Characterisation and ontogeny of natural killer cells in Xenopus laevis

Stewart, Rebecca

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Rebecca Stewart

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The initial aim of the work described in this Thesis was to investigate the lymphoid organ distribution, phenotype and function of the lymphocyte population identified by candidate anti-*Xenopus* natural killer (NK) cell monoclonal antibodies (mAb's). Since removal of the thymus gland early in larval life (thymectomy) results in the eradication of T-cells and subsequent increase in the proportion of candidate NK cells, thymectomised (Tx) *Xenopus* were integral in the study of this subset of lymphocytes. Phenotypic and functional studies respectively demonstrated that mAb-defined candidate NK cells do not belong to the B- or T-cell lineage and display cytotoxic activity towards MHC class-Ia-deficient tumour target cells, strengthening the contention that these cells represent the NK subset in *Xenopus*.

The ontogeny of NK cells was investigated in relation to the emergence of the NK cell inhibitory ligand, MHC class-I. Splenic NK cells were found to emerge in 6-7 week-old larvae (stage 56-58), which is ≈5 weeks after T- and B-cells become detectable, and some 2 weeks after MHC-Ia is first detected. However, these cells do not appear to be functionally competent until 6 months of age. The expression and ontogeny of recently cloned β2m (the molecule essential for MHC class-I expression) was also briefly investigated. β2m (both RNA and protein) was detectable in all adult tissues and cell lines, even class-I-deficient tumour cells; β2m transcripts were found in 5 week-old larvae that lack MHC class-I.

The emergence of NK antigen on a population of T-cells following *in vitro* stimulation of splenocytes with PMA and calcium ionophore presented the opportunity to biochemically characterise (through immunoprecipitation) the mAb-defined NK antigen. Proteins precipitated using the anti-NK mAb were either surface labelled with biotin, or metabolically labelled with ^{35}S. Both techniques resulted in the detection of a protein 55kDa in size.

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Contents

Tables and illustrations

Declaration

I confirm that no part of the material offered has previously been submitted by me for a degree in this or any other University. Material generated through joint work has been acknowledged and the appropriate publications cited. In all other cases material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

Signed: ..... R Stewart .....

Date: ..... 11/2/03 .....

Statement of copyright

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Abbreviations

^{3}H-TdR	:	tritiated thymidine
A_{260}	:	absorbance at 260nm
ABC	:	avidin-biotin-peroxidase complex
ADCC	:	antibody dependent cellular cytotoxicity
APBS	:	amphibian phosphate buffered saline
BSA	:	bovine serum albumin
Ca^{2+} ion	:	calcium ionophore
CD	:	cluster of differentiation
CMF	:	calcium/magnesium free
Con-A	:	concanavalin A
CTL	:	cytotoxic T-lymphocyte
DAB	:	3'3'-diaminobenzidine
DAG	:	diacylglycerol
ddH_20	:	double distilled water
dH_20	:	distilled water
DNA	:	deoxyribonucleic acid
dNTP	:	deoxynucleoside triphosphate
E:T	:	effector to target ratio
EDTA	:	ethylenediaminetetra-acetic acid
FACS	:	fluorescence activated cell sorting
FADD	:	Fas-associated death domain
FCS	:	foetal calf serum
FITC	:	fluorescein isothiocyanate
FS	:	forward light scatter
GFM	:	growth factor-rich medium
HBSS	:	Hank's balanced salt solution
HRP	:	horseradish peroxidase
IEL	:	intraepithelial lymphocyte
IFN	:	interferon
Ig	:	immunoglobulin
IgSF	:	immunoglobulin superfamily
IL	:	interleukin
ILT	:	immunoglobulin-like transcripts
IP_3	:	inositol 1,4,5-triphosphate
ITAM	:	immunoreceptor tyrosine-based activatory motif
ITIM	:	immunoreceptor tyrosine-based inhibitory motif
kDa	:	kilodalton
KIR	:	killer cell inhibitory receptor
L	:	litre
LIR	:	leukocyte immunoglobulin receptors
mA	:	milliamperes
mAb	:	monoclonal antibody
MACS	:	magnetic cell sorting
mg	:	milligram
MHC	:	major histocompatibility complex
ml	:	millilitre

MS222	:	3-aminobenzoic acid ethyl ester
ng	:	nanogram
NK	:	natural killer
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
PE	:	phycoerythrin
PHA	:	phytohaemoagglutinin
PI	:	propidium iodide
PIP_2	:	4,5-biphosphate
PKC	:	protein kinase C
PLC	:	phospholipase-C
PMA	:	phorbol 12-myristate 13-acetate
PS	:	phosphatidylserine
Rag-1	:	recombination activating gene-1
RBC	:	red blood cell
RNA	:	ribonucleic acid
rpm	:	revolutions per minute
RT-PCR	:	reverse transcriptase polymerase chain reaction
SDS-PAGE	:	sodium dodecyl sulphate polyacrylamide gel
SS	:	side scatter
TBS	:	Tris-buffered saline
T_c	:	cytotoxic T-cell
TCR	:	T-cell receptor
T_h	:	helper T-cell
TNF	:	tumour necrosis factor
Tx	:	thymectomised
UV	:	ultra-violet
V	:	volts
α-MM	:	α-methyl mannoside
μg	:	microgram

CHAPTER 1

General Introduction

1.1 Mammalian natural killer cells

1.1.1 Innate and adaptive immunity

Responses of the immune system can be described as being either innate or adaptive. Innate (non-specific) immunity is the first line of defence against viral, bacterial and parasitic pathogens, and its components include leukocytes such as macrophages and natural killer (NK) cells, and complement proteins. The adaptive immune system is antigen-specific and, unlike innate immunity, improves with repeated exposure to a particular antigen. The adaptive system "remembers" the encounter with the pathogen, producing antigen-specific long-term immunity, thereby preventing it from causing any future disease. Both B- and T- cells are adaptive immune system components, the former responding to extracellular pathogens by producing antibodies. T-cells demonstrate a wide range of functions and can be further divided into two subsets, helper T-cells (T_h) and cytotoxic T-cells (T_c). These subsets can be functionally subdivided further on the basis of their cytokine secretions e.g. $T_{helper1}$ cells, which secrete IL-2 and IFN-γ, and $T_{helper2}$ cells which secrete IL-4 and IL-10. T_{h2} cells are involved in promoting antibody production by B-cells, whereas T_{h1} cells help phagocytes destroy ingested pathogens and also promote activation of T_c cells. Helper T-cells interact with antigenic peptides delivered to the cell surface by MHC (major histocompatibility complex) class-II molecules whereas the cytotoxic T-cells function to lyse virally-infected cells through interactions with MHC class-I molecules. Should a cell become infected with a virus for example, the MHC-I proteins present peptides of viral origin at the cell surface. These foreign proteins are detected by T-cell receptors (TCR's), which signal to the T-cells to destroy the infected cell. In some cases, virally-infected or tumourous cells will down-regulate their MHC-I expression, rendering T-cells unable to detect the dangerous cell. In such cases, the innate immune system comes into play by recruiting NK cells, whose duty is to destroy cells which have down-regulated class-I molecules. It is this subset of lymphoid cells which is the main focus of this Thesis.

