of 100 μgml^{-1}. Cells from treated cultures were recovered 5 days later and stained with mAb TcOm15 to be evaluated by FCM according to Section 2.2.4.

5.2.1.2 **Evaluation of TcOm15+ cells with three doses of Con A/LPS on HCA assays**

The PBLs from five fish were pooled together and prepared for culture according to methodology outlined in Sections 2.2.1 and 2.2.2. Mitogens Con A from *Canavalia ensiformis*, type IV-S and LPS from *E. coli* (055:B5) were added separately at three different final concentrations of 15 μml^{-1}, 30 μml^{-1}, and 45 μml^{-1}. Cultures with mitogens were prepared in two batches of three replicate samples for analysis on Day 1 and Day 6 post-stimulation. Staining with mAb TcOm15 was evaluated by FCM on these days.

5.2.2 Detection of TcOm15+ activated PBLs from individual fish

5.2.2.1 Evaluation by FCM of TcOm15+ PBLs with one dose of Con A/LPS

Three fish were bled and leukocytes isolated as described in Section 2.3.1. Cells were cultured separately according to Section 2.3.2. Two different mitogens were used by separate for stimulation of the leukocytes; Con A from *Canavalia ensiformis*, type IV-S was added to obtain a final concentration of 30 μgml^{-1}, and a final concentration of 100 μgml^{-1} with LPS from *E. coli* (055:B5). Samples were prepared and evaluated by FCM 5 days later according to Section 2.2.4.

5.2.2.2 Evaluation by FCM and WB of TcOm15+ PBLs with six different mitogens

Leukocytes from 4 fish were isolated as described in Section 2.3.1, and cells were cultured separately according to Section 2.3.2. Six different mitogens were used individually to promote activation. Accordingly, separated batches with mitogens were prepared as follows: Con A from *Canavalia ensiformis*, type IV-S was added at a final concentration of 30 μgml^{-1} and 90 μgml-1, LPS from *E. coli* (055:B5) at a final concentration of 100 μgml^{-1}, PMA (Sigma-Aldrich) at a final concentration of 5 ng, PHA-P (Sigma-Aldrich) at 20 μgml^{-1}, PWM (Sigma-Aldrich) at 10 μgml^{-1} and Poly I:C (Sigma-Aldrich) at 100 μgml^{-1}. Samples were evaluated by FCM 3 days later according to Section 2.2.4.

A second experiment was performed under the same conditions with 6 fish and samples evaluated on Day 1 and Day 3 post-stimulation. Cells were fixed with Cytofix solution (BD Biosciences) before staining. A batch of cells from these cultures was also prepared for analysis by SDS-PAGE and Western blot (Section 3.2.4).

5.2.3 TcOm15 staining of activated PBLs by IIF

5.2.3.1 **Confocal microscopy evaluation of TcOm15+ PBLs activated with mitogens**

Peripheral blood leukocytes from 4 fish were isolated as described in Section 2.2.1, and cells were cultured according to Section 2.2.2 in 8 wells LabTek chamber glass slides (Nunc, Fisher Scientific, UK). Three different mitogens were used to promote activation by separate. Con A from *Canavalia ensiformis*, type IV-S was added at a final concentration of 90 μg ml^{-1}, LPS from *E. coli* (055:B5) at a final concentration of 100 μg ml^{-1}, and PMA at 5 ng ml^{-1}. Samples were evaluated by confocal microscopy after 20 min, and 1 hour post- stimulation.

Slides were washed two times with 200 μl of PBS/0.5 % BSA by inverting to remove the liquid. First they were incubated with 200 μl of mAb TcOm15 at 10 μg ml^{-1} in PBS/BSA for 30 min and then washed twice with this buffer. A further incubation of 30 min was performed with a second antibody anti-mouse conjugated with Texas Red (20 μlml^{-1} of PBS/BSA) and again washed twice as above. Subsequently, the slides were incubated with a solution to permeabilize and fix the cells (*i.e.* with 2 % paraformaldehyde (Sigma-Aldrich), 3 % sucrose (Sigma-Aldrich), 0.5 % Triton X-100 (Sigma-Aldrich), and 2.5 % of methanolic solution of Alexa Fluor 488 Phalloidin (Invitrogen) in PBS (pH 7.4) for 12 min). Finally, Vector mounting shield with DAPI (stain for DNA) was added and slides were maintained in the dark until observation using a Leica TCS SP2 AOBS confocal scanning laser microscope. A series of twenty sections through the image was acquired and digitalised from two randomly selected fields in each sample (4 fish x 4 treatments x 2 times post stimulation). The 20 images were taken from top to bottom, using the slide surface as point of reference to start the

series. A total of 20 μm at the zy position were obtained for the spatial image series. Relative mean intensity was evaluated in the collapsed 20 series section for every series scanned using the programme ImageJ (http://rsbweb.nih.gov/ij/).

5.2.4 Detection of TcOm15+ leukocytes from different organs

5.2.4.1 Evaluation by FCM and WB of TcOm15+ leukocytes isolated from four organs and stimulated with mitogens

Leukocytes from 4 fish were isolated from thymus, spleen, head kidney and peripheral blood as described in Section 3.2.1, and cells were cultured separately according to Section 3.2.2. Mitogens were added as follows: (1) PBLs were stimulated with PWM at a final concentration of 10 μgml^{-1}; (2) Thymus leukocytes were stimulated with 20 μgml^{-1} of PHA-P; (3) Spleen leukocytes were stimulated with 100 μgml^{-1} of LPS from *E. coli* (055:B5); (4) Head kidney leukocytes were stimulated with 5 $ngml^{-1}$ of PMA.

Stimulated cells were fixed with Cytofix solution (BD Biosciences) on Day 1, Day 3 and Day 6 post-stimulation and evaluated by FCM according to Section 3.2.4.

Stimulated cells were also harvested on Day 6 for analysis by SDS-PAGE and Western blot. The cells collected were washed twice with PBS. The cells were lysed for IP which was performed to isolate proteins binding with mAb TCOm15 according to Section 3.2.5. Electrophoresis and Western blot was carried out to analyze differences in protein expression and antibody binding between treatments according to Section 3.2.4 and 3.2.5.

5.2.4.2 Evaluation of TcOM15+ head kidney leukocytes stimulated with mitogens

Leukocytes from the head kidney of one fish were isolated as described in Section 2.3.1, and cells cultured according to Section 2.3.2. Three different mitogens were utilised to promote activation. Con A from *Canavalia ensiformis*, type IV-S was added at three final concentrations of 15 μgml^{-1}, 30 μgml^{-1} and 45 μgml^{-1}, LPS from *E. coli* (055:B5) at two final concentrations of 60 μgml^{-1} and 100 μgml^{-1} and Poly I:C at a final concentration of 100 μgml^{-1}. Samples were evaluated by FCM on Days 3 and 6 post-stimulation according to Section 2.2.4.

5.2.5 Effects of immunostimulation <u>in vivo</u> with Ergosan and β-glucan on detection of TcOm15^{+} cells.

The expression of the protein detected by mAb15 on Rainbow Trout leukocytes, was examined after feeding the fish with two different immunostimulant diets. A total of 90 fish were randomly separated into 6 experimental groups (15 fish $tank^{-1}$). Commercial pellets for trout were moistened with gelatine (SuperCook, UK) (at 8 g l^{-1} of water) as a binder and powdered immunostimulants were added to the pellets at the concentrations indicated below, before allowing the treated pellets to air dry. Two groups of fish receive a diet of Ergosan (*Laminaria digitata* 99 % and *Ascophillum nodosum* 1 %, from Schering-Plough Aquaculture, UK), using a dose of 0.5 % of diet weight according to the manufacturer´s recommendations. Another two groups received a diet with 10 gkg^{-1} β-glucan (from *Saccharomyces cerevisiae*) prepared using Biolex tablets (Lieber, Denmark) and another two groups were fed with the same commercial trout diet without immunostimulants as a control group. Fish were fed

with the diets for 15 days feeding twice daily to satiation throughout the experiment period.

Three fish were sampled from each experimental group before treatment (*i.e.* D0). After starting the treatment, three fish from each tank *i.e.* six fish per treatment and six control fish (18 fish in total) were sampled on Days 3, 7, and 14 after feeding with the experimental diets. Sampled fish were anaesthetized with benzocaine 10 % w/v in ethanol by immersion, bled from the caudal vein and sacrificed by over-anaesthetizing them. Leukocytes from thymus, spleen, head kidney and peripheral blood were isolated from each sampled fish according to the methods described in Section 2.3.1. Isolated leukocytes were fixed and preserved in the fridge (4^{o}C) and the next day stained with mAb TcOm15 for FCM according to Section 2.2.4.

5.3 Results

5.3.1 Detection of TcOm15+ cells on multiple way HCA assays by FCM

5.3.1.1 Evaluation of one dose effects of Con A/LPS on HCA assays

Data were collected from the area gated in R1 or RLymph, corresponding to the lymphocyte region as shown in Figure 4.4. When pooled PBLs were cultured in a mHCA small differences could be seen in the RLymph region between the different treatments (Figure 4.5 a & b). The basal mHCA reaction showed that 27.7 ±3.6 % of cells gated in the RLymph region of the scatter plot from 10,000 cells counted (Figure 4.5). Of these, 72.0 ±0.5 % were positively stained with mAb TcOm15. For the cells treated with LPS, a total of 24.2 ±8.8 % of cells were gated and of these 74.5 ±0.9 % were positively stained with the mAb, while for the Con A treated cells, a total of 24.7 ±4.0 % were gated in the RLymph region and 75.5 ±0.8 % of these were positive with

the mAb. An increase in the total number of positive cells (*i.e.* gated and not gated) was detected in the presence of mitogens (32.1 ±2.5 in the basal reaction, 38.8 ±1.2 plus LPS and 40.8 ±0.2 plus Con A).Thus there was a tendency to detect higher numbers of TcOm15+ cells in the mHCA treated with LPS and Con A compared to the basal reaction (Figure 4.5 b). A slight increase was also noted in the number of positives cells inside the RLymph gate when the mHCA with the mitogens (72.0 ±0.5 in the basal reaction, 74.5 ±0.9 with LPS and 75.5 ±0.8 with Con A).

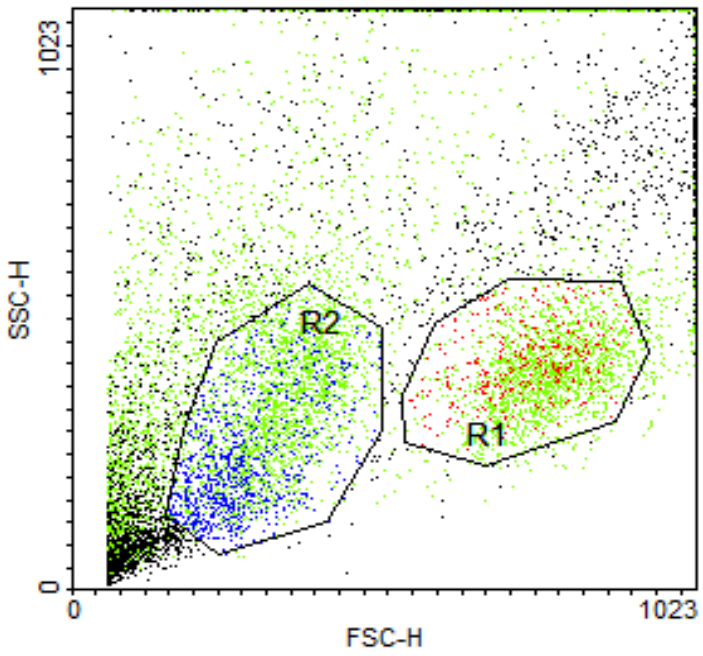
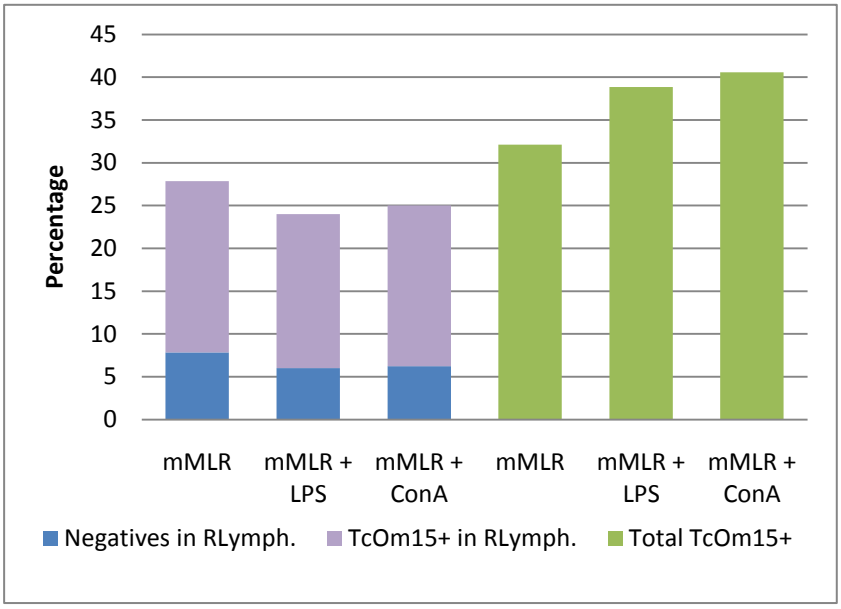


Figure 5.4 Flow cytometry analysis of rainbow trout leukocytes from mHCA culture after 5 days of culture. Example of a dot plot showing the R1 or RLymph region, red dots indicate gated cells in R1, and blue dots indicate gated cells in R2; green dots indicate positive cells.

a)



b)

	mHCA	*mHCA+LPS*	*mHCA+Con A*
% Gated in RLymph	27.7 ± 3.6	24.2 ± 8.8	24.7 ± 4.0
% Positives in RLymph	72.0 ± 0.5	74.5 ± 0.9	75.5 ± 0.8
% Total positives	32.1 ± 2.5	38.8 ± 1.2	40.8 ± 0.2

Figure 5.5 Flow cytometry analysis of rainbow trout leukocytes from mHCA culture after 5 days of culture. (a) Percentage (± s.d.) of total gated cells (positive in purple and negative in blue) in lymphocyte region (RLymph.), and positive cells from the total number of cells counted (10,000) in green, (b) values are mean percentages (± s.d.) of triplicate sample cultures from one mHCA assay.

5.3.1.2 Evaluation of TcOm15+ cells with three doses of Con A/LPS on HCA assays

Pooled leukocytes (from five fish) were stimulated in the mHCA and the basal stimulation response was enhanced with Con A or LPS. Data were collected from the gated R1 region (RLymphocytes), and the R3 region (Total positive cells). For R1, a mark (M1) was set during analysis on the histograms to obtain the percentage of positive cells inside the gate and, two more marks were set (M2 and M3) due to the detection of two groups of cells showing different intensities between treatments (example for data analysis, Figure 4.6).

Samples of mHCA stimulated cells after 1 day of culture with Con A and LPS showed a reduction in the population of total positive cells that was inversely

proportional to the amount of mitogen added when compared to control cells (Figure 4.7 & Table 4.1). Conversely, a slight increase was observed in the total number of cells gated in RLymph with respect to the control; however the number of positive cells inside the gate was inversely proportional to the dose of Con A added. The number of cells in M2 was also inversely proportional to the dose of mitogen used, with Con A appearing to have a greater effect than LPS. A decrease in the intensity of fluorescence was observed in the mitogen-enhaced reactions when compared to the basal reaction (Figure 4.7 & Table 4.1). For the cells in mHCA enhanced with mitogens in M3, lower intensities than the control or basal reaction were observed (Figure 4.7 & Table 4.1).

After six days of culture, a slight increase in the percentage of total positive cells was observed in the mHCA enhanced with Con A compared to levels seen after only three days of culture (Figure 4.8 & Table 4.2). However, when compared to the control for same day (*i.e.* Day 6), the reduction was proportional to the dose of mitogen used, reaching a 50 % reduction of labelled cells for those cells treated with 45 $\mu g ml^{-1}$ LPS. The cells gated in the RLymph region stimulated with either Con A or LPS, showed a decrease of positive cells in relation to the concentration of Con A or LPS used. At lower concentrations of Con A or LPS, a higher number of positive cells were gated in the RLymph, while an inverse was observed for the total number of cells gated inside RLymph region (*i.e.* negative + positive cells), showing major detection occurring with cells stimulated with 45 $\mu g\ ml^{-1}$ LPS compared to control cells (Figure 4.8). When histograms comparing the dose of Con A used after 1 day of culture were analysed, it was possible to observe a migration of peaks in marks M2 and M3; the higher the dose used the lower the intensity of fluorescence seen, as shown in Figure 4.9. After 6 days of stimulation with Con A, the intensities were comparable to the controls indicating

an up regulation of the TcOm15+ molecule (Figure 4.9). The number of cells in mark M3 had doubled with 45 μgml^{-1} of Con A indicating a increased detection of positive cells (Figure 4.9). For histograms of LPS stimulated cells histograms showed a slight down regulation of the TcOm15+ expression and slight increase of cells after 6 days of stimulation (Figure 4.10).