1.1.2 The major histocompatibility complex (MHC)

The MHC is a region of highly polymorphic genes (reviewed in Owen, 1998), originally discovered in the 1940's by Gorer and Snell (Abbas *et al.*, 1991; Janeway, 1993), the gene complex playing a crucial role in histoincompatibility (transplantation reactions). The human MHC is known as the human leukocyte antigen (HLA) system and murine MHC is termed the H2 system (Parham, 1996). The gene products, the MHC antigens, are highly polymorphic cell surface proteins of which there are three classes, I, II and III. MHC class I and II proteins both function to present antigens on the cell surface, whereas the class-III MHC proteins are structurally and functionally distinct from class-I and II molecules and are involved in antigen processing and the complement system (Owen, 1998). Classical class I molecules (class-Ia), encoded for in humans by the HLA genes A, B and C, are expressed by nearly all cells of the body (Janeway, 1993), and present antigenic peptides arising from cytosolic compartments on the cell surface. Should the presented peptide be foreign (of viral origin for example), it is detected by $CD8^{+ve}$ cytotoxic T-cells (Monaco, 1992; Neefjes and Ploegh, 1992), which then proceed to lyse the infected cell *(Fig. 1.1)*. Unlike class-I, MHC class-II expression is restricted to immune response cells, such as B-cells, certain dendritic cells and macrophages (Janeway, 1993). MHC-II molecules bind to, and present on the cell surface, bacterial antigens which have been engulfed by macrophages or B-cells and are residing inside the cell within vesicles. Such MHC-II/antigen complexes are recognized by T-cells expressing the CD4 marker (helper T-cells) (Monaco, 1992; Neefjes and Ploegh, 1992) *(Fig. 1.1)*. Unlike cytotoxic T-cells, helper T-cells (e.g. T_{h1}) do not directly lyse the infected cell themselves, but instead may stimulate the activation of the infected cell, which is then able to deal with the bacteria within its own vesicles. Alternatively, T_{h2} cells mediate target cell destruction through the recruitment of other lymphocytes such as B-cells.

MHC-I molecules are comprised of a glycosylated 45kDa heavy chain and possess three extracellular domains, each of approximately 90 amino acids, denoted α_1, α_2 and α_3, together with a transmembrane region and cytoplasmic tail (Owen, 1998).

The α_1 and α_2 domains form the protein binding groove, a polymorphic region which binds peptides approximately 9 amino acids in length. This MHC-encoded heavy chain non-covalently interacts with the 12kDa polypeptide β_2m (Parham, 1996), a molecule which is invariant in humans (Owen, 1998), polymorphic in mice and which is encoded outside of the MHC gene complex (reviewed in Shum *et al.*, 1996).

The class II gene products are heterodimers denoted DR, DQ and DP in humans. They are comprised of a heavy (α) chain of 30-34kDa and a light (β) chain of 26-29kDa. Class-II molecules possess an extracellular region of two domains, α_1 and α_2 or β_1 and β_2, a transmembrane region and cytoplasmic domain. The peptide binding groove formed between α_1 and β_1 is able to bind longer peptides (approximately 17 amino acids in length) compared to MHC-I.

Class-I molecules can be further subdivided into classical (Ia) (described above) and non-classical (Ib) molecules, the latter encoded by the genetic loci HLA-E,-F and -G, are non polymorphic and restricted to cells of the thymus, liver, intestine and placenta (reviewed in Hughes *et al.*, 1999). A further set of genes have been discovered, termed the MIC genes, of which only MICA and MICB are expressed, even in the absence of bound peptide or the molecule β_2-microglobulin (β_2m), which is essential for class Ia expression (Hansen *et al.*, 1988; Vitiello *et al.*, 1990). These MIC proteins have been termed class-Ic molecules (Hughes *et al.*, 1999) and are encoded by gene loci within the MHC complex or at least on the MHC chromosomes. Although it is known that MICA is stress-induced (Wu *et al.*, 1999), the specific functions of this molecule are yet to be determined (Hughes *et al.*, 1999). Additionally, class-Id proteins exist, which are encoded for by genes located on chromosomes other than those related to MHC. They are non-polymorphic proteins and include the molecule CD1 (Calabi and Milstein, 1986), which presents lipid molecules to T-cells (Beckman *et al.*, 1994). Both classical and non-classical class-I molecules are ligands for NK cells.

1.1.3 Characterisation of mammalian NK cells and NKT-cells

1.1.3.1 NK cells

Morphologically, NK cells are relatively large (12-15μm in humans, 8-10μm in mice) and possess cytoplasmic azurophilic granules; they are consequently often termed "large granular lymphocytes" or "LGL's" (reviewed in Valiante and Parham, 1996). Derived from lymphoid progenitors in the bone marrow, they form heterogeneous populations with regard to both phenotype and specificity (Allavena and Ortaldo, 1984) and are distinct from T- and B-cells, lacking T-cell receptors/surface CD3 complex and Ig gene rearrangements respectively (reviewed in Valiante and Parham, 1996). NK cells account for approximately 10-15% peripheral blood lymphocytes and can also be found in the liver, mucosal tissues, and in the red pulp and marginal zone of the spleen (Warren, 1996). Unlike T-cells, NK cells do not recirculate between blood and lymph (Rolstad *et al.*, 1986), and it is therefore surprising that they also reside in the lymph nodes, albeit at very low levels (Warren, 1996).

NK cells express a variety of cell surface markers, the majority of which are not exclusive to NK cells, as seen in *Table 1.1.* NK cells also express selectins, β1 integrins (VLA-4 and VLA-5) and β2 integrins (LFA-1 and Mac-1), facilitating NK cell and endothelial cell associations (Warren, 1996). Surface expression of CD31 is also integral in NK movement through endothelial cells and therefore the migration from the blood into tissues (Berman *et al.*, 1996).

An integral part of the innate immune system, NK cells function to spontaneously lyse and destroy cells which have down-regulated MHC class-I protein expression, such as tumour cells or bacterially/virally infected cells (reviewed in Valiante and Parham, 1996). The way in which NK cells carry out this function was originally described by the "missing-self hypothesis" (Ljunggren and Karre, 1990) which suggested the existence of inhibitory receptors on the NK cell surface

capable of associating with MHC class-I molecules. When MHC is down-regulated, the inhibitory receptors no longer have a ligand to interact with, resulting in the activation of the NK cells and the subsequent lysis of the dangerous cell.

1.1.3.2 NKT-cells

There is a distinct lineage of lymphocytes in humans and mice which express both NK and T-cell markers. These cytotoxic cells are termed NKT-cells and are, in the main, characterised by expression of activatory/inhibitory NK receptors together with TCR's of a restricted repertoire.