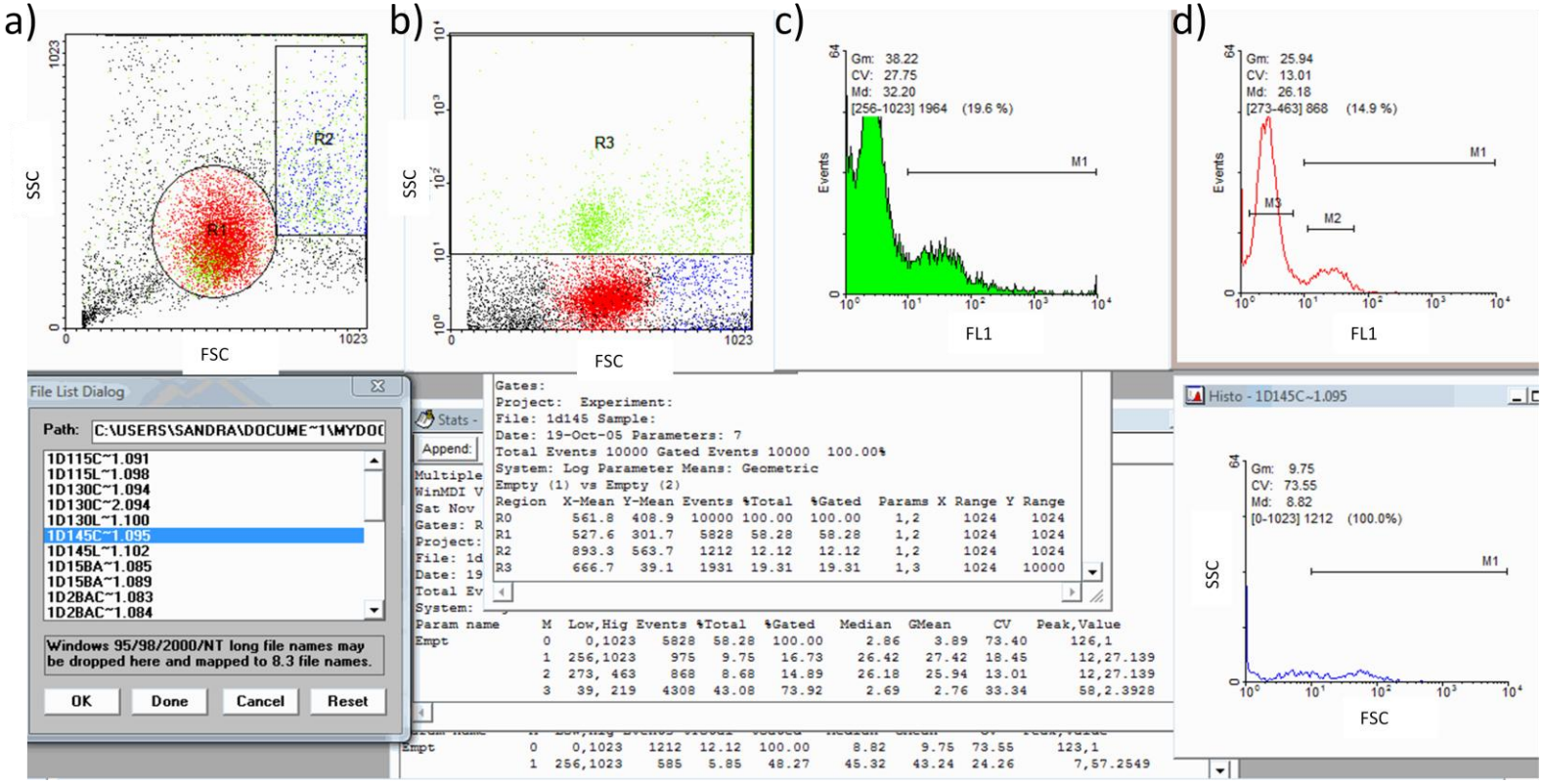


Figure 5.6 Example window in the WinMDI program to analyse data obtained by flow cytometry for mHCA experiments. a) gate R1 or RLymphocyte, b) R3 or total positive TcOm15 cells, c) histogram in green showing total positive cells showing mark 1 (M1) to determine the number of gated positive cells, and d) histogram in red showing mark 1 (M1) to determine the number of gated positive cells, and mark 2 (M2) and mark 3 (M3) to determine percentage of cells with two different peaks of intensity inside R1.

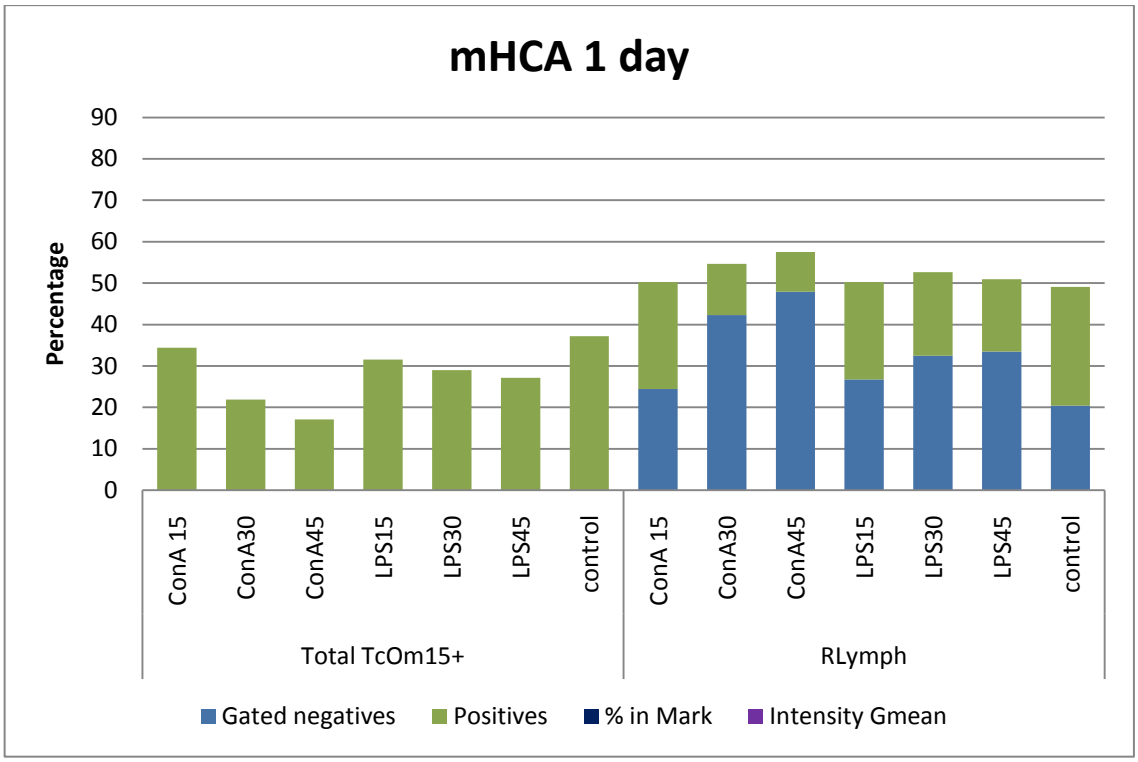


Figure 5.7 Flow cytometry analysis of rainbow trout PBLs from mHCA after culture for 1 days. Percentages of total positive TcOm15 cells (positives and negatives) from the total counted (10,000), percentage of gated cells in RLymph (negatives in dark blue and positives in green), (One assay with 5 fish pooled PBLs).

Table 5.1 Flow cytometry analysis of rainbow trout PBLs from mHCA after cultured for 1 day.

1 Day	*% Total*	*% Gated from total in RLymph*		*M2 in RLymph*		*M3 in RLymph*	
	TcOm15+	**Negatives**	**TcOm15+**	**% in Mark**	**Intensity Gmean**	**% in Mark**	**Intensity Gmean**
Con A15	34.4 ±1.0	24.4 ±0.6	25.9 ±0.7	27.5 ±1.8	44.7 ±2.5	35.1 ±2.8	8.6 ±3.2
Con A30	21.9 ±0.8	42.3 ±0.4	12.3 ±0.1	16.8 ±2.0	36.0 ±1.0	64.4 ±1.9	4.4 ±0.1
Con A45	17.0 ±1.3	47.9 ±0.9	9.6 ±0.9	12.7 ±3.1	32.0 ±8.7	51.5 ±25.8	4.0 ±1.7
LPS15	31.5 ±0.8	26.8 ±0.6	23.5 ±1.2	21.8 ±3.5	47.7 ±2.1	40.1 ±0.6	8.7 ±0.1
LPS30	29.0 ±0.5	32.5 ±3.2	20.2 ±0.3	22.2 ±1.3	45.6 ±2.0	48.6 ±2.7	7.1 ±0.4
LPS45	27.1 ±0.5	33.5 ±0.4	17.4 ±1.0	21.3 ±1.0	43.2 ±0.7	50.5 ±2.2	6.6 ±0.4
Basal reaction	37.2 ±0.5	20.4 ±0.6	28.7 ±0.0	17.6 ±0.7	56.4 ±0.4	43.3 ±1.3	12.0 ±0.1

Mean percentages (± s.d.) obtained for total positives cells (R3) and gated cells in Rlymph. Geometric mean (± s.d.) for records of intensity fluorescence in M2 and M3 are shown. Data obtained from duplicated sample wells and 10,000 events analysed from mHCA without mitogen as control or basal reaction, mHCA stimulated separately with three different doses of Con A (15, 30 and 45µg/ml) and mHCA stimulated with three different doses of LPS (15, 30 and 45µg/ml), (One assay with 5 fish pooled PBLs).

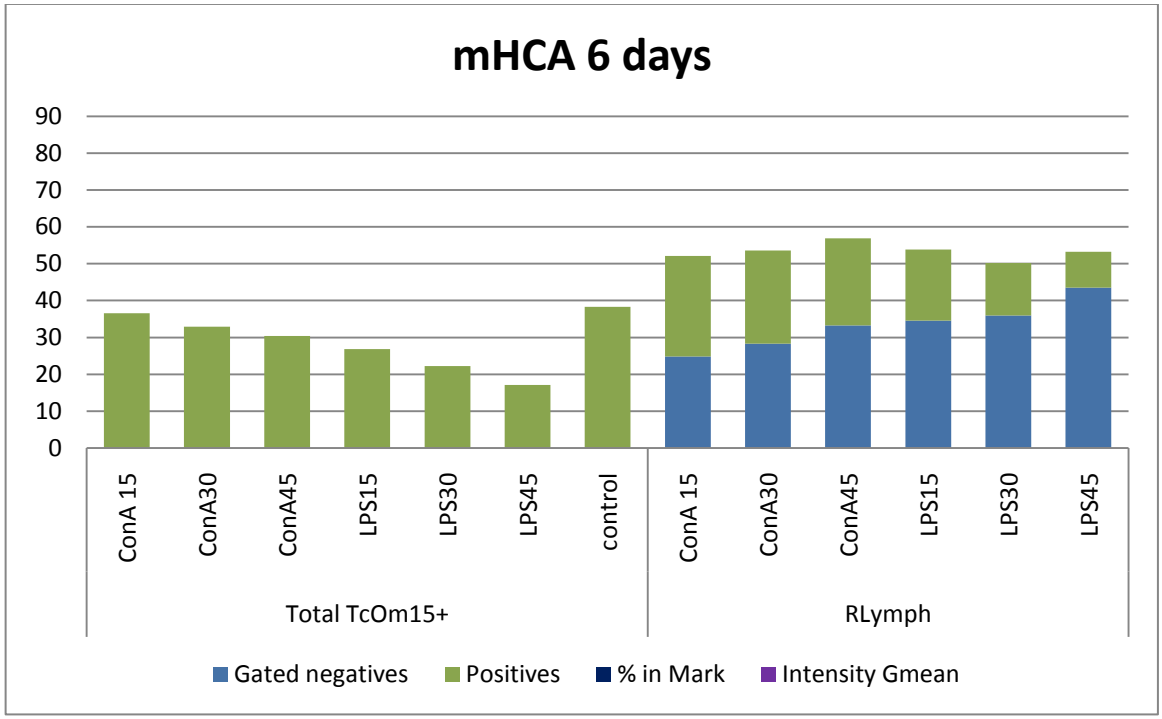


Figure 5.8 Flow cytometry analysis of rainbow trout PBLs from mHCA after cultured for 6 days. Percentages of total positive TcOm15 cells (positives and negatives) from the total counted (10,000), percentage of gated cells in RLymph (negatives in dark blue and positives in green), (One assay with 5 fish pooled PBLs).

Table 5.2. Flow cytometry analysis of rainbow trout PBLs from mHCA after cultured for 6 days.

6 Days	*% Total*	*% Gated from total in RLymph*		*M2 in RLymph*		*M3 in RLymph*	
	TcOm15+	**Negatives**	**TcOm15+**	**% in Mark**	**Intensity Gmean**	**% in Mark**	**Intensity Gmean**
Con A15	36.6 ±1.7	24.9 ±0.9	27.3 ±0.5	11.7 ±0.4	62.8 ±4.2	55.6 ±0.3	12.4 ± 0.4
Con A30	32.9 ±0.9	28.3 ±0.1	25.3 ±0.6	11.8 ±1.2	51.0 ±4.0	52.0 ±0.1	10.8 ±0.3
Con A45	30.4 ±2.2	33.3 ±1.5	23.6 ±1.3	10.5 ±2.6	47.3 ±3.1	64.0 ±11.1	8.9 ±0.7
LPS15	26.8 ±2.3	34.6 ±0.2	19.2 ±0.2	13.7 ±0.2	56.4 ±2.2	66.6 ±1.6	7.3 ±0.4
LPS30	22.2 ±1.0	35.9 ±2.5	14.3 ±0.3	18.4 ±3.8	56.2 ±1.1	63.0 ±4.9	6.3 ±0.0
LPS45	17.1 ±0.4	43.5 ±0.6	9.8 ±1.6	12.5 ±0.4	44.5 ±4.6	70.7 ±2.0	5.1 ±0.5
Basal reaction	38.3 ±1.6	19.7 ±0.8	28.7 ±1.1	16.8 ±1.0	72.3 ±2.9	49.4 ±1.6	13.4 ±0.7

Mean percentages (± s.d.) obtained for total positives cells (R3) and gated cells in RLymph. Geometric mean (± s.d.) for records of intensity fluorescence in M2 and M3 are shown. Data obtained from duplicated sample wells and 10,000 events analysed from mHCA without mitogen as control or basal reaction, mHCA stimulated separately with three different doses of Con A (15, 30 and 45µg/ml) and mHCA stimulated with three different doses of LPS (15, 30 and 45µg/ml), (One assay with 5 fish pooled PBLs).

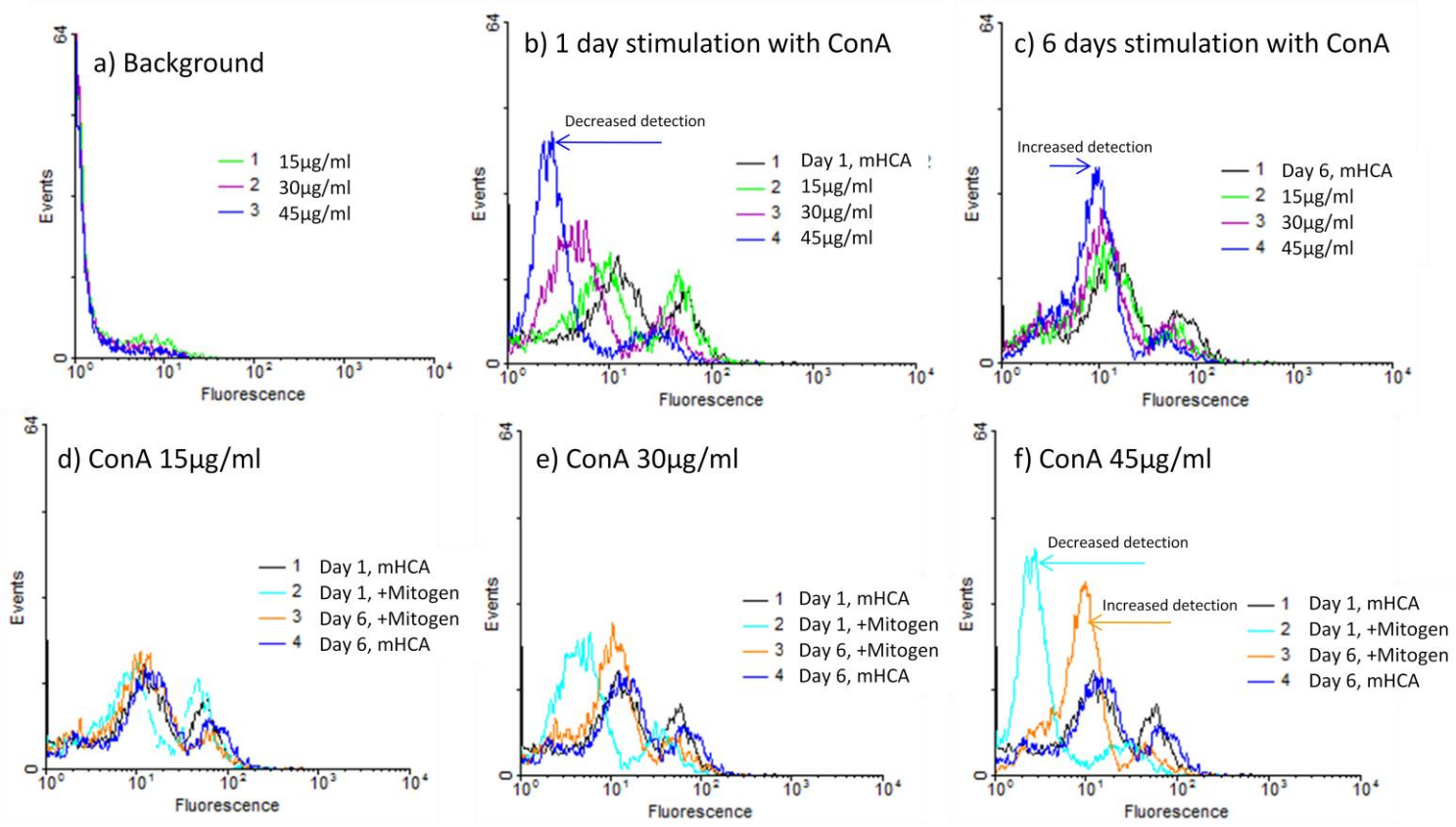


Figure 5.9 Flow cytometry analysis of rainbow trout PBLs from mHCA after culture for 1 and 6 days. Histograms showing data from 10,000 events analysed in gate RLymph and during stimulation with Con A. a) shows data from cells stained with 2nd antibody without 1st antibody to evaluate background, b) data comparing the different doses of mitogen at day 1 indicating increase in the number of unlabeled cells and migration of events to a lower intensity when comparing to control (black line) with a blue arrow, and c) comparison of three doses after 6 days of culture (blue arrow indicates an increase in intensity and numbers compared to controls). In d), e) and f) histograms comparing single doses over time are shown with arrows indicating down regulation in the first day and up regulation for the highest dose on day six, (One assay with 5 fish pooled PBLs).

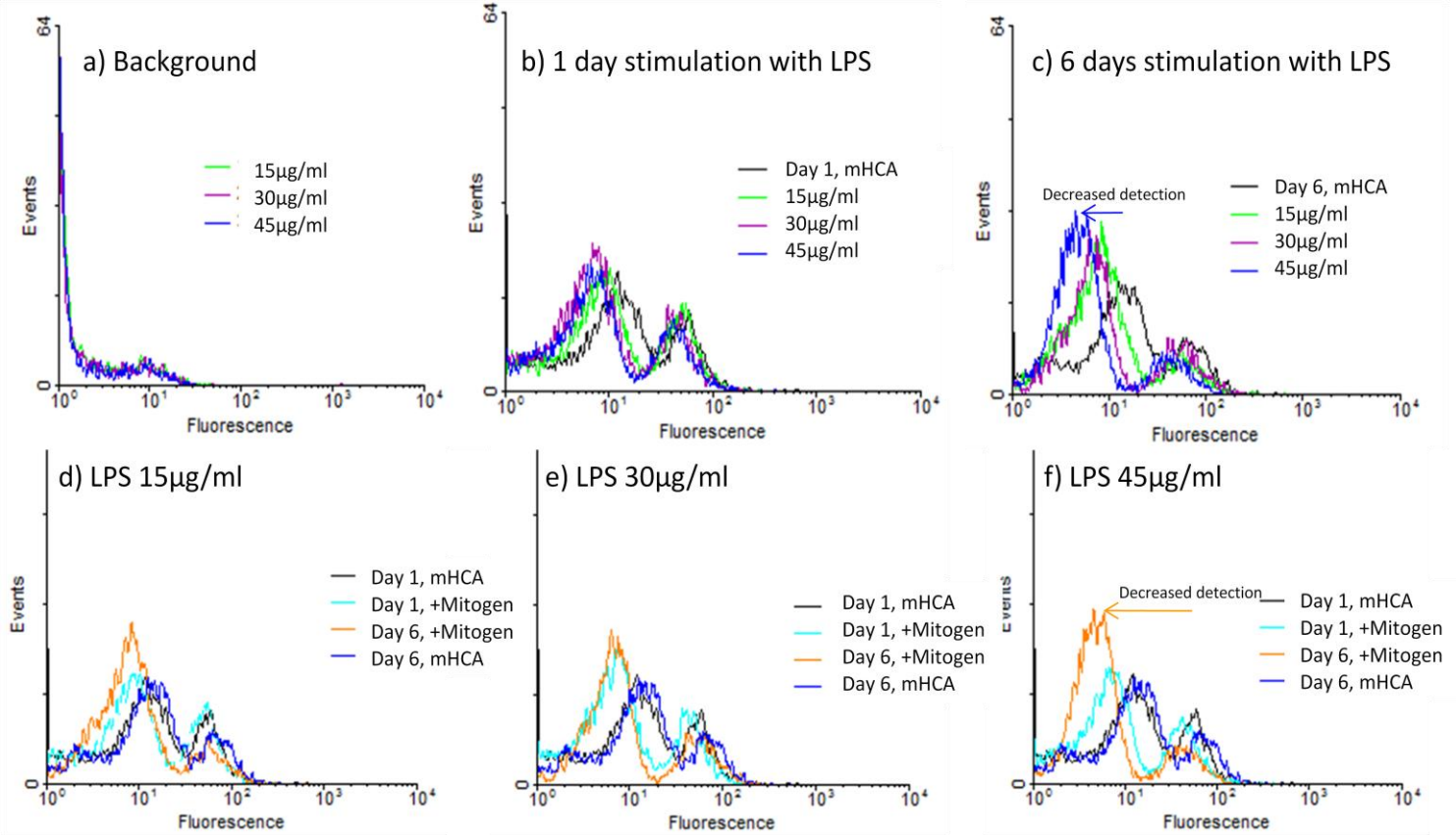


Figure 5.10 Flow cytometry analysis of rainbow trout leukocytes from mHCA cultured for 1 and 6 days. Histograms showing data from 10,000 events analysed in gate RLymphocytes and their intensity during stimulation with LPS (a) data from cells stained with 2nd antibody without 1st antibody to evaluate background; (b) data comparing different doses of LPS after day 1, and (c) comparison of three doses after 6 days of culture with the blue arrow indicating slight decreased detection of labelled cells of events. In d), e) and f) histograms with single doses over time are compared, the orange arrow shows an increased number of unlabelled cells on the first day compared to day six, (One assay with 5 fish pooled PBLs).

5.3.2 Detection of TcOm15+ activated PBLs from individual fish by FCM

5.3.2.1 **Evaluation of TcOm15+ PBLs with one dose of Con A/LPS**

Leukocytes from peripheral blood of three fish were stimulated separately with Con A (30 μg ml^{-1}) or LPS (100 μg ml^{-1}). Cells were stained with mAb TcOm15 on day 5 after stimulation and the level of fluorescing cells determined in gate R1 (RLymph), and R3 (Total positive cells). For R1, the mark (M1) was used to obtain the percentage of cells fluorescing inside the gate (Figure 4.11).

The mean percentage of labelled cells from the three fish evaluated showed an increase of 4 % in cells stimulated with 100 μg ml^{-1} LPS and 6 % for leukocytes stimulated with 30 μg ml^{-1} Con A compared to unstimulated cells in which 32 % of cells were stained (Table 4.3).

By separate analysis of the three fish evaluated, an increase of approximately 8 % and 10 % was observed in positive cells gated in the RLymph region following stimulation with LPS or Con A, respectively, for two of the three fish (Figure 4.12). One fish did not respond to stimulation with either LPS or Con A. For Fish 2, an increase in the number of labelled cells inside the gate in the RLymph region of 5 % and 10 % approximately was observed for cells stimulated with LPS and Con A respectively. For Fish 3, an increase in the percentage of cells labelled inside gate RLymph of 6 % was detected for leukocytes stimulated with LPS, while those stimulated with Con A showed a lower percentage of gated and fluorescent cells inside the gate compared to the control.

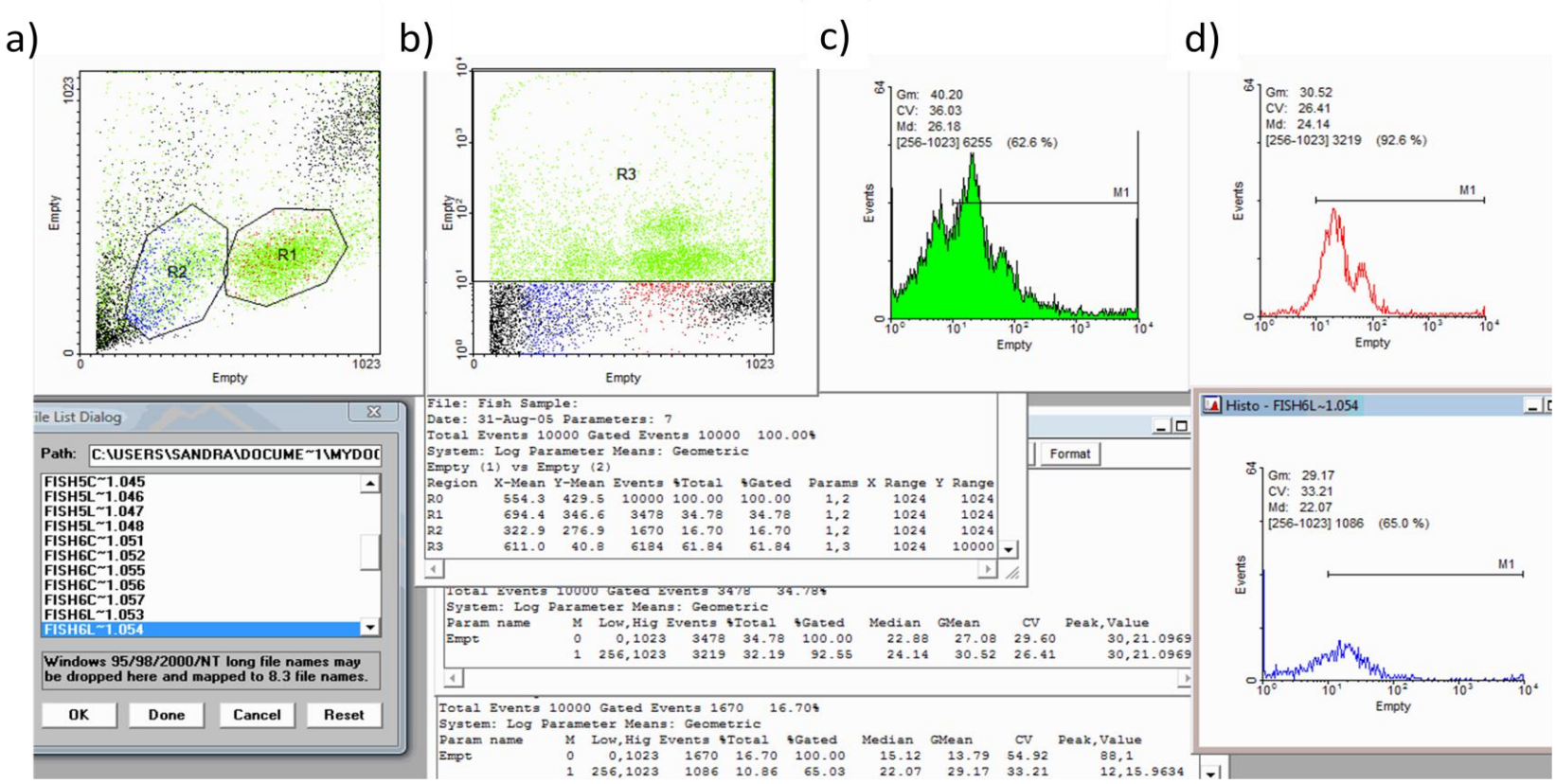


Figure 5.11 Example window in WinMDI program to analyse data from stimulation experiments with Con A and LPS in single fish obtained by flow cytometry. Gate R1 or RLymphocyte in a), R3 (total positive events) is shown in b) and c), mark 1 (M1) to obtain the number of cells positives inside gate RLymph or R1.

Table 5.3 Percentage of leukocytes of three rainbow trout labelled with mAb TcOm15 after culturing for five days with either LPS or Con A. Data are shown as mean percentages (± s.d.).

	% of Total TcOm15+	*% Gated in RLymph*	
		Negatives	Positives
Control	32.2 ± 14.1	2.3 ± 2.3	14.7 ± 6.2
LPS	36.1 ± 18.6	3.0 ± 1.5	17.1 ± 8.2
Con A	38.3 ± 17.8	2.4 ± 1.3	14.5 ± 5.3

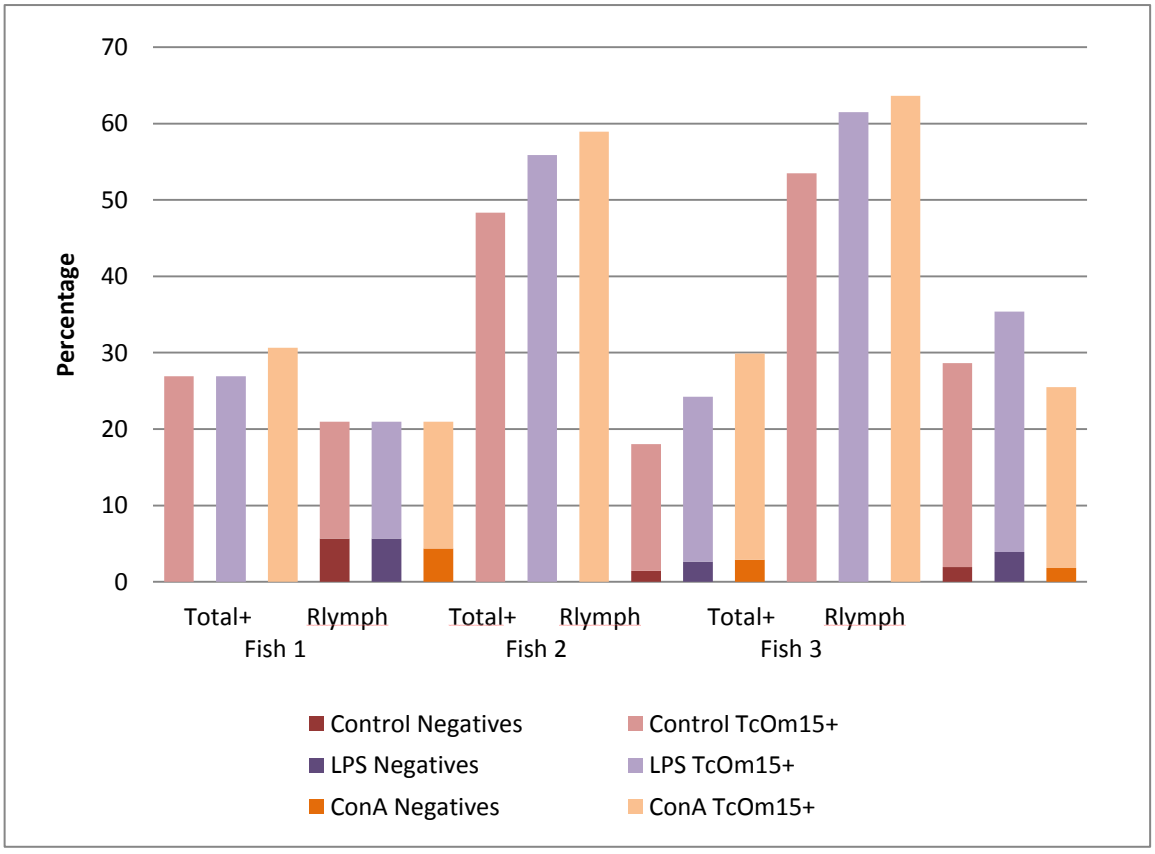


Figure 5.12 Percentages of PBLs from rainbow trout cultured without mitogen as control and stimulated with Con A (30 µg/ml) and LPS (100 µg/ml). Percentages of total positive cells are shown in light colour and percentage of gated cells in RLymph, negatives in dark colour and positives in light colour. Data obtained after 5 days of stimulation from duplicated samples from three fish.

5.3.2.2 **Evaluation by FCM and WB of TcOm15+ PBLs with six different mitogens**

Six different stimulants were added separately to PBLs from 4 fish: Con A45 (45 μgml-1) and Con A90 (90 μgml-1) to promote stimulation of T-cells; LPS to stimulate B-cells; PMA to stimulate granulocytes; PHA for T-cells; PWM for B-cells and Poly I:C as a monocyte stimulator. It was found that gated cells in the RLymph region increased in number when stimulated with PWM by 70 % compared to the control with 55 % gated cells. In contrast, PMA reduced the number of gated cells to 18 % (Figure 4.13 & Figure 4.14). Cells stained with the mAb inside the gate RLymph had a tendency to increase under mitogenic stimulation, increasing by more than 2 % for LPS, PHA and PWM when compared to the labelled cells in gate RLymph from control wells with a value of 21 %. However, the number of labelled cells in the gate decreased to 11 % when stimulated with Poly I:C and almost disappeared in the presence of PMA to 1 %. From the total number of labelled (or fluorescing) cells, the controls showed a mean of 21 % (Figure 4.14). This mean number was increased by approximately 37 % with PWM stimulation and decreased by up to 15 % in cells stimulated with PMA.