Murine NKT-cells express the activatory NK marker NK1.1 (Rohrer *et al.*, 2000; Shi *et al.*, 2001) and possess an invariant TCR consisting of Vα14 and Jα281 segments, which associate with Vβ2, -7 or –8 segments (Rohrer *et al.*, 2000). (Vβ8 is not expressed on conventional T-cells (Taniguchi *et al.*, 1996). Murine NKT-cells appear to have a specific distribution and are found in high levels in the thymus, liver and bone marrow in comparison to the spleen, peripheral blood (reviewed in Ishihara *et al.*, 2000) and lymph nodes (Rohrer *et al.*, 2000). Unlike conventional T-cells, NKT-cells associate with glycolipid antigens (Shi *et al.*, 2001) presented by the non-classical MHC molecule CD1 (Hong *et al.*, 1999), expressed by APC's such as dendritic cells (Burdin *et al.*, 1998). The majority of murine NKT-cells are $CD4^{+ve}CD8^{-ve}$ or $CD4^{-ve}CD8^{-ve}$ (Bendelac *et al.*, 1994), although a small proportion are $CD8^{+ve}$ (Rohrer *et al.*, 2000). Such $CD8^{+ve}$NKT-cells also express Ly49 molecules (Rohrer *et al.*, 2000), γδ or αβTCR (Emoto *et al.*, 2000) and display a memory phenotype (Coles *et al.*, 2000). They are not however considered "classical" NKT-cells as they do not have a restricted TCR repertoire and instead express TCR's similar to conventional $CD8^{+ve}$ T-cells (Rohrer *et al.*, 2000). Stimulated $NK1.1^{+ve}$T-cells produce cytokines such as IFN-γ and IL-4 (Arase *et al.*, 1993) and are thought to be involved in T_h-cell differentiation (Vicari and Zlotnik, 1996) and in NK cell proliferation (Carnaud *et al.*, 1999; Erbel and MacDonald, 2000). Murine NKT-cells are cytotoxic towards various tumour cell lines (Takeda *et al.*, 1996) and are also thought to play a role

in the prevention of autoimmunity. Evidence in support of this comes from studies into the nonobese diabetic (NOD) mouse, which is prone to autoimmunity and consistently has low levels of NKT-cells. Over-expression of Vα14-Jα281 or introduction of NKT-cells seems to lessen the severity of the disease (Lehuen *et al.*, 1998).

Human NKT-cells express both inhibitory and activatory receptors. An example of an NK inhibitory receptor expressed by T-cells is p58, a member of the killer cell inhibitory receptor (KIR) family (discussed in section 1.1.4.1) (Ferrini *et al.*, 1994). Such T-cells are $CD8^{+ve}$ (Mingari *et al.*, 1996), and like murine $CD8^{+ve}$NKT-cells possess a memory phenotype (Mingari *et al.*, 1998b). KIR^{+ve}T-cells also show a restricted TCR repertoire, of one or two TCR Vβ segments only (Mingari *et al.*, 1996), although in contrast to murine NKT-cells, KIR^{+ve}NKT-cells are not detectable in the thymus (Mingari *et al.*, 1997a). The presence of KIR molecules on cytotoxic T-cells may regulate NK-like cytotoxicity exhibited by this T-cell subset, and may also prevent autoreactivity (Mingari *et al.*, 1998b). It has been established that patients who have successfully undergone transplant operations mismatched by one haplotype, possess high levels of $p58^{+ve}$T-cells (Cambiaggi *et al.*, 1997). However, there is also the possibility that KIR expression may prevent cytotoxic T-cells from lysing virally-infected/tumour cells which still express MHC molecules (Ikeda *et al.*, 1997). Indeed, the masking of T-cell KIR molecules by mAb's, results in the restoration of lytic activity and the destruction of the infected cell, indicating the inhibitory effect of the KIR (reviewed in Mingari *et al.*, 1998b). Some $CD8^{+ve}$T-cells in humans also express the inhibitory CD94/NKG2A complex (Mingari *et al.*, 1997a), (discussed in section 1.1.4.1), which is induced *in vitro* by the cytokines IL-15, IL-10 and TGF-β (Mingari *et al.*, 1997b; Mingari *et al.*, 1998b).

The human NK activatory receptor NKR-P1 (discussion in section 1.1.4.2), the human homologue of murine NK1.1, is also expressed on a subset of T-cells (Lanier *et al.*, 1994). These T-cells are mostly $\alpha\beta TCR^{+ve}$, $CD8^{+ve}$, and almost all express the activation marker CD69, demonstrating the active state of these NKT-cells *in vivo* (Ishihara *et al.*, 2000). In contrast to a variety of murine NKT-cells,

the TCR repertoire of NKR-P1^{+ve}T-cells is not skewed to Vα24 TCR (the human homologue of murine Vα14 TCR) (Ishihara *et al.*, 1999). The liver has been shown to be a good source of NKR-P1^{+ve}T-cells; approximately one-third of hepatic lymphocytes express NKR-P1, in comparison with only 4% in peripheral blood (Ishihara *et al.*, 2000). The high numbers of these cells in the liver may be due to the presence of Kupfer cells (reviewed in Ishihara *et al.*, 2000), macrophages which produce significant amounts of IL-12 (Cavaillon, 1994), a known up-regulator of NKR-P1 on T-cells (Azzoni *et al.*, 1998). NKT-cells of the CD8^{+ve}NKR-P1^{+ve} phenotype produce TNF-α and IFN-γ (Ishihara *et al.*, 2000) and display even higher levels of cytotoxicity towards various tumour cell lines than conventional T-cells (Ishihara *et al.*, 1999).

1.1.4 NK cell regulatory receptors

In 1995, Yokoyama described the "two receptor hypothesis" *(Fig. 1.2)* suggesting killing activity by NK cells is attributable to complex interplay between signals from both activating and inhibitory receptors. Once an activating receptor has bound its target cell ligand, the transduction of the activation signal is initiated. This results in lysis of the target cell, unless the target is healthy and expressing MHC-I molecules. The inhibitory signals transduced by interaction between the MHC-I on the target cell and the inhibitory receptor of the NK cell overrides the activation signals, subsequently saving the healthy cell from unnecessary lysis.

1.1.4.1 NK inhibitory receptors *(Fig. 1.3)*

It is widely known that the ability of NK cells to lyse target cells is inversely correlated to MHC-I expression on the target cell, a feature which prevents the destruction of healthy cells (Sentman *et al.*, 1995). However, some virally-infected and transformed cells still expressing surface MHC-I are shown to remain NK-sensitive, implying the existence of further mechanisms for recognising dangerous cells (Raulet, 1996).

It has been established that resistance to NK killing is conferred by the α_1 and α_2 domains of the MHC-I on the target cell. Specifically, amino acid residue 74 on HLA-A has particular importance with regard to resistance to NK lysis (Storkus *et al.*, 1991) and similarly residues 77 and 80 on HLA-C molecules (Colonna *et al.*, 1993) and residue 80 on HLA-B molecules (Cella *et al.*, 1994). It is worth noting that MHC-I may also serve a regulatory role in NK cytokine production; the interaction of MHC-I with NK cell clones prevents IFN-γ production. It does not, however, affect NK proliferation (reviewed in Scott and Trinchieri, 1995).