It was noted during analysis that the data from the four fish showed differences in their response to the various stimulants (Figure 4.14). However, even though the total number of gated cells in RLymph region and the gated positives varied, similar patterns of reaction were seen between fish according to the mitogen used (see Figure 4.14). The histograms for percentage of TcOm15+ gated cells, indicated that the values were similar between lower responder fish and higher responder fish to stimulation see TcOm15+ in RLymph in Figure 4.14.

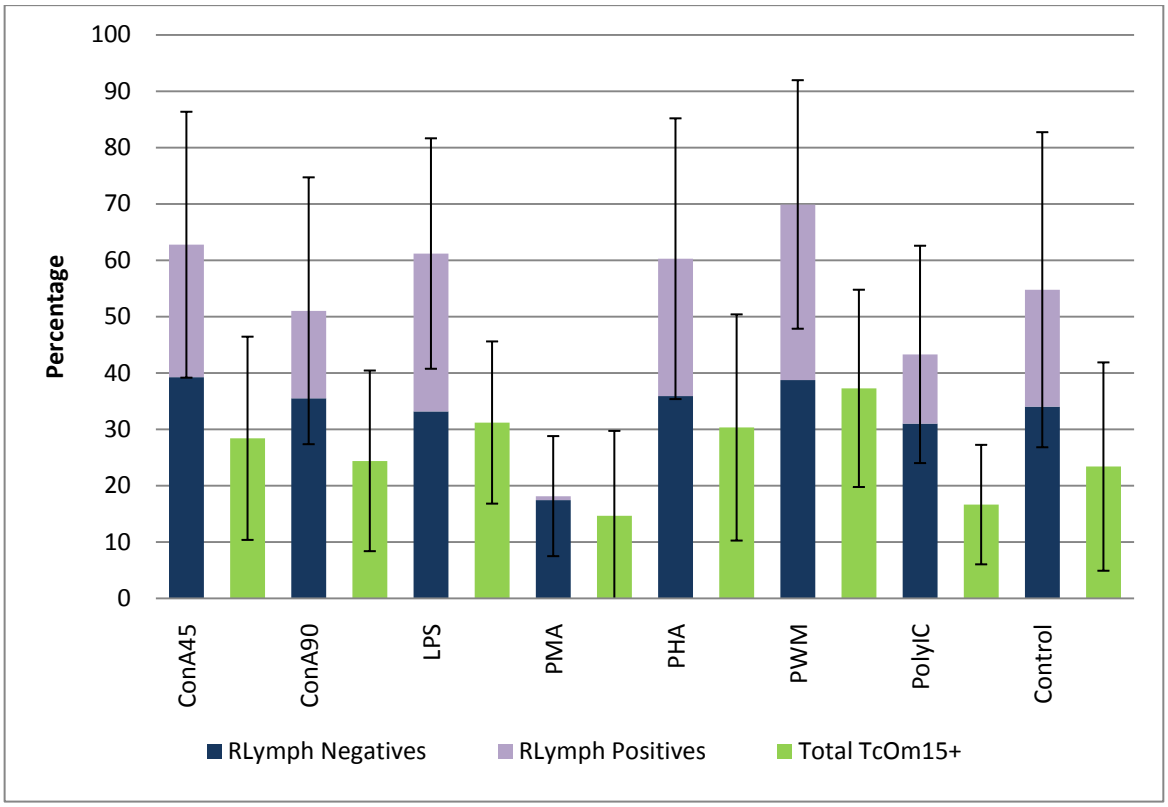


Figure 5.13 Flow cytometry analysis of rainbow trout leukocytes from peripheral blood cultured with six different mitogens for 3 days. Percentages of gated cells (± s. d.) in RLymph were positives are in purple and negatives in dark blue, and total TcOm15+ cells (± s. d.) in green from 10,000 events (n=4).

However, stimulation was detected (increase in the number of gated cells compared to the control cells) for PWM stimulated cells, inhibition (decrease in the number of gated cells in RLymph compared to the control cells) with Poly I:C, up regulation (increase in the Gmean intensity of fluorescence compared to the control) for LPS stimulation, and down regulation (decrease in the Gmean of intensity compared to the control) in PMA stimulated cells. These patterns of stimulation were detected for all four fish (Figure 4.15).

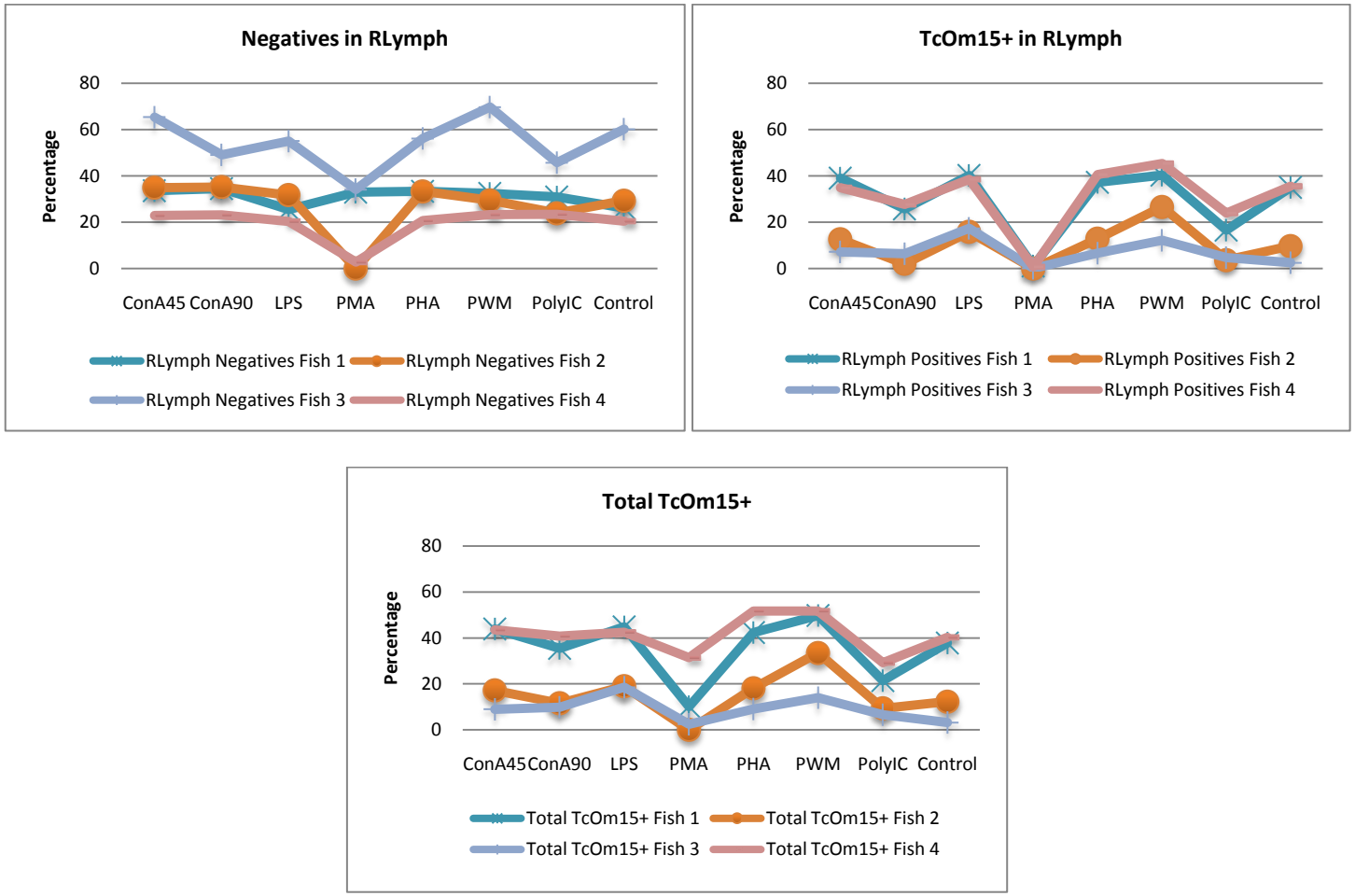


Figure 5.14 Flow cytometry analysis for leukocytes from peripheral blood cultured separately for 3 days with 6 mitogens from four rainbow trout. Data are shown in separated graphs to show for every fish the pattern of detection of negative cells gated in RLymph, TcOm15+ cells gated cells in RLymph, and total TcOm15+ cells.

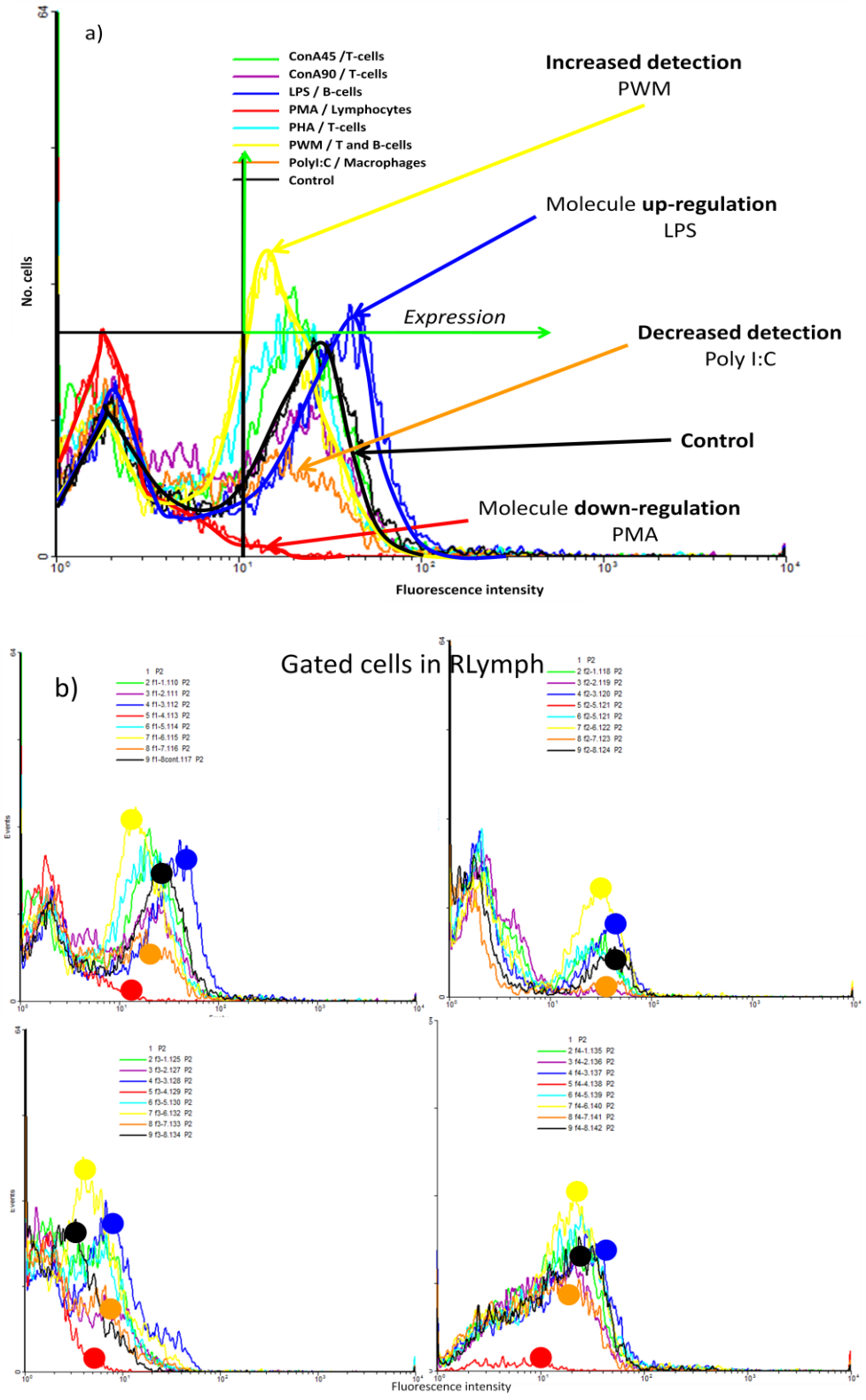


Figure 5.15 Flow cytometry analysis of rainbow trout leukocytes from peripheral blood cultured for 3 days in gate RLymph. (a) example of one fish (b) principal features in the response to different mitogens for four separate fish analysed: yellow dots increased detection with PWM; blue dots up regulation with LPS; orange dots decreased detection with poly I:C, and red dots down regulation with PMA. Positives from 10^1 of fluorescence intensity (x axis).

A second experiment was performed under the same conditions with PBLs from six fish. However, due to an error in the methodology with samples for flow cytometry (*i.e.* they were fixed with formalin before staining), no data were obtained. However a batch of cells from one fish (not fixed) were treated for protein extraction, and SDS-PAGE and Western blot was performed. No differences were found between treatments by mitogens after six days by SDS-PAGE and Western blot analysis. Protein bands were weakly detected by mAb TcOm15 at 250, 160 and 105 kDa (Figure 4.17b).

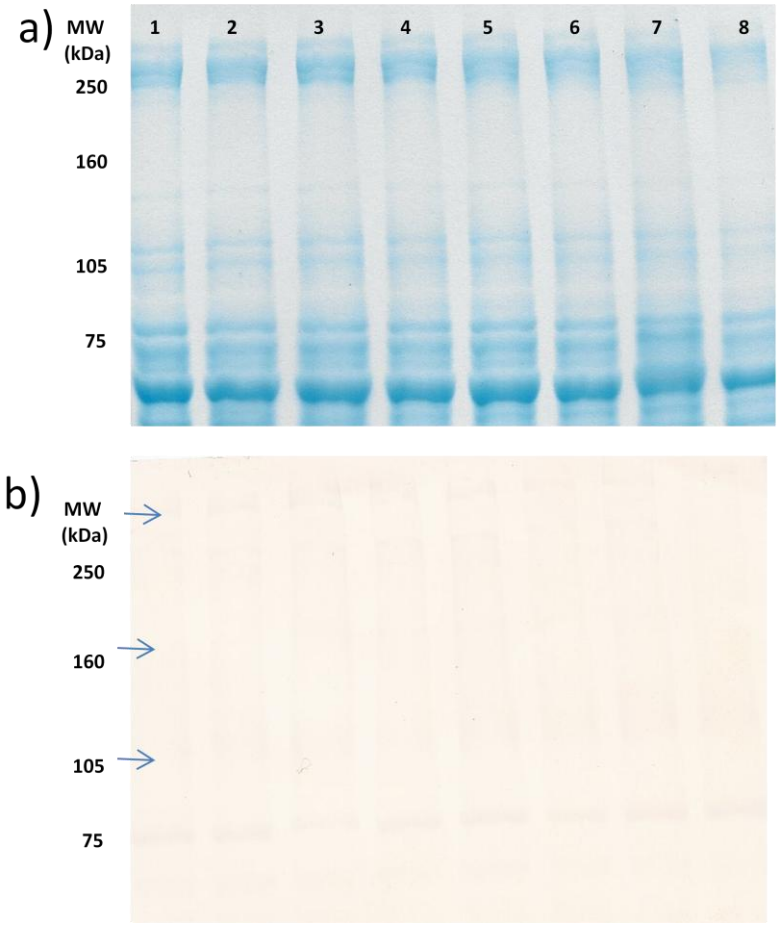


Figure 5.16 a) SDS-PAGE for protein samples extracted from PBLs from one rainbow trout cultured for three days with (1) Con A45; (2) Con A90; (3) LPS; (4) PMA; (5) PHA; (6) PWM; (7) Poly I:C; (8) control; b)Western blot with mAb TcOm15.

5.3.3 TcOm15 staining of activated PBLs by IIF

Leukocytes cultured in the slide chambers covered with poly-L-lysine (*i.e.* PBLs from 4 fish) were stimulated either with Con A, LPS or PMA. A sequence of twenty images was randomly taken from two sites on every slide (analysed by the ImageJ program). The mean intensity was obtained for every field from four fish, four treatments (including control), at two different times (Example Figure 4.18).

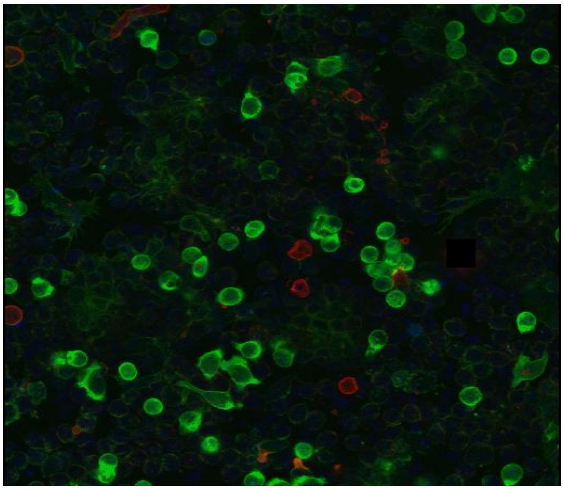


Figure 5.17 Example of collapsed 20 series image of confocal microscopy analysed with ImageJ programme for mean intensity of Texas red conjugate binding to mAb TcOm15. MAb TcOm15 in red, nuclei in blue and F-actin in green.

From the confocal analysis a slight increase in the mean intensity for the leukocytes treated with mitogens was detected after 20 min when compared with the control (see Figure 19 & Table 4.4). For cultures 24 hours old, no differences were observed in the intensity detected for Texas red (Figure 4.19). In order to relate mean intensity of nuclei fluorescence with DAPI and mean intensity of fluorescence detected for Texas red conjugate binding to mAb TcOm15 a ratio was calculated. The results indicated that a positive trend in the relation was observed only for ConA (0.09 ±0.03) after 20 minutes of stimulation when compared to the control (0.04 ±0.02), a slight

negative trend was registered after 24 hours for samples treated with LPS (0.05 ±0.02) compared to the control (0.08 ±0.06) (Table 4.4).

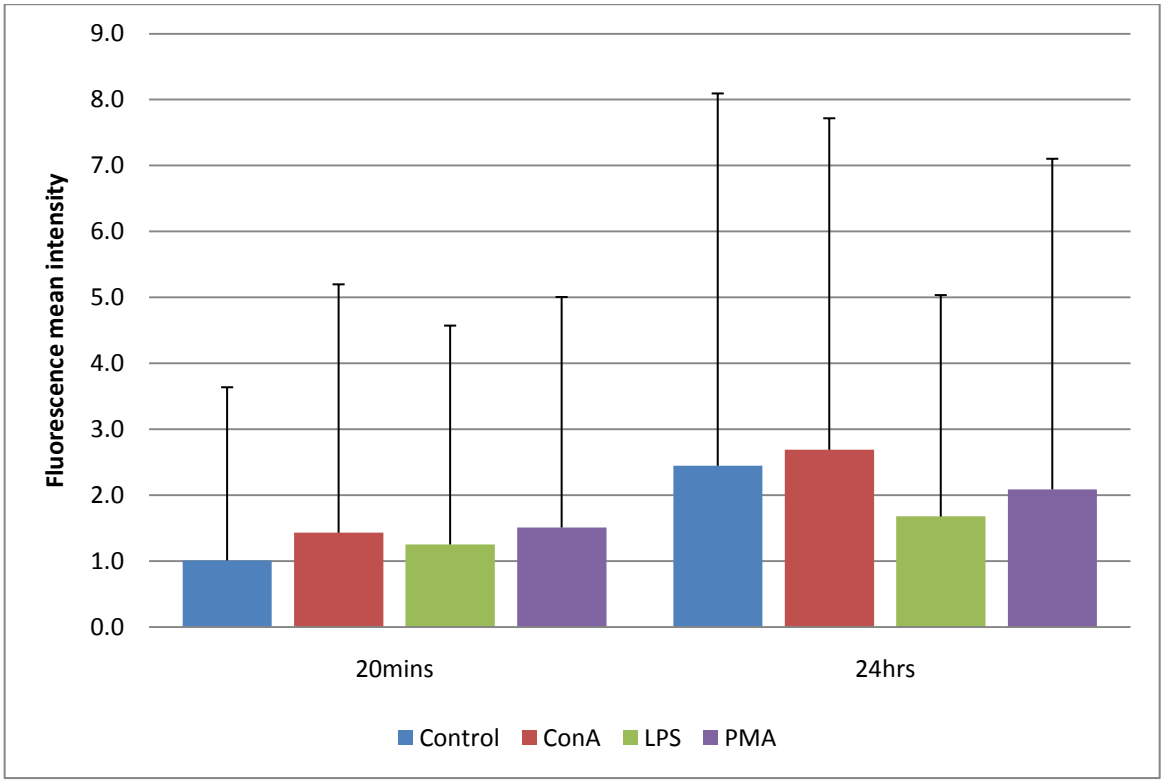


Figure 5.18 Mean intensity fluorescence detected for TcOm15 + rainbow trout PBLs with Texas red conjugated second antibody stimulated with 3 different mitogens and control. Confocal images were analysed for Texas red channel after 20 min, and 24, 72 and 124 h of culture. Data were obtained from collapsed 20 series of images every treatment and time, (n=4).

Table 5.4 Mean intensity of fluorescence detection for Texas red channel and relation of texas red respect to the mean fluorescence of DAPI as control detected on TcOm15+ rainbow trout PBLs.

		20 min	*24 hrs*
Control	Texas red	1.01 ± 2.63	2.45 ± 5.65
	TR/DAPI	0.04 ± 0.02	0.08 ± 0.06
ConA	Texas red	1.43 ± 3.77	2.69 ± 5.03
	TR/DAPI	0.09 ± 0.03*	0.06 ± 0.01
LPS	Texas red	1.26 ± 3.32	1.68 ± 3.36
	TR/DAPI	0.05 ± 0.02	0.05 ± 0.02
PMA	Texas red	1.51 ± 3.50	2.09 ± 5.02
	TR/DAPI	0.05 ± 0.02	0.07 ± 0.06

Data were obtained from confocal images from stimulation assays with 3 different mitogens. Samples were analysed after 20 min, 24, 72 and 124 h, and data obtained from a 20 series colapsed image for every treatment and time, (n=4).

5.3.4 Detection of TcOm15+ leukocytes from different organs

5.3.4.1 **Evaluation by FCM and WB of TcOm15+ leukocytes isolated from four organs and stimulated with mitogens**

Leukocytes were isolated from different organs of rainbow trout and cultured with one of four different mitogens. PBLs were stimulated with PWM, thymus leukocytes with PHA, spleen leukocytes with LPS and head kidney with PMA and cultured for 1, 3 and 6 days with the mitogens. Cultures were stained with mAb TcOm15 and then analysed by FCM.

Data were obtained from the gated cells in the RLymph and RGran regions of the dot plot. PBLs were seen to have undergone stimulation indicated by the increase in the number of gated cells (48 % - 53 %) when compared with the unstimulated cells (25 % - 48 %). Stimulation with the mitogen over time, in contrast led to a decrease in the number of thymus leukocytes seen in the RLymph gate. Overall, the highest number of cells was seen with the control cells at Day 1 (62 %) and the lowest with thymus leukocytes at Day 6 with PHA (*i.e.* 31 %). Spleen and head kidney leukocytes also showed some inhibition with mitogen stimulation over time (Figure 4.20). Gated cells in the granulocyte region were very low for PBL, thymus and spleen populations of leukocytes (a maximum of approximately 5 %) but higher with head kidney leukocytes (*i.e.* 25 %). However, the levels of gated cells appeared similar with stimulated cells from each organ over the course of the experiment (Figure 4.20).

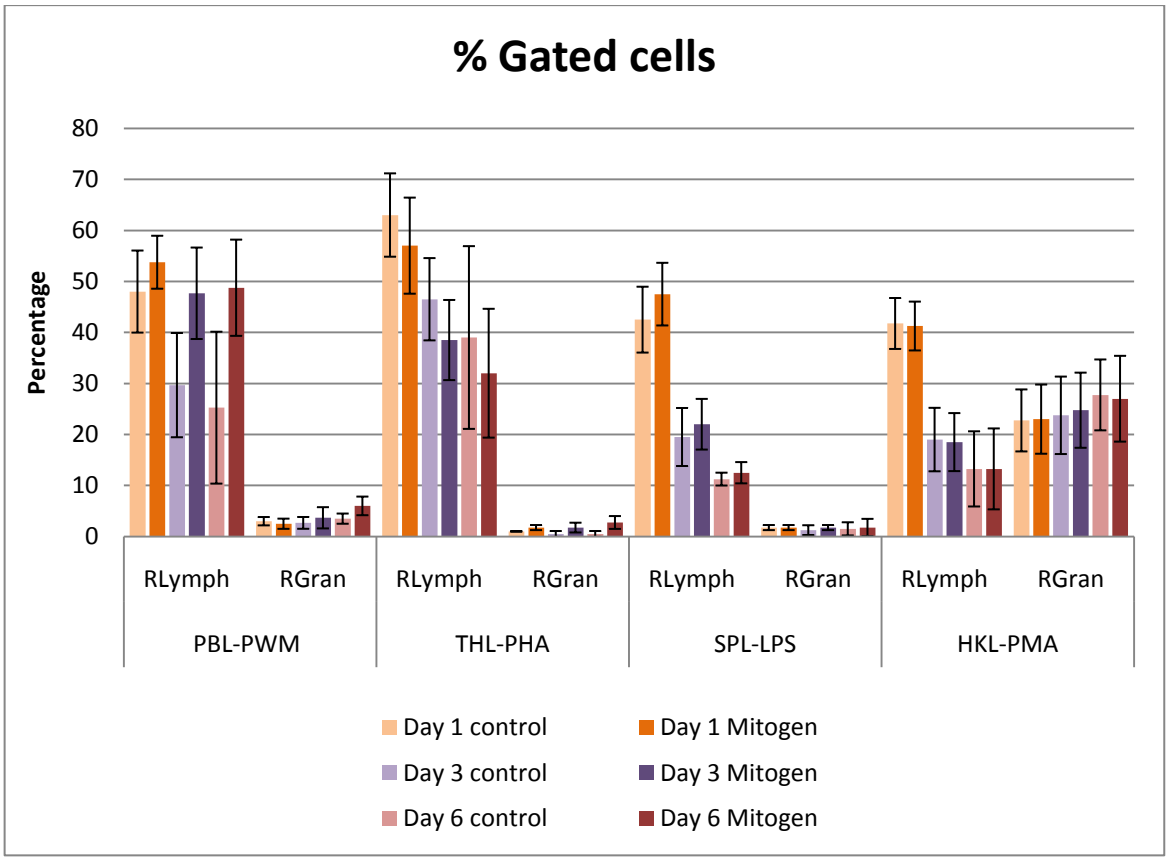


Figure 5.19 Percentages of gated leukocytes from peripheral blood (PB), thymus (THL), spleen (SPL) and head kidney (HKL) of rainbow trout analysed by flow cytometry in RLymph and RGran. Analysis was performed on day 1, 3 and 6 of culture with 4 different mitogens. Controls are indicated by lighter colour and stimulated cells by the darker, (n=4).

The total number of cells labeled with mAb TcOm15 in PBL and thymus leukocytes samples had a tendency to increase under mitogen stimulation and over time (dark red and dark blue bars in Figure 4.21), while no evident increase of staining occurred with mAb TcOm15 with spleen and head kidney leukocytes with mitogen stimulation over the course of the trial (dark brown and dark orange in Figure 4.21).

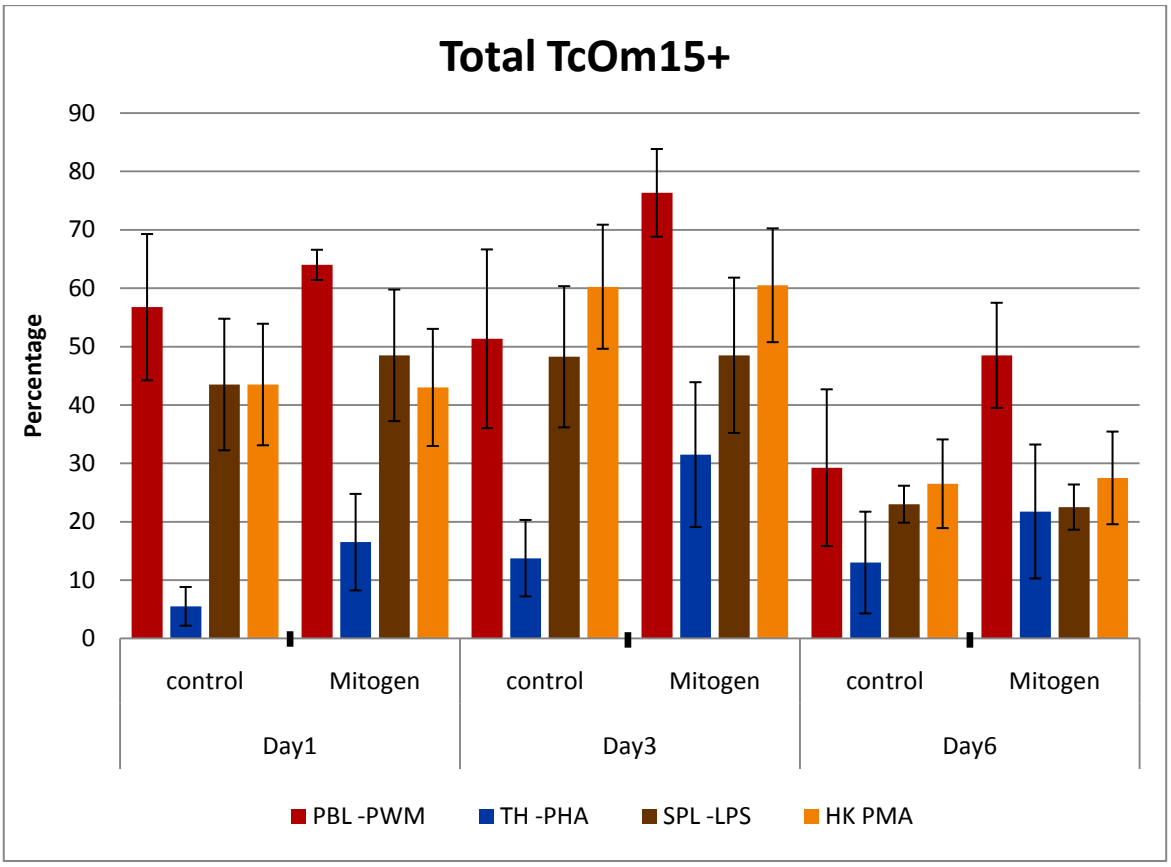


Figure 5.20 Percentage of total TcOm15+ cells from trout leukocytes from 4 organs cultured with 4 mitogens during 1, 3, and 6 days. Percentages for leukocytes from peripheral blood (PBL) are shown in dark red, thymus (THL) in dark blue, spleen (SPL) in brown, and head kidney (HKL) in orange, (n=4).

A set of samples, cultured under the same conditions for 6 days, were lysed, and immunoprecipitated with mAb TcOm15. The proteins that were obtained were analysed by SDS-PAGE and Western blotting. No differences were observed between leukocytes treated with the mitogens and untreated cells from the different organs. Proteins bands observed in SDS-PAGE and Western blotting from whole lysates and IP were comparable between treated and untreated cells for all organs (Figure 4.22). Faint bands were detected at 250 kDa on the Western blot by mAb TcOm15 (Figure 4.22 c).

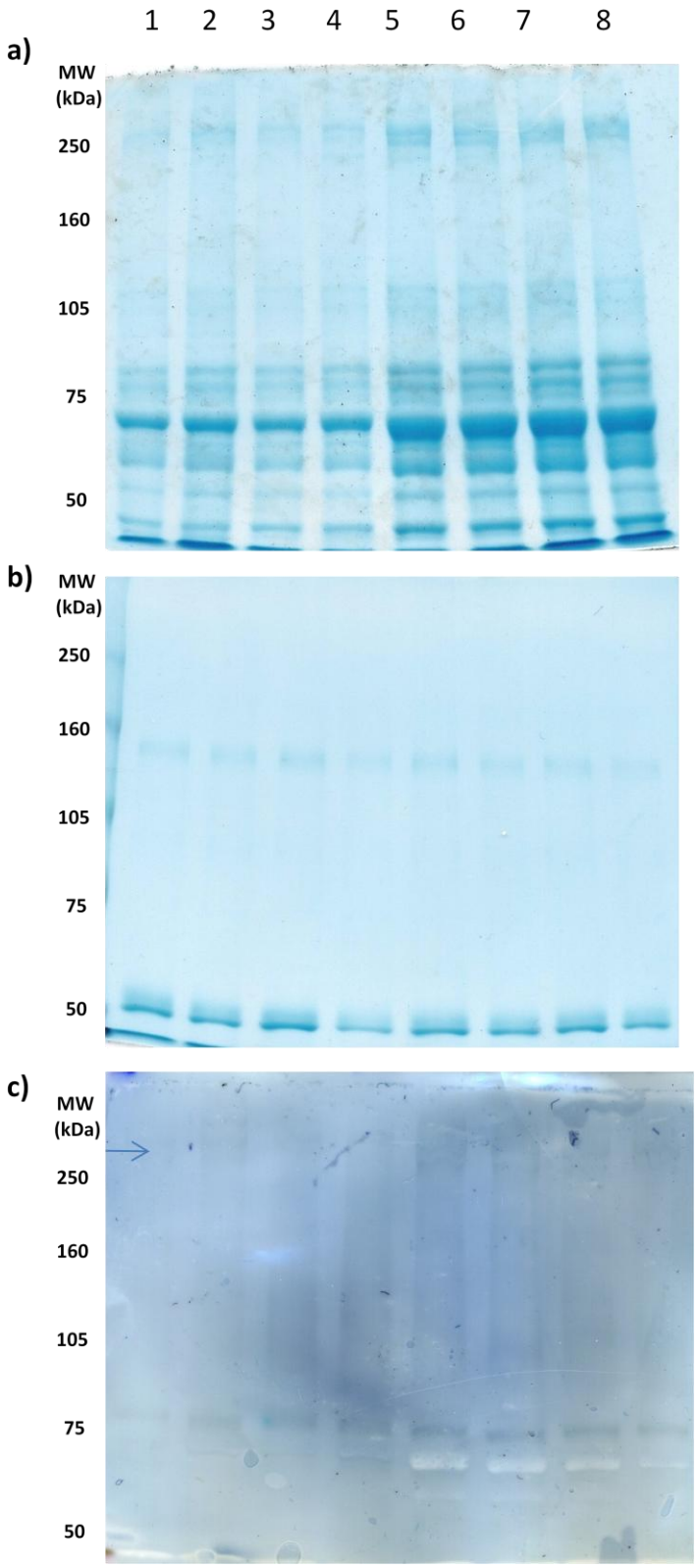


Figure 5.21 SDS-PAGE of a) whole lysates from rainbow trout leukocytes, b) for IP, and c) Western blot of IP with mAb TcOm15. Protein samples were extracted from leukocytes obteinaed from a single fish cultured for 6 days. Lanes (1) PBL with PWM, and (2) PBL control; (3) thymus leukocytes with PHA, and (4) thymus leukocytes control; (5) spleen leukocytes with LPS, and (6) spleen leukocytes control; (7) head kidney with PMA, and (8) head kidney leukocyte control.