NK receptors used to inhibit reactivity to class-I MHC are heterogeneous (Scott and Trinchieri, 1995) and highly species specific (Moretta *et al.*, 2000). They belong to one of two main groups, the immunoglobulin superfamily (IgSF) and the C-type lectin superfamily.

- **Immunoglobulin superfamily receptors**

A major sub-group of the IgSF is the KIR (restricted killer cell immunoglobulin receptor) family, relatively non-polymorphic type-I transmembrane glycoproteins expressed on human lymphocytes (Karre and Colonna, 1998). By using techniques such as DNA cloning, some 30 members are known to exist (Raulet, 1996), although all are not exclusive to NK cells; a subset of T-cells have been identified which also express KIR's (Phillips *et al.*, 1995) (see earlier section "NKT-cells"). KIR's are clonally distributed and are encoded for by at least 12 genes on human chromosome 19 (Wende *et al.*, 1999) which do not undergo re-arrangements. Any individual NK cell may express more than one KIR on the cell surface (Warren, 1996). Human NK cells are known to express both p58 KIR's and p70 KIR's. p58 receptors are type I membrane glycoproteins (Raulet and Held, 1995) and possess two immunoglobulin domains. In the main, they are monomeric and may appear as 50kDa or 58kDa molecules (Lanier, 1997). p58 receptors have been proven to distinguish allelic subsets of HLA-C (Moretta *et al.*, 1993). In 1995, Yokoyama demonstrated that should targets of $p58^{+ve}$NK cells be transfected using HLA-C cDNA, resistance to lysis results. Such resistance can be reversed with α-p58 monoclonal antibodies (e.g. GL183, EB6, Moretta *et al.*,

1990), therefore establishing the interaction between p58 and HLA-C (Yokoyama, 1995). The p70 KIR has three immunoglobulin domains and recognises polymorphic determinants of HLA-B. In the main, this KIR is monomeric, however, a p70 has been identified, which exists as a homodimer (disulphide-linked), which recognises HLA-A (Lanier, 1997).

Leukocyte immunoglobulin receptors (LIR's), also known as immunoglobulin-like transcripts (ILT's) are additional IgSF NK receptors found on human cells (Ugolini and Vivier, 2000). These receptors are located on human chromosome 19 along with KIR's mentioned above.

Human KIR displays structural homology with the murine receptor gp49B, a monomeric type-I transmembrane glycoprotein of 49kDa which has previously been found on activated NK cells, macrophages and mast cells (Lee *et al.*, 2000; Wang *et al.*, 2000), and is thought to act as a co-receptor for Ly49 (Karre and Colonna, 1998). Another isoform is known to exist (gp49A) although unlike gp49B, this isoform does not possess typical inhibitory receptor features such as immunoreceptor tyrosine-based inhibitory motifs (ITIM's – see section entitled "Inhibitory Signal Transduction") and does not seem to perform similar functions (Lee *et al.*, 2000). Expression of gp49 on NK cells can be induced *in vitro* by IL-2 stimulation or *in vivo* through CMV infection, but is not found on resting NK's and other lymphocytes of spleen and liver (Wang *et al.*, 2000).

- **C-type lectin superfamily receptors**

One sub-group of this superfamily is the Ly49 family of class-I receptors, which were first detected in mice and are encoded by at least 14 distinct genes (Brooks, 1998). Although the Ly49 and KIR families are functionally homologous, they remain biochemically distinct. Ly49 receptors are relatively polymorphic type II integral membrane homodimeric glycoproteins (disulphide-linked) of 44kDa subunits (Yokoyama *et al.*, 1989). Ly-49A receptor was the first to be characterised and eight Ly-49 proteins are now known to exist (Ly-49A to H). It has been established through cytotoxicity experiments that this molecule binds

directly to murine $H2D^d$ MHC-I molecules and causes a subsequent transduction of the inhibitory signal (Karlhofer *et al.*, 1992; Correa *et al.*, 1994). Approximately 80% of NK cells express at least one Ly49 receptor, most express several. Modulation of expression occurs *via* extracellular interactions between the Ly49 receptors themselves and MHC-I on host tissues (Sykes *et al.*, 1993). These receptors are not exclusive to NK cells and share specificity with subsets of T-cells (Warren, 1996).

A second sub-group of the C-type lectin superfamily has been identified in both humans and mice and is known as the CD94 family. Both human and rodent CD94 exists as a heterodimer comprising a CD94 chain (43kDa, (Jaso-Friedmann *et al.*, 1997) and an NKG2 molecule (Lazetic *et al.*, 1996) such as NKG2A/B (Rohrer *et al.*, 2000). CD94-NKG2 heterodimers favour binding to non-classical MHC class-Ib molecules (e.g. HLA-E and Qa-1 in human and mouse respectively). This contrasts KIR's and Ly-49 receptors which bind to classical MHC class-Ia molecules (Ugolini and Vivier, 2000).

- **Inhibitory signal transduction**

The cytoplasmic tails of some KIR's, Ly-49 and NKG2 molecules contain immunoreceptor tyrosine-based inhibitory motifs (ITIM's) of the sequence (I/V)xYxx(L/V), which become phosphorylated on NK cell association with its target. KIR's and NKG2 molecules possess two ITIM's (Bruhns *et al.*, 1999), approximately 23 amino acids apart, whereas Ly49 molecules contain only one per chain, which may in part explain the reason behind Ly49 dimerisation. Higher levels of ITIM phosphorylation result from the cross-linking of the KIR (McVicar and Burshtyn, 2001). ITIM phosphorylation is mediated by members of the Src family kinases (McVicar and Burshtyn, 2001) and results in the recruitment of protein tyrosine phosphatases such as SHP-1 and SHP-2 (Burshtyn *et al.*, 1997b; Bruhns *et al.*, 1999), the former being essential for KIR and Ly49 ITIM phosphorylation (Gupta *et al.*, 1997). SHP-1 contains an Src homology 2 (SH2) domain, whose steric inhibition is removed on binding to the phosphorylated ITIM (Burshtyn *et al.*, 1997b; Barford and Neel, 1998). This enhances the activity of

the phosphatase which proceeds to dephosphorylate substrates involved in NK activation (McVicar and Burshtyn, 2001), such as ZAP-70 and syk family kinases and those involved in the phospholipase-C (PLC) pathway (Burshtyn and Long, 1997a). Inositol triphosphate (IP_3) production is subsequently blocked, therefore hindering the mobilisation of intracellular calcium which is necessary for activation of the NK cell (Valiante and Parham, 1996).

1.1.4.2 NK activatory receptors *(Fig. 1.4)*

NK activatory receptors have proven more difficult to characterise than their inhibitory counterparts, and there is subsequently less information available with regard to these receptors which trigger NK cytolytic activity.

NK cells are known to effectively lyse and destroy MHC-deficient cells (reviewed in Valiante and Parham, 1996). The fact that masking MHC-I molecules with monoclonal antibodies (Moretta *et al.*, 1996) results in target lysis, coupled with the known heterogeneic specificity of NK cell clones, implies the existence of MHC-independent NK activatory receptors (Moretta *et al.*, 2000).