5.3.4.2 Evaluation by FCM of TcOM15+ head kidney leukocytes stimulated with mitogens

In order to determine if activated granulocytes expressed the protein detected by mAb TcOm15, head kidney leukocytes were stimulated with Con A, LPS and Poly I:C and data were acquired by flow cytometry for cells cultured for 3 and 6 days. Cells were gated in three main groups for analysis, R1 (or RLymph), the region for granulocytes R2 (RGran), and a R3 for small precursor cells (RPrecursor) (Figure 4.26).

Few leukocytes were gated (negatives + positives) in RLymph region, but higher levels of gated cells were observed in samples stimulated with Poly I:C (8 %) and LPS (9 %), while the samples stimulated with Con A for three days showed approximately 4 % of cells gated in this region (Figure 4.27, 3 days). The numbers of labelled cells inside the RLymph gate were small. From these, the higher numbers of positives cells were obtained with LPS and Poly I:C stimulation (7 % and 4 % respectively), while Con A stimulated cells were labelled at about 2 %, with no differences between the concentrations of Con A used. On the other hand granulocytes represented the highest numbers of gated cells. Following stimulation with Con A, the number of total cells in gate RGran increased according to the dose (about 2 % between Con A concentrations). Gated granulocytes stimulated with poly I:C showed a high percentage of 66 % when compared to the cells stimulated with Con A or LPS. It was noticed that the numbers of cells in region 3, where precursors and debris occur, were highly labelled at about 22 % for Con A15 (15 μg/ml), while stimulation with LPS100 (100 μg/ml) gave approximately 5 %. The number of cells observed in R3 appeared to change according to the dose of Con A used, with a higher percentage of gated cells at

the lowest concentration used. The total number of fluorescent cells was more or less maintained between 20 and 30 % (Figure 4.27, 3 days).

After six days of culturing head kidney leukocytes with Con A, there was a low level of gated cells in RLymph (0.2 – 1.4 %), while percentages for cells in this region stimulated with LPS and Poly I:C were comparable to that of the control cells (Figure 4.27, 6 days). Gated cells in the granulocyte region showed a decrease of approximately 20 % for cells stimulated with LPS, while cells stimulated with 30 and 45 μgml^{-1} of Con A showed a 10 % decrease compared with control cells. However, a large increase in the percentage of cells labelled with the mAb was seen with cells stimulated with Poly I:C, of approximately 20 % with respect to the control (Figure 4.27, 6 days). Numbers of labelled cells in RGran were comparable to the control (2.5 %) with the exception of Poly I:C stimulated cells where 6 % was recorded in this region (Figure 4.27, 6 days). When cells gated in the RPrecursor region were analysed, it was found that between 47 and 57 % of cells were gated in this region for leukocytes stimulated with Con A 30 and 45, and LPS 60 and 100, which was equivalent to increases of between 12-20 % with respect to the control. The fluorescent cells inside the RPrecursor region were only 2 % for Poly I:C stimulated cells, 10 % for the control cells and 21 % for Con A45 stimulated cells. For Poly I:C treated cells, the percentage of gated cells in RPrecursor was very low (i.e. 10 %) or 25 % lower than the control. The total percentage of labelled cells registered between 28 % for the Poly I:C treated cells, 34 % for the control, and a maximum of 41 % for the Con A30, 45 and LPS 60 treated cells (Figure 4.27, 6 days).

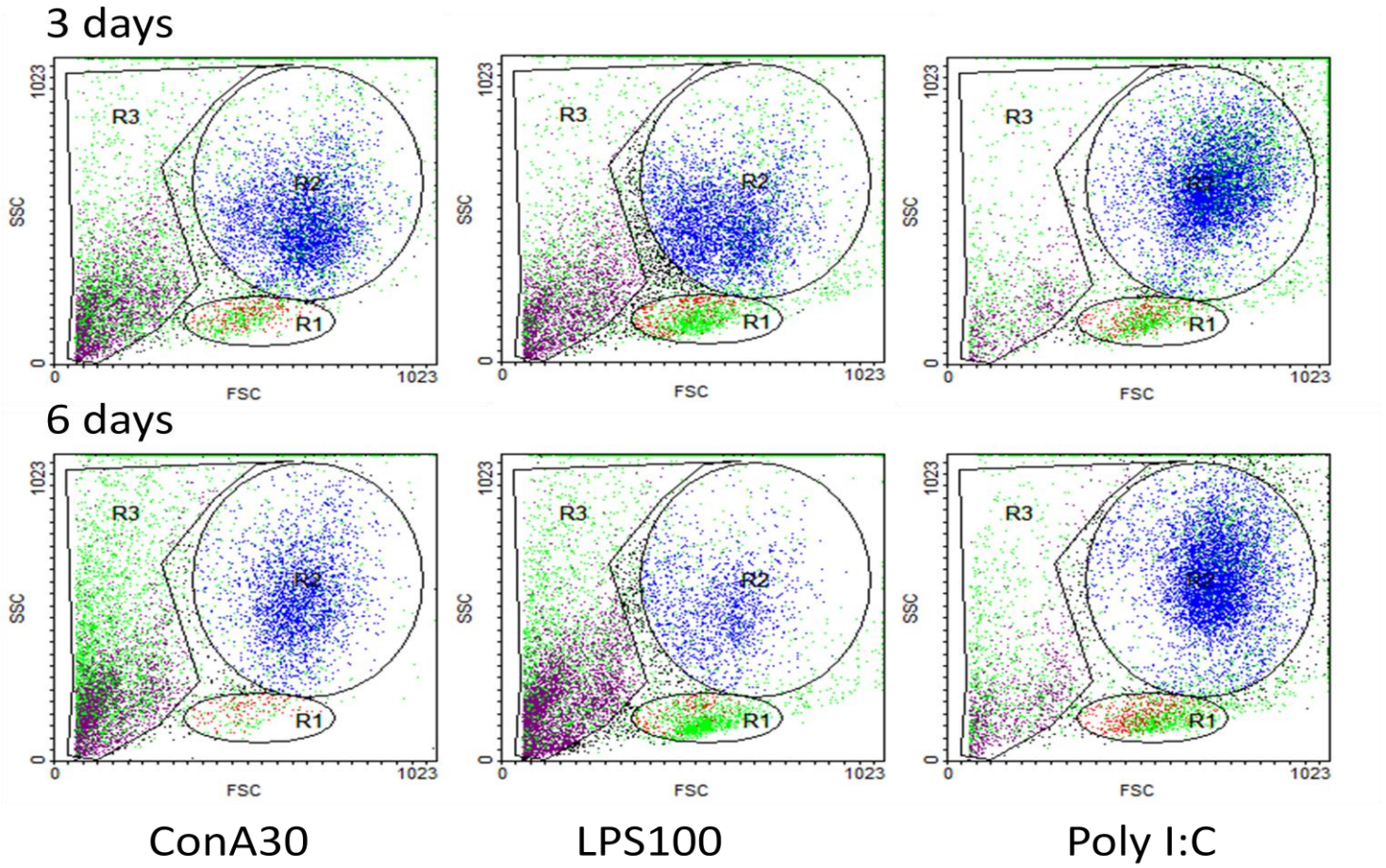
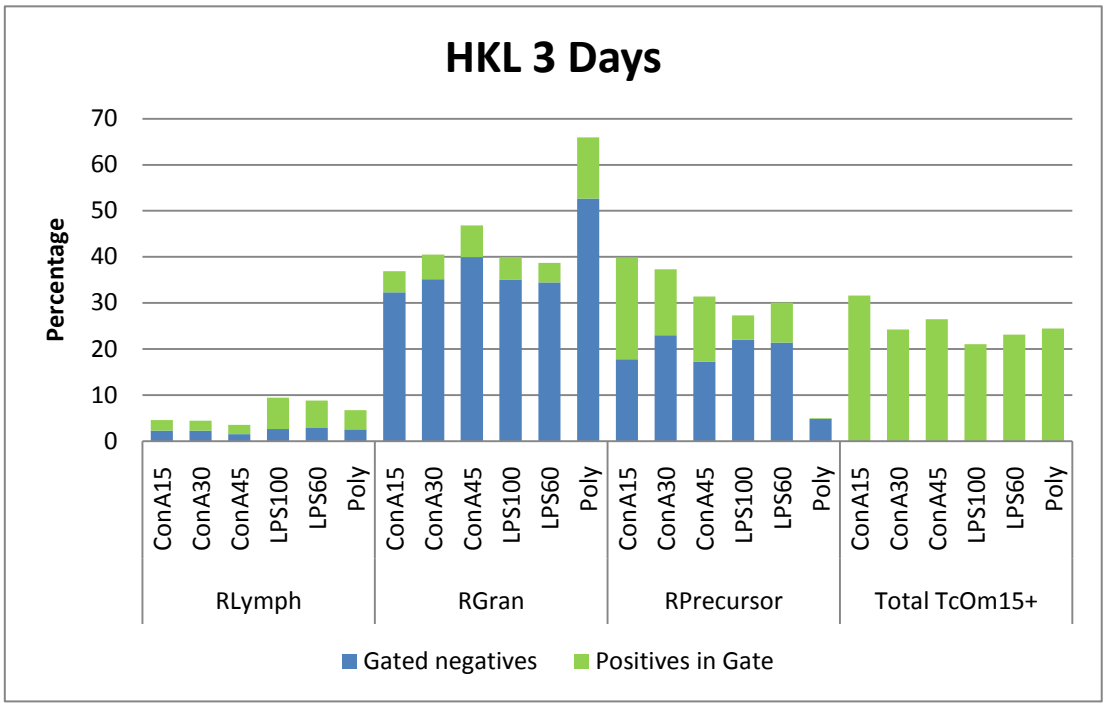


Figure 5.22 Example dots plots of flow cytometry analysis of leukocytes from rainbow trout stained with mAb TcOm15 after 3 and 6 days of culture with Con A (30 µg ml-1), LPS (100 µg ml-1) or Poly I:C (100 µg ml-1). The delimitation for the regions R1 (or RLymph), R2 (or Rgran), and R3 (precursors and debris) is shown. Dots in green indicate positive cells, red dots indicate gated cells in R1 (RLymph), blue dots indicate gated cells in R2 (RGran), purple dots indicate gated cells in R3 (precursors or debris).



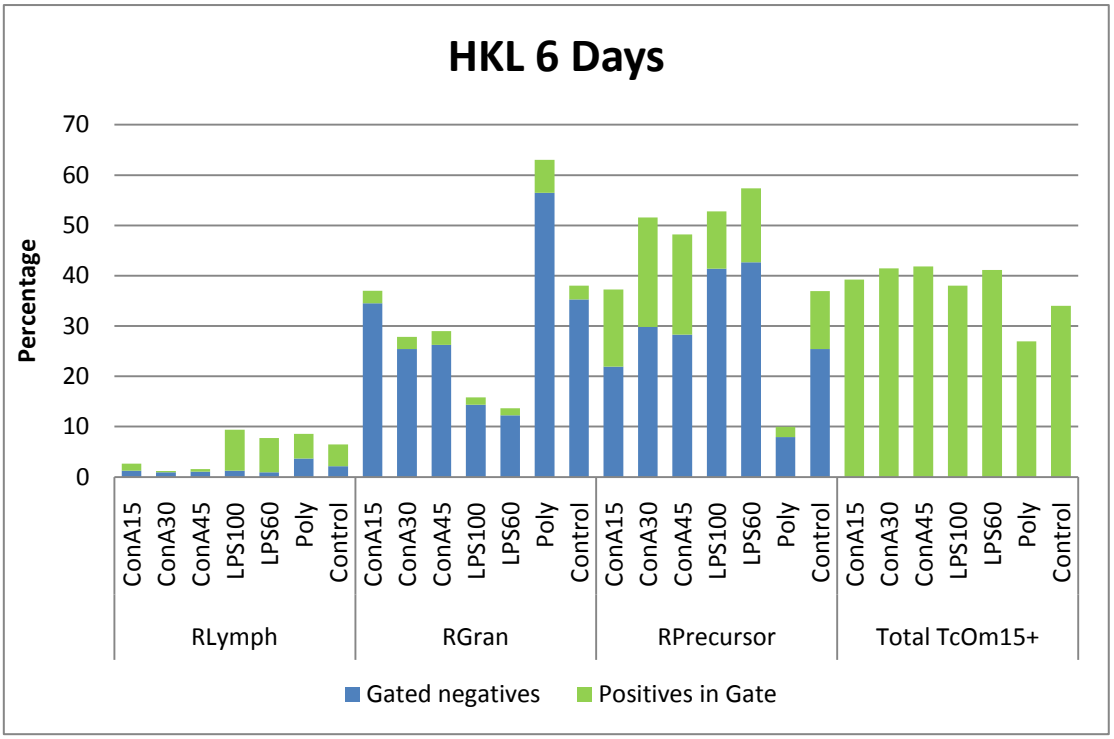


Figure 5.23 Flow cytometry analysis of rainbow trout leukocytes from head kidney (HKL) cultured for 3 and 6 days. Percentages of total positive cells (in green), gated cells in RLymph, and gated cells in RGran (negatives in blue and positives in green) from 10,000 events. Data from cultures with three different concentrations of Con A (15, 30 and 45 µg/ml), two of LPS (60 and 100 µg/ml), one concentration of Poly I:C (100µg/ml), and controls are shown.

When comparing the level of stimulation obtained over time, the main findings are that the number of cells in RLymph did not show an increase in the number of cells nor in the percentage of fluorescence. The mitogens Con A and LPS induced a decrease of the population gated in RGran, while Poly I:C was a strong promoter of activation for granulocytes. However, the number of cells labelled inside gate RGran showed a tendency to decrease from 13 % to 8 % following stimulation where the highest labelling was found for Poly I:C (Figure 4.24). The number of cells gated in the RPrecursor region increased by about 10 % over time for all mitogens except for Poly I:C, where very low levels were maintained. An increase of about 10 % was observed in the total number of fluorescing cells, attributable mainly to the precursor population (Figure 4.24).

5.3.5 Effects of immunostimulation <u>in vivo</u> by Ergosan and β-glucan on expression of TcOm15^{+} cells.

A total of 234 leukocyte samples isolated from 4 organs (PBL, thymus, spleen, and head kidney) were prepared from 56 fish sampled. However, unfortunately the samples were fixed in formalin/PBS prior to staining for FCM. This fixation step may have altered the staining obtained by denaturing the antigen binding to the mAb. The data obtained in FCM did not appear consistent, and it was difficult to differentiate between the various cell populations. Thus, only the total percentage of TcOm15+ cells was analysed. When the percentage of total fluorescing cells were analysed from the different organs, one fish fed with Ergosan and one fish fed with yeast for three days showed a high percentages of labelled cells, 22 % for the fish fed Ergosan (Figure 4.28) and 14 % for the one fed yeast (Figure 4.25). After 14 days of immunostimulation with Ergosan, two out of five fish showed a high percentage of

staining in their thymocytes (40 % and 21 % respectively), while fish from day 0 and controls showed a maximum of 6% of staining (during the whole experiment) (Figure 4.25 & Figure 4.26). No statistical differences were found between treatments or time in any of the populations of leukocytes.