Although molecules such as CD16 (Lanier *et al.*, 1988), CD69 (Moretta *et al.*, 1991), CD2 (Bolhuis *et al.*, 1986) and 2B4 (Mathew *et al.*, 1993) may trigger NK cells, they do so in an indirect manner, the latter two molecules possibly acting as a co-receptor (Moretta *et al.*, 2000). In the early to mid 1980's, a notion was put forward describing the involvement of carbohydrate in NK activation. Although carbohydrate molecules are not thought to specifically affect the formation of NK-target conjugates, the various carbohydrates expressed on target cells bind to NK activatory receptors, resulting in cytolytic activity (Yokoyama, 1995).

A variety of NK cell activatory receptors are known to exist :-

- **NKR-P1**

The study of rat NK cells heralded perhaps the most widely studied NK activatory receptor. All rat NK cells were found to express a homodimeric (disulphide-linked) type II integral membrane protein (30kDa subunits) known as NKR-P1 (Yokoyama, 1993). This protein is of the C-type lectin superfamily and was the first protein of this type to be cloned and sequenced (Giorda *et al.*, 1990). There are thought to be many different isotypes of the NKR-P1 receptor co-existing on rat and mouse NK cells; this may explain why blocking with α-NKR-P1 mAb's does not inhibit killing (Yokoyama, 1993).

The role of NKR-P1 as an activatory marker was implied when it was demonstrated that cross-linking of the receptor with antibodies results in fluxes of intracellular calcium (Ryan *et al.*, 1991). Interest therefore gathered into prospective ligands for the NKR-P1 NK receptor. The generation of a soluble recombinant form of NKR-P1 (denoted sNKR-P1) has been expressed in *E. coli* and has aided in such a search for the characterisation of NKR-P1 ligands. Studies showed that the soluble form binds with high affinity to carbohydrates, and does so in a calcium-dependent manner (Bezouska *et al.*, 1994b; Raulet and Held, 1995). Such association results in target cell recognition and transduction of activatory signals to the NK cell nucleus (Bezouska *et al.*, 1994a). NKR-P1 molecules are known to be more promiscuous with respect to carbohydrate specificity compared to other C-type lectin family members and have particular affinity for β-N-acetyl-D-galactosamine, gangliosides and glycosaminoglycans (Bezouska *et al.*, 1994a). The fact that ligands such as these are found on NK-susceptible tumour cells gives further evidence in support of the major involvement of carbohydrate/NKR-P1 interactions in NK cytolytic activity (Bezouska *et al.*, 1994a). There are possible clinical implications from study into this activation receptor. Tumour cells previously resistant to NK cells can be made vulnerable by incubating with liposomes containing ligands for the NKR-P1 receptor. It is feasible that these liposomes loaded with NKR-P1 ligands could be

directed to the required target cell through antibody-mediated mechanisms (Bezouska *et al.*, 1994a).

- **The NCR's**

NKp46 (Pessino *et al.*, 1998), NKp44 (Cantoni *et al.*, 1999) and NKp30 (Pende *et al.*, 1999), termed "natural cytotoxicity receptors" ("NCR's"), are three novel activatory receptors of the Ig superfamily, exclusively expressed by all activated human NK cells; NKp46 and NKp30 are also present on the surface of resting NK cells. The three receptors have little homology with each other, and indeed with other human NK cell markers (Moretta *et al.*, 2000). All NCR's play an integral role in NK cytotoxicity as masking with respective monoclonal antibodies results in the inhibition of NK function. There is evidence for NCR co-operation as it has been established that on antibody-inhibition of NKp44, simultaneous masking of NKp46 results in heightened inhibition of killing. NCR's are down-regulated on NK cells whose KIR's have associated with HLA (Moretta *et al.*, 2000). The ligands for these cytotoxicity receptors have yet to be defined in detail.

NKp46

NKp46, the 46kDa prototype NCR, is expressed by all NK cells, whether resting or activated (Pessino *et al.*, 1998). Mapped to human chromosome 19, it is a type-I transmembrane glycoprotein possessing two Ig-like extracellular domains, a transmembrane region with a positively charged arginine residue and a cytoplasmic region of 30 amino acids (Pessino *et al.*, 1998). NKp46 is the only NCR capable of recognising murine ligands (Pessino *et al.*, 1998), a finding which prompted the successful search for murine and rat NKp46 homologs (Biassoni *et al.*, 1999), indicating conservation between species. Indeed, the gene encoding murine NKp46 is located on mouse chromosome 7, which is syntenic to human chromosome 19 (Pessino *et al.*, 1998). NKp46 receptor plays an integral part in NK cytotoxicity against normal or tumourous, autologous, allogeneic or xenogeneic target cells (Sivori *et al.*, 1999) and is capable of triggering NK-killing independently (Moretta *et al.*, 2000). The importance of NKp46 in NK cell-

mediated cytotoxicity is indicated by the fact that cells of the phenotype NKp46^{dull} (determined by brightness of fluorescence) are not as effective killers as those of the phenotype NKp46^{bright}. Following NKp46 cross-linking, the cytotoxic signal is transduced resulting in NK activation and secretion of cytokines (Sivori *et al.*, 1997).

NKp44

NKp44 is a 44kDa protein and encoded for by a gene located on human chromosome 6 (Cantoni *et al.*, 1999). Unlike the remaining NCR's, NKp44 is expressed by activated NK cells only. It is not however detectable on activated T-cells and should therefore not be regarded as an activation marker (Moretta *et al.*, 2000). NKp44 is also absent on NK cells *ex vivo*, although expression can be induced following culture with IL-2 (Vitale *et al.*, 1998). It is interesting to note, that two γδTCR^{+ve} cell clones taken from a patient with melanoma, did in fact express NKp44, but only at very low levels (Vitale *et al.*, 1998).

NKp30

NKp30 is the gene product of *1c7* located in the HLA class III region of human chromosome 6 (reviewed in (Moretta *et al.*, 2000). As with NKp46 expression, NK cells may be of the phenotype NKp30^{dull} or NKp30^{bright} (Moretta *et al.*, 2000). These cells are responsible for the destruction of certain tumour target cells which are not affected by NK cells expressing NKp46 or NKp44 (Pende *et al.*, 1999).

- **Other activatory receptors**

These include the C-type lectin superfamily members Ly49-D, -H and –P (McVicar and Burshtyn, 2001), and the heterodimer CD94/NKG2C/E (Jaso-Friedmann, *et al.*, 1997; Rohrer *et al.*, 2000), which contain a positively charged amino acid within the trans-membrane domain, characteristic of activatory members (Campbell and Colonna, 1999). NKG2D, another C-type lectin family member (Houchins, *et al.*, 1991), is a 42kDa transmembrane molecule which

binds to, and is activated by, the non-classical class-I protein MICA (Bauer *et al.*, 1999), a stress-inducible molecule expressed by epithelial tumour cells (Groh *et al.*, 1996). This restricted expression of MICA may account for the fact that the transduction of the cytotoxic signal by NKG2D takes priority over that induced by KIR interactions with HLA class-I.

IgSf members such as certain human KIR's also function to activate the NK cells (Brooks, 1998) and are therefore termed KAR's (killer cell activation receptors (Olcese *et al.*, 1997; Campbell and Colonna, 1999). These receptors lack ITIM's through truncation of the COOH terminal (McVicar and Burshtyn, 2001) and instead possess the characteristic charged residues (e.g. lysine) within their transmembrane regions, which are able to associate with other receptor chains such as DAP-12 (see below) (Olcese *et al.*, 1997; Vely and Vivier, 1997).

- **Activatory signal transduction**

NK receptors involved in activation do not possess ITIM's like their inhibitory counterparts, but instead contain charged residues within the transmembrane domains, which mediate associations with other receptor chains (Vely and Vivier, 1997) and proteins, such as the CD3ζ/FcRγ family member, DAP-12 (Smith *et al.*, 1998). This is a 12kDa type-I transmembrane protein which exists as a disulphide-linked homodimer (Campbell and Colonna, 1999), each chain containing a single immunoreceptor tyrosine based activation motif (ITAM) (Lanier, *et al.*, 1998) comprising two copies of the Yxx(I/L) motif, approximately 6-7 amino acids apart (McVicar and Burshtyn, 2001). On cross-linking of the activatory receptors, the ITAM becomes phosphorylated by as yet undetermined kinases (src family kinases are the prime candidates) (McVicar and Burshtyn, 2001), which leads to further recruitment and phosphorylation of syk/Zap70 family kinases, PLCγ1 and c-Cbl, and to the activation of mitogen-activated protein (MAP) kinases (McVicar *et al.*, 1998). Mobilisation of intracellular Ca^{2+} subsequently leads to NK cytotoxic activity and secretion of cytokines (Sivori *et al.*, 1997).

NKG2D however, has been shown by co-immunoprecipitation (Wu *et al.*, 1999), to complex with DAP-10 (Rohrer *et al.*, 2000), a type-I membrane adaptor protein, which has approximately 20% sequence homology with DAP-12 (Wu *et al.*, 1999). The cytoplasmic domain of DAP-10 contains a motif of the sequence YxxM, an SH2 domain binding site, which when activated, transduces signals and subsequently activates PI-3-kinases (Wu *et al.*, 1999).

1.1.5 NK functions

1.1.5.1 Production of cytokines

Although spontaneous cytolytic activity is one of the main functions of NK cells, it is by no means their only function. On activation, NK cells release cytokines such IFN-γ (involved in T_{h1} regulation, (Scharton and Scott, 1993; Scott and Trinchieri, 1995), GM-CSF (Cuturi, *et al.*, 1989) (implicated in haematopoiesis regulation), TNF-α and IL-1 (Murphy *et al.*, 1992), -3 and –8 (Somersalo *et al.*, 1994), the latter facilitating inflammatory responses (Warren, 1996). IL-5, required for eosinophil differentiation is also secreted by NK cells which are proliferating or have been stimulated with IL-2 (Warren *et al.*, 1995). In addition, the NK population have the ability to activate other cells (for example, macrophages), and may also play an indirect role in adaptive immunity by producing cytokines which influence the development of cells such as the T_{h1} subset (Scharton and Scott, 1993; Scott and Trinchieri, 1995).

NK activity is enhanced by cytokines such as TNF-α (Scott and Trinchieri, 1995), IFN-α (Trinchieri and Santoli, 1978), and IL's-1, -2 (Trinchieri *et al.*, 1984), -10 (Warren *et al.*, 1995), -12 (Aste-Amezaga *et al.*, 1994) and -15, the latter also promoting NK proliferation (Carson *et al.*, 1994).

1.1.5.2 Cytolytic activity

NK cells spontaneously lyse and subsequently destroy cells which have become deficient in surface expression of MHC-I antigens. Primary targets include susceptible tumour cells, bacterially/virally infected cells and bone marrow cells, all of which may be killed without prior sensitisation (Herberman and Ortaldo, 1981) through perforin-dependent mechanisms, interactions with death receptors such as FAS (Arase *et al.*, 1995) and production of lymphokines (reviewed in Van Den Broek *et al.*, 1998). NK cells also exhibit antibody-dependent cell-mediated cytotoxicity (ADCC), through interactions between the NK CD16 receptor and the Fc region of IgG-coated target cells. In humans, this form of cytotoxicity is mediated by $CD56^{dim}CD16^{+ve}$ NK cells (Warren, 1996). NK specificity is a clonally distributed characteristic; the cytotoxic potential of an NK population is the summative effect of the different specificities of NK subsets within the population (Allavena and Ortaldo, 1984). Although both NK and ADCC killing may be displayed, it may be true that some NK subsets may only be able to perform one of these activities (Allavena and Ortaldo, 1984).

Once activated, mammalian NK cells employ a variety of mechanisms (some of which are described below) to induce suicide in the target cell, a process termed apoptosis.

1.1.6 Apoptosis

Apoptosis (programmed cell death) occurs when specific environmental or developmental stimuli trigger an intracellular self-destruction program, resulting in the cell committing suicide (White, 1996). The manifold roles of this process include the deletion of potentially harmful immune cells, such as those which are autoreactive or which fail to undergo necessary gene re-arrangements (Nagata, 1997), sculpting the body (e.g. digit formation, Raff, 1998), and the maintenance of homeostasis between cell death and cell division (Steller, 1995; Osborne, 1996). Apoptosis is both deliberate and genetically controlled, unlike necrosis (accidental cell death), which occurs following physical damage to the cell (Savill,

et al., 1993). There are distinct and important differences in the characteristics of these two modes of cell death. Necrosis results in cytoplasmic organelle disruption and breaching of the plasma membrane (White, 1996). The cell effectively swells and bursts, causing significant leakage of the dying cells contents, subsequently damaging neighbouring cells. An inflammatory response ensues (Savill *et al.*, 1993; Raff, 1998). Apoptosis is typified by heterochromatin condensation and fragmentation of nuclear DNA (Savill *et al.*, 1993; White, 1996). In contrast to necrosis, apoptosing cells form apoptotic bodies (Savill *et al.*, 1993) and are very rapidly phagocytosed whilst the integrity of membranes remains intact, therefore avoiding inflammatory responses or damage to neighbouring cells. Such rapid disposal and therefore lack of evidence of the apoptosed cell may explain why details of apoptosis remained somewhat elusive for many years (Raff, 1998).