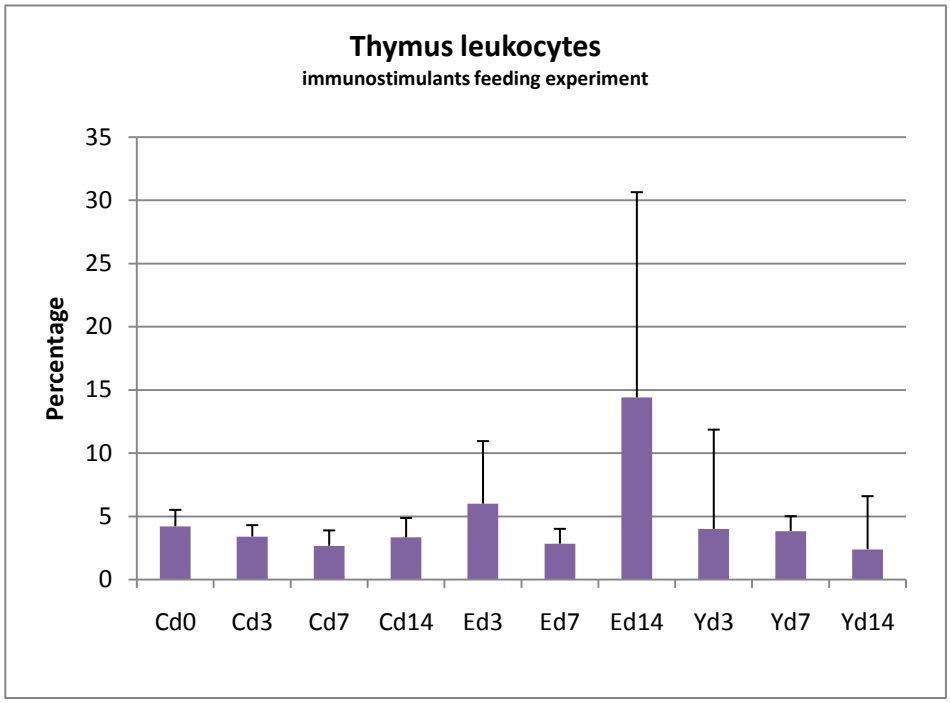


Figure 5.24 Percentage (± s.d.) of total TcOm15 positive cells from thymus leukocyte from rainbow trout fed with Ergosan (E) or β-glucan (Y) during 15 days. C, control; d, days post treatment. Six fish were evaluated for every sampling treatment and time point (n= 6 fish per treatment).

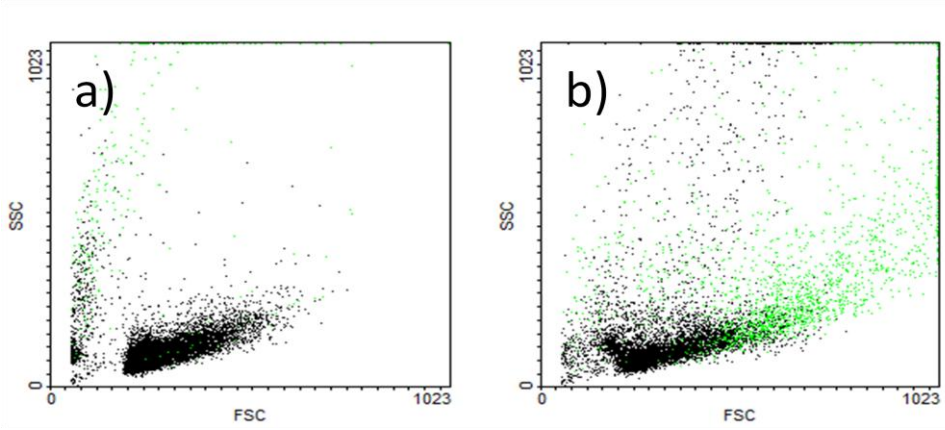


Figure 5.25 Example dot plots for total cells labelled from thymus leukocytes of a) control trout fish after 14 days (3 %), and b) Ergosan fed trout after 14 days (30 % of staining).

5.4 Discussion

Activation assays are widely used in mammals to evaluate the ability of organisms to respond to stimulation (Schwager & Schulze, 1997). For teleosts, activation has been observed for leukocytes from different tissues and different fish species (Passer *et al.*, 1996; Scapigliati, Romano & Abelli, 1999; Sarmento *et al.,* 2004; Moulana *et al.,* 2008). In the present chapter the ability of TcOm15 mAb to detect modulation of the labelled molecule on Rainbow trout leukocytes by the activity promoted of different mitogenic conditions was evaluated.

5.4.1 mHCA experiments

In the mHCA, enhanced with LPS and Con A, detection of cells was very low (2 % above control for LPS and 4 % for Con A). Although there was little detection, the values of TcOm15+ cells obtained from three replicate wells were constant for each treatment, suggesting consistent expression of the antigen recognised by this mAb during culture (6 % above control for LPS and 8 % for Con A). While the results from the different experiments cited in the literature are not directly comparable, due to differences in the treatments used, variations in culture conditions, and the disease status of the fish are helpful for reference as these give an interesting comparison. Wang *et al.*, (1997) found that Con A and LPS promoted effective activation of whole blood leukocytes from percichthyid bass indicated by a stimulation index of 4, measure by ^{3}H-thymidine. Conversely, Scharsack *et al.* (2001) observed by FCM that after 5 days of culture leukocytes from Rainbow trout with Con A (30 $\mu g ml^{-1}$), the numbers of cells gated in the lymphoid region remain constant (431 cells for control and 429 for Con A). The studies by Gauthier *et al.* (2003) showed that activation of leukocytes from

Rainbow trout revealed a maximum stimulation index of 2. In the present study, the analysis of data from mHCA experiments shows that cell activation was not easily distinguished by just examining the total number of gated cells in a region. Detailed analysis of cells stained with mAb TcOm15 allowed different peaks of staining intensity to be detected (*e.g.* an intensity of 12.0 for the basal cell reaction in M3 and 4.0 for Con A stimulated cells) (Table 4.2). The specific detected population in M3 showed a characteristic behaviour or modulation by the different concentrations of Con A, and due to the specific activity on T-cells, it is possible that this type of cells were involved. After one day of stimulation with 45 μgml^{-1} of Con A, the populations in the M3 were seen to increase in cell numbers but a considerable decrease in the antigen detection by mAb TcOm15, possibly due to a down regulation of the molecule, was also observed. After six days TcOm15+ cells were detected again (*i.e.* acquiring an intensity close to that of the controls, 8.9). When the same cells were stimulated with LPS, a down regulation of the detected molecule with the mAb was observed by the third day (*e.g.* 12.0 Gmean intensity for basal reaction cells in M3 and 6.6 for LPS 45 μgml-1, Table 4.2), but TcOm15+ cells were not detected after six days (control Gmean 13.4 and LPS 5.0). However, it is possible in this case that the dose of LPS used was not high enough to obtain activation. Higher concentrations have been shown to be needed to obtain activation of leukocytes (Wang *et al.*, 1997; Kehrer, Hannan & Raison, 1998; Morrison *et al,.* 2004; Araki *et al.*, 2007). The group of cells in M2 did not show stimulation, so there appears to be a lack of stimulation of these cells by Con A and LPS. Added to that, there appeared to be no effect of mitogens on the expression of the antigen detected by mAb TcOm15, so it is possible that the TcOm15 molecule is conserved in cells that are not stimulated by Con A or LPS. Meloni *et al.* (2006)

reported that the time necessary to obtain a two fold increase of T-cells in a HCA was two weeks. Taking this into account, it could be assumed that the time used for the experiments with mHCA (3 and 6 days) was not long enough to obtain higher peaks of response. Arkoosh, Clem & Casillas (1994), obtained similar negative or borderline positive activation of leukocytes in studies with *Pleuronectes vetulus* using a three way HCA, evaluated by ^{3}H-thymidine after 5 and 7 days.

In early studies on human immunology, identification of subpopulations of leukocytes was based on activation caused by Con A in MLR and in proliferation assays, and proved to be a very useful tool for the description of lymphocyte subpopulations (review Dwyer & Johnson, 1981). In the present study Con A and LPS on activation *via* foreign histocompatibility complex assays promoted stimulation and variation of TcOm15 molecule detection on the cells in M2, but a strong decreased detection in another group of cells (M3) by Con A stimulation that was recovered with the time.

5.4.2 Stimulation of cells from individual fish

To better understand the effects of activation on TcOm15 molecule detection by FCM, analysis of data from individual animals was performed. In general, it was observed that PWM and PMA had a more pronounced effect on TcOm15+ peripheral blood leukocytes from Rainbow trout. Because of different patterns of stimulation on leukocytes were detected on individual fish stimulated with mitogens, it is necessary to emphasized that the Rainbow trout used in these studies were families from a farm where records of breeds, diseases or treatments, that could have give an explanation for the individual variations, was not obtained. The results obtained agrees with the those observed by Kehrer, Hannan & Raison (1998) who found PWM to be a better

stimulator of peripheral blood trout leukocytes than mitogens such as Con A, LPS or PHA. PWM has the ability to stimulate both B and T-cells in mammals, and not just the specific populations stimulated by Con A, LPS or PHA (Ashraf & Khan, 2003). Even though individual fish showed different levels of activation and stimulation with the various mitogens used, a similar pattern of response was seen between fish. It is possible that the difference in the proportions of stimulation and expression is related with the immune status of the individual fish, but similar patterns of response, consistent within cell populations, were observed upon stimulated by the mitogens. Replication of assays, managing same periods of time under stimulation during experimentation, was a weak element during the thesis research where results obtained from experiments evaluated with confocal microscopy are no supported by FCM approach. Such sort, is the finding of two fold increased registration of mean intensity of Texas red conjugate on ConA stimulated TcOm15+ cells after 20 mins in relation with the mean intensity of DAPI staining the nuclei compared to the control. With this ratio calculation it was expected to indirectly find a relation of TcOm15+ staining with the amount of DAPI fluorescing in nuclei of cells. It was considered that this approach could be valid, in the absence of a reliable method to count single cells in a localised tridimensionally on the slide.

5.4.3 PMA down-regulation

An interesting reaction was observed with PMA stimulation for which the expression of TcOm15 molecule was totally lost. Phorbol esters act on biological membranes and have affinity for phospholipid membrane receptors and are activators of the protein kinase C enzyme (Ron & Kasanietz, 1999; Silinsky & Searl, 2003). In the present study a dose of 5 ng PMA was used to stimulate leukocytes from peripheral

blood and head kidney, however no obvious stimulation was obtained and this may be explained by the lack of addition of a cofactor such as Ca+ for PKC to enhance activation, though some studies have demonstrated that the addition of phorbol ester compounds can effectively activate leukocytes in fish (Lin *et al.,* 1992; Blohm *et al.,* 2003). For mammals, activation has even been reported in a Ca+ independent pathway (Gelfand *et al.,* 1985; Burnet & Schwarz, 1994). For the present study, the addition of PMA without Ca+ did not have any effect on activation, but strongly down regulated the expression of TcOm15. This down-regulation suggests that possibly the TcOm15 molecule is related with changes promoted by PMA, either through the PKC pathway or an alternative phorbol receptor. It has been shown in humans that important receptors or molecules are down regulated by PMA. For instance, it is argued that the receptor for TNFα is down-regulated by PMA due to the redistribution of receptor caused by modifications in the cytoskeleton or by phosphorylation of the receptor (Aggarwal & Esssalu, 1987; Winzen *et al.,* 1992; Kilpatrick *et al.,* 2000). Another example molecule down-regulated by PMA is L-selectin; it is a molecule that mediates leukocyte homing through binding to ligands on vascular endothelial cells. This molecule, which is constitutively expressed on all virgin or naive leukocytes, but absent in a subset of effector or memory cells, is rapidly shed after activation with PMA (Kansas, 1996, Fors *et al.,* 2001). The possibility that the TcOm15 molecules resemble one of these molecules (known down-regulated in humans) needs to be further investigated.

5.4.4 Modulation of expression in organs

The TcOm15+ leukocytes from peripheral blood and the thymus under the experimental conditions used were most sensitive to the action of stimulators. An

interesting result was the detection of an almost two fold increase of TcOm15+ cells with mitogens. Thymus leukocytes from fish have shown the ability to activate in the presence of PHA, as was observed by Rombout *et al.,* (1997). However, differences in the amount of PHA exist between the dose from the present study (20 μgml^{1-}) and the one cited by Rombout *et al.,* (1997) (1 μgml^{1-}). In humans, the culture of the thymic cells has been used to identify subpopulations of thymic cells (Forsdyke, 1969) and to examine how these subpopulations respond to stimulation to PHA. For instance, up-regulation of CD4 molecules by PHA has been shown to be a marker to identify activated CD8+ cells, a controversial subpopulation of double positive T cells (DP T) (Sullivan *et al.,* 2001).

The high percentages of TcOm15+ found *in vivo* in some fish in the present study, may be a random finding not related to the immunostimulants administered, yet the thymic cells of these fish appeared to be stimulated with levels comparable to those found *in vitro*.

Attempts to study the expression of the TcOm15 antigen from different organs with different mitogens by SDS-PAGE and Western blot failed probably because of the existence of mixed populations of cells and the ability of the mAb to detect cytoskeletal proteins. The presence of several leukocyte populations expressing TcOm15+ at different levels could mask the results. Alternatively, is possible that the dilution of the mAb TcOm15 was not appropriate to detect the proteins in the Western blots assays. The FCM results reflect the variation of the TcOm15 molecule during different treatments because the staining was used to detect molecules on the membrane of the cell and not internal antigens which would have required permeabilization of the cells.

The series of assays performed in this chapter should be considered as preliminary because of the low number of samples. used. However, the results obtained provide enough evidence to confirm that FCM is an ideal method to obtain valuable information as to the role of the molecule detected by the mAb TcOm15 and its apparent involvement in the activated state of leukocyte populations of Rainbow trout. In fact much of the research involving detection of markers on cells are initiated with assays that, by the continue experimentation, are improved. Further experimentation is necessary to corroborate the findings.

Chapter 6 General Discussion

The differentiation of cell phenotype and function during an immune response has been made possible in mammals thanks to the availability of mAbs against cell surface markers (Mak & Saunders, 2006). For example, the activation of leukocytes is an important step in the inflammatory response in which the cells become primed to respond. Increased expression of some antigens or expression of new antigens occurs on the surface of the cells as they become activated, while the expression of other antigens is down-regulated.

Unlike mammalian immunology, there are few mAbs to identify different cell markers on teleost leukocytes. However, great progress has been achieved in the knowledge of fish immune response thanks to advancements in molecular biology. Genes involved in the immune reaction of teleosts have been sequenced and characterised, allowing better interpretation of the mechanisms which occur during physiological changes in the humoral and cellular immune responses so as to maintain homeostasis. The main features of the immune response found in mammals have been also identified in teleosts, such as cellular responses (e.g. B-cells, T-cells, CD8, CD4, plasma cells, phagocytes, killer cells, *etc.*), and humoral mediators (antibodies, cytokines, lymphokines, chemokines, *etc*) (Castro *et al.,* 2010; Maisey & Imarai, 2010).

The aim of this research project was to develop a cell marker for the activation of Rainbow trout leukocytes that could be used to provide information about the function of leukocytes upon stimulation. To achieve this, the thymus of rainbow trout was selected as a source of leukocytes and used to immunise mice for MAb production. The thymus is important as a haematopoietic tissue and contains both

immature and mature forms of leukocytes (mainly T-cells). The leucocytes isolated from the thymus were first stimulated with a potent T-cell mitogen (e.g. Con A) prior to immunising the mice with the cells. The intention of this was to promote changes in the expression of functional molecules on the surface of the cells and against which the mouse antibodies would be produced.

Selection of the MAbs during the screening process was based on the ability to detect surface antigens on thymic cells using a dot blot. Further screening was performed by FCM using PBL stimulated allogeneically with incompatible leukocytes in the presence of Con A. It was believed that this would allow the selection of mAbs that could recognise activated T-cells. The mAb TcOm15 was selected from the screening process and its ability to stain activated leukocytes assessed. Three main bands of protein were detected in Western blotting by this mAb, and these were identified by mass spectrometry as a cytoskeleton-related molecular complex consisting of non-muscle myosin, spectrin and actinin, and also as a immunoglobulin-like protein. Assays were evaluated by FCM in order to explore the ability of mAb TcOm15 to stain cells were performed, showing that cytometry is a valuable technique to detect changes associated with activation of Rainbow trout leukocytes.

As explained above, the mAb of particular interest, produced during the study (*i.e.* mAb TcOm15) was originated from mice immunisated with thymic cells activated with Con A. The thymus is an organ where T-cells experience an ideal microenvironment for maturation, during which they acquire specific receptors (TcR and CD3) and antigens of differentiation (*i.e.* CD8+ or CD4+) on their surface, thus enabling them to respond to foreign antigens (Picchieti *et al.,* 2009). Con A was used to activate mature T-cells so that they would express surface markers that were

phenotypically different to naive T-cells. The aim was to produce MAbs against these expressed antigens by immunising the mice with the activated cells.

During the initial screening, only thymic cells were used to detect positive MAbs. In hindsight it may have been more useful to also have screened with spleen cells as well as the thymic cells, to improve the chances of selecting mAbs against only T-cells (*i.e.* mAbs that stained thymus but not spleen cells). Thus there was of an obvious risk that other potentially useful MAbs were missed during the screening process.

The differences observed during screening by FCM with a basal or enhanced allogeneic reaction (multiple way histocompatibility activation, mHCA), stimulated with Con A, were important for the selection of mAb TcOm15, with differences possibly related with up-regulation of molecules on the surface of activated cells. The activation *via* foreign histocompatibility detection (HCA) for screening was a modified MLR reaction for which little information is available as a comparison. It is possible, that a better defined reaction may have allowed the detection of mAbs to specific cell populations (*e.g.* cytotoxic, non-cytotoxic cells). Even though the reaction has not been fully described elsewhere, it should still be considered as a useful assay for this study, especially because of the reproducibility of the results obtained within assays. Constant results were obtained between replicates, and mAb TcOm15 was shown to detect differences between the doses of Con A used (*i.e.* the more Con A used the fewer positive cells obtained). However, between assays there were differences, explained possibly because the proportions of participating cells during the HCA (modified MLR) reaction could have changed according to the number of responsive fish during the multiple way reaction. Unfortunately it was not possible to identify the cells

responding during this multiple-way reaction. However, the participation of cytotoxic T-cells would be expected during a allogeneic reaction, based on the findings of other authors (Meloni *et al.,* 2006; Picchietti *et al*., 2008; Araki *et al*., 2008). The first impression obtained during initial cell screening by FCM was that a small population restricted to the lymphocyte region increased in number when stimulated with Con A. However, further work showed that the staining was not restricted to this small population of leucocytes. Lymphocyte-like, granulocytes-like and monocyte-like cells were stained and observed using confocal microscopy where it was possible to observe a mesh-like pattern (Lymphocyte-like cells) and dotted patterns of staining (granulocytes-like and monocyte-like cells).

It appears that the mAb identified in this study did not recognise phenotypic T-cells, but instead the molecule recognised by mAb TcOm15 seemed to be up-regulated during the allogeneic response enhanced by Con A stimulation. The role of the TcOm15 molecule in the MLR could be investigated further by isolating TcOm15+ cells and investigating the functional activity of these cells, such as cytotoxicity *i.e.* by blocking the antigen site with mAb TcOm15+ in order to stop its functional activity (*e.g.* by observing inhibition of cytotoxic activity). The incubation of cells with mAbs to evaluate functional activity of the molecules on the surface of cells has been used previously to investigate the inhibitory effects of anti-aggregatin found during phagocytosis by seabream macrophages (Mulero *et al.,* 2001).

The application of mAbs already available for rainbow trout leukocytes (as shown earlier in Table 1.3) to screen the cell populations labelled with mAb TcOm15 may help provide information on the cells identity. This could permit separation or

enrichment of cells in order to evaluate their functional activity and allow relating the modulation observed with the labelled molecules on specific populations of cells.

The detection of mAbs during the present study could have been improved by specifically looking for differences in functional cell populations during the screening with the mHCA. Assessments of functional activity should be included in future work aimed at developing mAbs against different cell populations, *e.g.* using phagocytic assays whereby the bacteria are labelled with fluorescence to monitor phagocytosis by macrophages, neutrophils, or even B-cells, and also screen to find potential markers by double fluorescence cytometry (Li *et al.*, 2006; Overland *et al.*, 2010). The importance of developing markers against functional states of cells would provide valuable tools to evaluate immunological states *in vivo*.

A key factor preventing the development of mAbs against fish immune cells is the absence of relevant cell lines representing cellular components of the fish's immune system. These would provide a means of standardising the assays used for screening and for comparing results between experiments and laboratories. In the present study, inconsistencies were found in the recognition of the molecule TcOm15, where cells were obtained from Rainbow trout from fish farms. For instance, where comparisons were made between individual fish or experiments repeated, differences in the proportions of cells labelled with the mAb cells were observed. The use of farmed fish in these experiments is not ideal because their history of disease or exposure to pathogens is unknown; their genetic background differences between fish and the environments the fish are cultured in also varies. In early studies performed for human cell marker development, great achievements were made using available cell lines. For example, the K562 human cell line (natural killer activity) (Moretta *et al.,* 1981), T cell

lines CEM, HSB-2 and MOLT-4 (Reinherz *et al.,* 1980), or Epstein-Barr virus-transformed B lymphoblastiod cell line (Engleman *et al.,* 1981) all contributed to such work. Although there have been advances in the number of mAbs against components of the immune response of catfish (Passer *et al.,* 1996; Moulana *et al.,* 2008) using cell lines for this species (Miller, Chinchar & Clem, 1994), there are few cell lines for teleosts. This is clearly an important area for future research.

Staining of cells with mAb TcOm15 indicated that distinct cell types were recognised by the mAb, but the level of staining did not occur to the same extend with every cell. Stained cells showed defined staining in confocal analysis which appeared to co-localise with F-actin confirming the results obtained from mass spectrometry. According to the identification of the proteins that bind to mAb TcOm15 (*i.e.* a 200 kDa molecule identified as Myosin; a 160 kDa molecule identified as non-erythroid α Spectrin and membrane bound Ig-like; and a 105 kDa molecule identified as α Actinin-4), the cytoskeleton is highly implicated as the site where the antigen is detected. The results showed co-localization of the mAb with F-actin confirm this relationship and it was possible to observe changes on the pattern of staining (capping, polarization or dots) during mitogen stimulation; however no experiments to further probe this relationship were performed due to time constraints.

The explanation given for the results of the confocal microscopy work is complicated by the fact that the proteins identified by spectrometry are ubiquitous for almost every type of cell present in fish, but it would appear that the reaction of mAb TcOm15 was restricted to only some cell populations. One explanation proposed is that mAb TcOm15 detects a specific site on a complex of proteins that it is being expressed or exposed during stimulation. Studies in mammals have demonstrated that

multi-proteins are related with important cellular functions of the cytoskeleton. The existence of a teleost multi-protein like the Trio complex in mammals may help to explain the detection of multiple cytoskeletal proteins by mAb TcOm15. Trio is a complex protein that contains two guanine nucleotide exchange factor domains, each one associated with pleckstrin homology domains (pleckstrin is a protein domain of approximately 120 amino acids that occurs in a wide range of proteins involved in intracellular signalling or as a constituent of the cytoskeleton), a serine/threonine kinase domain, two SH3 domains, an immunoglobulin-like domain, and spectrin-like repeats (spectrin repeats are found in several cytoskeletal proteins involved in the cytoskeletal structure). Originally, this Trio multi-protein was identified as a protein involved in actin remodelling, cell migration and cell growth (LAR tyrosine phosphatase-binding) (Debant *et al.,* 1996; Medley *et al.,* 2000). Different isoforms of the Trio complex have been described, for instance one expressed during leukaemia, or ones specifically found in the brain (*i.e.* Trio 9S and Trio 9L) (McPherson, Eipper & Mains, 2004). It possible that the antigen detected by TcOm15 could be a protein showing a conformation comparable to the Trio type, *i.e.* a multiprotein complex, that mAb TcOm15 is able to label at a specific antigenic site available in some leukocytes under specific conditions. Attempts to identify this multi-protein complex were unsuccessful (2D Blue Native PAGE, Chapter 3), and it was therefore necessary to analyse the proteins separately in mass spectrometry. It is possible that samples of protein lysates obtained for mass spectrometry analysis were not appropriate for identification of a multi-protein complex. Alternatively, relatively recent interpretation of results on mammal cellular activation confirms that cytoskeletal reorganization is essential for cell activation (Smith-Garvin, Koretzky & Jordan, 2009). This

rearrangement is necessary to polarize and form clusters of receptors at the stimulatory interface, where the identified cytoskeletal proteins for this study have been detected (example Figure 5.1). From another perspective, Ig superfamily domains are commonly found on the membrane surface of leukocytes as part of receptors, and have a high participation in cell to cell recognition (example Figure 5.2). Several studies have found that cytoskeletal proteins co-precipitate with receptors of leukocytes, and it is therefore possible that there is a strong association with some receptors and the group of proteins recognised by mAb TcOm15 (Pardi *et al.,* 1992; Joseph & Samanta, 1993; Pavalko *et al.,* 1995).

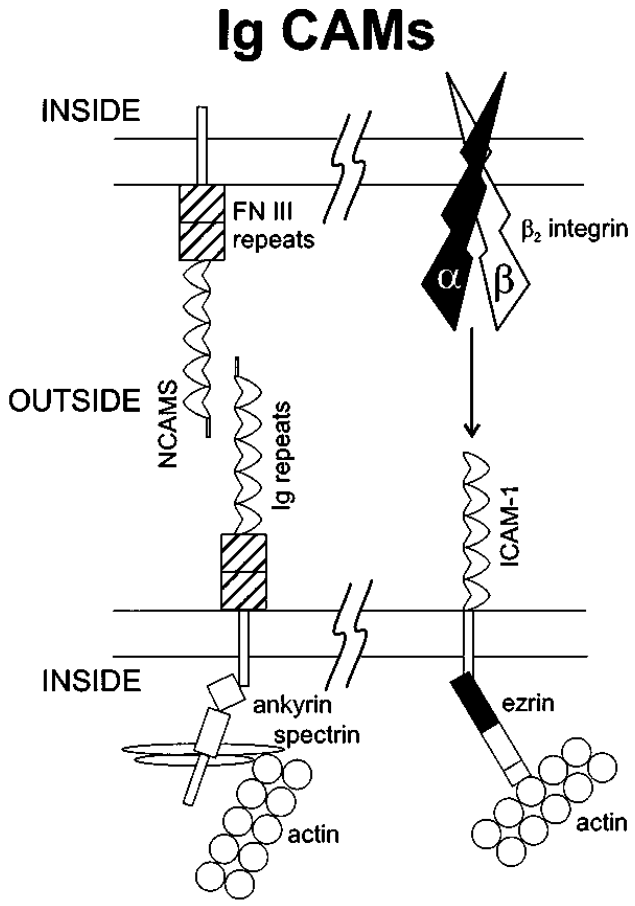


Figure 6.1 Model image of receptors of cell adhesion and associated components of the cytosekeleton. "Ig CAM cell adhesion receptors are depicted in association with their typical extracellular ligands and bound to the proteins that link them to the actin cytoskeleton" (from Barclay, 2003; page 221).

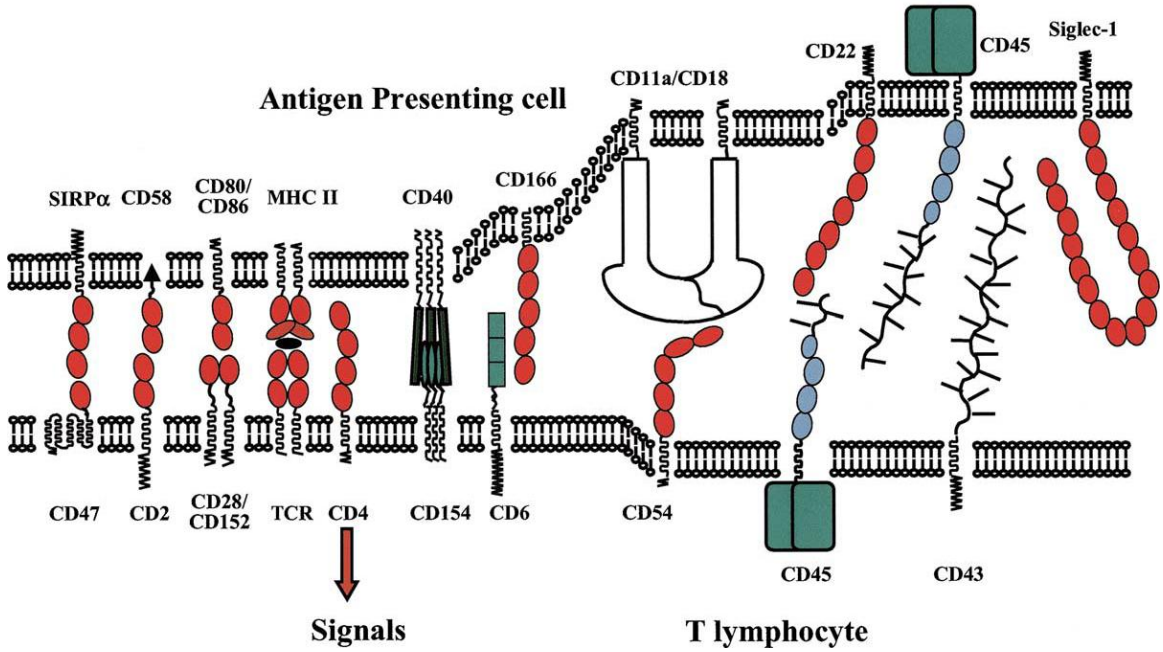


Figure 6.2 "Models for proteins interacting between T cells and antigen presenting cells. IgSF domains are indicated by red ovals. The sizes are based on structural data where available but otherwise the spatial arrangement of domains is speculation" (from Juliano, 2002; page 285).

Different mitogens were employed to promote activation, with the aim of stimulating specific types of cells, with mitogens specific for those cell types. However, due to the low numbers of fish used and replication of samples, the results obtained from FCM were not consistent and should only be considered as preliminary results. It could be that inconsistencies obtained were as a result of differences in the physiological conditions required for each cell type. In general, recognition of TcOm15+ cells by the mAb was observed on cells under specific stimulation of the mHCA and mitogenic stimulation with Con A. The use of mitogens, on their own to non-specifically stimulate cells showed that the TcOm15 molecule was mainly located on circulating lymphocytes and thymocytes. However, the level of TcOm15+ cells observed in lymphocytes isolated from tissues such as head kidney or spleen did not change very much during these experiments. Even though the results are inconclusive, it is

considered that they do provide a good insight in to the potential of mAb TcOm15 as a tool in FCM to evaluate the participation of the molecule detected under controlled conditions (cell lines, MLR assays, *etc*).

The cells detected in this thesis by mAb TcOm15 cannot be described in terms of a phenotype cell population; however, due to the modulation in the labelling of cells observed with mitogen stimulation, it may be possible to define the molecule that the mAbs bind to in terms of function. The flow cytometry results and the anatomical preference of staining in the thymus observed by microscopy could possibly indicate the participation of the TcOm15 molecule during differentiation. However, further studies are necessary to investigate this possibility.

Granulocytes are professional phagocytes that were poorly detected by mAb TcOm15, and no changes in the number of labelled cells were observed during mitogen stimulation. PMA and Poly I:C are substances that have been used to promote activation of macrophages or monocytes differentiation, and have been applied to peripheral blood leukocytes and head kidney cells (Tafalla & Novoa, 2000; Sarmento *et al.,* 2004; Sepulcre *et al.,* 2007). In the present study, low levels of staining of head kidney granulocytes with mAb TcOm15 was observed only with Poly I:C, while there was actually inhibition or down-regulation of the antigen recognised by the mAb in PBL. It is interesting to note that the results from mammalian studies showed PMA to activate monocytes when co-cultured with peripheral blood T-cells, and induced apoptosis in these T-cells (Wu *et al.,* 1995). This is based on a possible explanation that monocytes activated by PMA induce apoptosis of cells through cell-to-cell contact of receptors. Another possibility is that cells are being stimulated by cytokines secreted from other cell populations present during the culture, after they were directly

activated by the mitogens. For instance, PMA treatment effects on co-cultured granulocytes could have promoted the activation of the cells resulting in the release of factors (*e.g.* cytokines) that produced the down-regulation of the molecule in another co-culturing population of cells sensitive to these factors. Studies with activation factors like PMA and antagonist of activation through protein kinase C in leukocyte cultures to elucidate the cause of the strong down-regulation of TcOm15 molecule are required.

Development of markers for cellular components of the immune response of fish has been the subject of many studies over the past few decades (Secombes *et al.,* 1987). However, success in these endeavours has been poor, partly due to the diversity of fish species examined, and the numerous surface molecules that could be detected and described (without counting cytosolic or soluble molecules). The results suggest that this task needs to be performed by numerous groups of scientists, with the intention of characterising molecules that show modulation during the immune response. The search for key cell markers in fish that are equivalent to the markers known in mammals has been limited to research on molecules such as $CD8^+$, TcR, *etc.*, possibly restricting the development of novel markers for teleosts. Thus, potential markers for leukocyte function may have been overlooked, because they do not fulfil the characteristics of markers that phenotypically differentiate between populations of mammalian leukocytes. Paradoxically, new explanations are being given for mechanisms known in mammals and old paradigms have been renovated (*e.g.* double positive CD4 and CD8 populations, Zloza & Al-Harthi, 2006). Therefore, there is a risk in expecting the same structure and function of molecules as cell markers in fish to those found in mammals, even when their immune systems share many important

features. For example, the existence of B cells has been shown for both fish and mammals, but phagocytic behaviour has only been reported in B cells of fish (Li *et al.*, 2006; Overland *et al.*, 2010). Thus in retrospect, it may have been more advantageous to describe causes, effects or events related to specific molecules, based on the broad knowledge that already exists for mammals, looking for similarities but not equivalences. Once a collection of mAbs is available and used to elucidate the immune response of fish, equivalent mechanisms between fish and mammals may be found, and those specific for teleosts can be described.

Functional studies are a valuable source of information for the characterisation of new mAbs developed as cell markers for fish, and for elucidating the role of actively functioning molecules in the immune response of fish. The mAb TcOm15 developed in the present study has the potential to be a useful tool to detect changes related with cytoskeleton, but elucidation of the role of the molecule detected by the mAb TcOm15 still requires further research. In order to define the applicability of mAb TcOm15 as a marker, future research could be performed to determine regulation of the molecule during remodeling of the leukocyte cytoskeleton for synapses (*i.e.* cell to cell communication) or migration. FCM together with confocal microscopy are invaluable for investigating cytoskeleton modification upon leukocyte activation and would be ideal for use in future work.

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