1.1.6.1 Regulation of apoptosis

a) Apoptosis induction

Excitement surrounding research into apoptosis originated from studies of the nematode worm, *C. elegans*. Two genes were discovered, *ced-3* and *ced-4*, which encode for proteins integral to the regulation of the apoptotic pathway (Hengartner and Horvitz, 1994b; Raff, 1998). This finding prompted the search for the corresponding genes in humans (Yuan *et al.*, 1993; Hengartner and Horvitz, 1994b), which subsequently led to the discovery of the ICE (interleukin-1 converting enzyme) proteins, also known as caspases, a family of cysteine proteases whose inhibition results in the inhibition of apoptosis. On activation of the apoptotic pathway, these enzymes activate each other, generating a proteolytic cascade (Raff, 1998), which leads to cleavage of DNase precursors (Enari *et al.*, 1998) and cytoskeletal proteins, therefore resulting in DNA fragmentation and detachment of the apoptotic cell from its neighbours (Raff, 1998).

i) Apoptosis induced by NK cells

Cytolytic granule secretion

On detection of a virally-infected cell, NK cells (and CTL's) secrete calcium-dependent (Oshimi *et al.*, 1996) proteins known as perforins which polymerise in the extracellular space between effector and target cells, and insert into the target cell membrane, forming transmembrane channels of 5-20nm (Hogan *et al.*, 1999). This subsequently permits entry of other proteins into the cell, such as the granzyme B protease (a serine protease, Jenne and Tschopp, 1988), also secreted by the effector cell, which is thought to activate both cdc-2 (a G2 cell-cycle kinase) (Shi *et al.*, 1994) and CPP32, an ICE family member (Darmon, *et al.*, 1995), subsequently resulting in activation of the proteolytic cascade and apoptosis of the infected cell (Atkinson and Bleackley, 1995). It has been postulated that NK cells at an early stage of maturation employ the perforin/granzyme system rather than Fas-mediated cytotoxicity (described below), which appears to be more frequently utilized at later stages of development (Nakazawa *et al.*, 1997).

Death receptors

Death receptors, expressed on the cell surface, belong to the tumour necrosis factor receptor (TNFR) superfamily and are all structurally alike, possessing 25% conserved extracelluar domains rich in cysteine molecules (MacFarlane *et al.*, 1997) and homologous cytoplasmic regions termed the "death domain" (Tartaglia *et al.*, 1993). On binding with the appropriate ligand, it is this domain which is responsible for transmitting the death signal and activating the proteolytic cascade, resulting in apoptosis of the cell (Ashkenazi and Dixit, 1998). Three examples of such death receptors are Fas *(Fig. 1.5),* TNF-R1 and DR4/5. The Fas receptor system is described here as it is known to pertain to NK cell killing.

Fas is also known as CD95 or APO-1 (Osborne, 1996) and its ligand, FasL (expressed on NK (Arase *et al.*, 1995) and T-cells (Henkart *et al.*, 1997)), exhibits

the typical trimeric structure of TNF family members (Nagata, 1997), each trimer binding three Fas molecules on the cell surface.

Insight into the role of Fas/FasL interactions was provided by the discovery that mutations of genes encoding for Fas and FasL resulted in a build up of peripheral lymphocytes and autoimmunity, (Salbeko-Downes and Russell, 2000), further investigations revealing a range of further roles. Fas and its ligand are responsible for the removal of both redundant activated T-lymphocytes in the periphery (Osborne, 1996; Lee *et al.*, 1997), and inflammatory cells at immunopriviledged sites (Ashkenazi and Dixit, 1998). Deletion of autoreactive B-cells occurs via interactions between Fas on the B-cell surface and FasL on $CD4^{+ve}$ T-cells (Rathmell *et al.*, 1995). Cancer cells, or those virally infected are also removed by Fas/FasL interactions involving either NK or T-lymphocytes (Mori *et al.*, 1997; Salbeko-Downes and Russell, 2000). However, tumour cells expressing FasL may also use the system to their advantage by employing Fas/FasL interactions to delete immune cells threatening their removal (Hahne *et al.*, 1996). This receptor and its ligand are also effective in cell suicide. Human natural killer cells stimulated through their Fcγ receptor, up-regulate surface expression of FasL (Eischen *et al.*, 1996), which associates with the NK cell's own Fas receptors, resulting in autocrine cell death (Eischen *et al.*, 1996; Raff, 1998).

Nuclear magnetic resonance techniques and mutagenesis studies demonstrate that association of Fas receptor with its ligand results in the recruitment of intracellular adaptor proteins which associate with the death domain of Fas (Boldin, *et al.*, 1995; Osborne, 1996). One such protein is FADD (Boldin *et al.*, 1995), or Fas-associated death domain (also termed MORT1), which possesses a death effector domain, (Boldin *et al.*, 1996) required to recruit caspases such as caspase-8, which self-cleaves (Muzio *et al.*, 1998) and subsequently activates other caspases downstream, thereby initiating programmed cell death. The lack of Fas-induced apoptosis in FADD gene knockout mice (Osborne, 1996) demonstrates the importance of this protein in cell death, although this is by no means the only function of FADD. The gene knockout mice also exhibit compromised T-cell proliferation and embryonic lethality (Newton *et al.*, 1998). Both receptor

interacting protein (RIP) (Osborne, 1996) and the cytoplasmic protein Daxx also associate with Fas, although the signalling pathway of the latter differs to that of FADD (Yang *et al.*, 1997).

ii) In the absence of external stimuli

Specific external stimuli are not necessarily essential for activation of the proteolytic cascade. Should a cell become critically stressed or injured in some way, it has the capacity to self-activate procaspases within the cell. Build-up of misfolded proteins within the endoplasmic reticulum, DNA damage (Raff, 1998), viral infection, loss of cell/cell or cell/substrate contact (White, 1996), or the presence of certain toxins are all initiators of the apoptotic pathway, although the exact mechanism differs in each case (Raff, 1998).

b) Apoptosis inhibition

i) The Bcl-2 family

Following investigations into the regulatory *ced-9* gene product of *C. elegans*, the search for the corresponding human homologues revealed several proteins collectively termed as the Bcl-2 family (Nagata, 1997; Raff, 1998), members of which either inhibit or promote apoptosis (Raff, 1998) *(Table 1.2)* by forming complexes with other Bcl-2 proteins, or with molecules outside the family (Merry and Korsmeyer, 1997).

ii) IAP's (inhibitors of apoptosis)

The IAP family is a group of highly conserved anti-apoptotic proteins induced by a wide range of stimuli, which directly associate with caspases, subsequently inhibiting apoptosis (Roy *et al.*, 1997; Raff, 1998). The exact mechanism involved has not as yet been defined. IAP family members, such as human XIAP, c-IAP-1 and c-IAP-2 (Roy *et al.*, 1997), are structurally similar and constitutively possess the baculovirus IAP repeat (BIR) motif (Duckett *et al.*, 1996), which is

thought to be integral in apoptosis resistance (Roy *et al.*, 1997). The majority of IAP's also possess a carboxy-terminal RING domain, although the specific function of this remains enigmatic (Duckett *et al.*, 1996; Roy *et al.*, 1997).

1.1.6.2 Disposal of apoptotic cells *(Fig. 1.6)*

Rapid disposal of the apoptotic cell by phagocytes is essential to prevent unwanted escape of self antigens, toxic proteins, nucleosomes and degradative enzymes, and therefore ensure minimal damage to neighbouring cells and tissues (Savill *et al.*, 1993; White, 1996). There are thought to be a wide range of disposal processes available to phagocytes with which to deal with apoptotic cells and the external stimuli may be the determining factor as to which mechanism or mechanisms are employed (Savill *et al.*, 1993). Three such disposal mechanisms are described below:-

Phagocyte lectins

It has been established through scanning electron microscopy that cells undergoing apoptosis experience specific changes in their surface carbohydrates and suffer loss of sialic acid residues, effectively reducing cell migration. It is postulated that such events may result in the exposure of side-chain sugars which are then free to associate with lectin molecules expressed on the surface of macrophages, leading to the subsequent removal of the dying cell (reviewed in Savill *et al.*, 1993).

Phagocyte vitronectin receptor integrin/thrombospondin

The $\alpha_v\beta_3$ vitronectin receptor integrin (VnR) expressed by macrophages has also been implicated in the recognition of apoptotic cells. It consists of two subunits and is thought to function alongside the 88 kDa molecule CD36, also expressed on the surface of macrophages. Both receptors are thought to associate with thrombospondin (TSP), a glycoprotein secreted by the macrophages themselves (Savill *et al.*, 1993). Although the specifics of the interaction between these three

molecules has not been established, TSP is thought to form a "molecular bridge" between the dying cell and the macrophage. The mechanism behind the association between TSP and the apoptotic cell surface has not as yet been defined (Savill *et al.*, 1993).

Phagocyte receptors for phosphatidylserine

Within viable cells, the negatively charged phospholipid, phosphatidylserine (PS) is located asymmetrically on the inner surface of the cell membrane (Savill *et al.*, 1993), a distribution pattern maintained by Mg-ATP-dependent aminophospholipid translocases. However, once the apoptotic pathway has been initiated, prior to the onset of DNA fragmentation and cell lysis (Verhoven *et al.*, 1995), PS is translocated to the external surface, where it persists for the remainder of the apoptotic pathway. This migration process is mediated by down-regulation of aminophospholipid translocases (Verhoven *et al.*, 1995; Bratton *et al.*, 1997), together with the activation of a lipid scramblase; the specific membrane proteins involved have not as yet been identified (Verhoven *et al.*, 1995). The integrity of the cell membrane remains intact throughout this process and the externally expressed PS molecules are recognised by an as yet unidentified receptor on macrophages, which subsequently remove the apoptotic cells before rupture (Fadok *et al.*, 1992a; Fadok *et al.*, 1992b).

There are definite clinical implications for the study of phagocyte recognition of apoptotic cells. For example, investigations may lead to the discovery of methods to increase the effectiveness of phagocyte function or to design a method of apoptotic induction in dangerous cells (Savill *et al.*, 1993).

1.1.7 NK development

NK cells, believed to be evolutionary forerunners of T-cells (Janeway, 1989) develop in the main extrathymically from pluripotent hematopoietic stem cells (Raulet, 1999), with important developmental stages occurring in the bone marrow

(Moore *et al.*, 1995) and also briefly in the foetal thymus (Raulet, 1999). It has been suggested that putative T-cell/NK-cell progenitors are present within foetal liver and adult bone marrow and that a proportion of these cells receive thymic homing signals and subsequently migrate to the thymus to undergo TCR re-arrangements. The cells which remain are destined for the NK cell lineage and acquire the cell surface marker CD56 (Lanier *et al.*, 1992). More recently however, a progenitor cell restricted to either NK or T-cell development has been described through the study of FcγIII^{+ve} (Rodewald *et al.*, 1992) foetal thymocytes, a heterogeneous population (Carlyle *et al.*, 1997; Carlyle *et al.*, 1998) consisting of CD117^{-ve} cells (which differentiate into NK cells) and CD117^{+ve} cells, which are the progenitors for either NK or T-cell lineages. The FcγIII^{+ve} population do not give rise to myeloid cells or B-cells (Raulet, 1999). The cytokine IL-15, produced by bone marrow stromal cells, has been heavily implicated in NK differentiation. Evidence in support of this comes from investigations into NK1.1^{+ve} splenocytes of marrow-disrupted mice, whose lack of cytolytic functions (Hackett *et al.*, 1986) are reversed following *in vitro* culture with IL-15 (Puzanov *et al.*, 1996). The Ly49E receptor is also thought to play a role in NK development due to the finding that murine foetal NK cells do not express any Ly49 molecules, except for Ly49E (Toomey *et al.*, 1998).

NK cell surface expression of CD94 receptors has been established at an early stage in mammalian NK cell development (Raulet, 1999). CD94-positive NK cells have been located in human foetal liver (Jaleco, *et al.*, 1997), although it is not clear as to whether NKG2 proteins are also present (Raulet, 1999). Immature cells such as these do display cytolytic inhibition on association with MHC-I (Toomey, *et al.*, 1998), and as Ly49 expression gradually increases during the first few weeks of life, eventually reaching a plateau after 1 month (Sivakumar, *et al.*, 1997; Dorfman and Raulet, 1998), it is feasible that CD94/NKG2 receptors are also functional at this early stage (Raulet, 1999). In addition to playing a role in NK differentiation, IL-15 is also implicated in the induction of expression of CD94/NKG2 receptors on both NK cells (Mingari *et al.*, 1997b) and T-cells (Mingari *et al.*, 1997a). The cytokines responsible for the development of Ly-49

receptors and KIR's are yet to be established, although IL-15 is not thought to be one of them (Mingari *et al.*, 1997b; Raulet, 1999).

Interactions between inhibitory receptors expressed by developing NK cells and MHC class-I proteins expressed by autologous cells are thought to play a crucial role in the education of NK cells and their development of self tolerance (Karre and Colonna, 1998). However, in humans it has recently been shown (Sivori *et al.*, 2002) that immature NK cells express activatory receptors before MHC-specific inhibitory receptors, implying that ligands other than MHC may, in early ontogeny, be crucial in NK inhibition.

1.2 NK cell evolution and *Xenopus* immunobiology

1.2.1 Natural cytotoxic cells in lower vertebrates and invertebrates

Natural killer cells have been characterised in avian species following the generation of α-chicken NK mAb's, which identify a discrete population of NK cells expressing CD8αα homodimers, IL-2 receptors and cytoplasmic CD3 molecules. A high proportion is found to reside within the intestinal epithelium (Gobel, 1995).

NK-like cells have also been identified in channel catfish (Yoshida *et al.*, 1995; Hogan *et al.*, 1996), rainbow trout (*Oncorhynchus mykiss*, (Greenlee *et al.*, 1991) and damselfish (McKinney and Schmale, 1997).

In channel catfish, these NK-like cells are termed non-specific cytotoxic cells (NCC's) and spontaneously lyse xenogeneic targets also lysed by human NK cells (Harris *et al.*, 1991; Hogan *et al.*, 1999), implying the existence of a conserved target cell antigen and similar NK receptor molecules (Hogan *et al.*, 1999). Such cytotoxicity is dependent on cell movement (Carlson *et al.*, 1985) and ATP metabolism (Hogan *et al.*, 1999). Anti-NCC monoclonal antibodies e.g.5C6 and

6D34 have been generated and have been shown to detect a small lymphocyte population (5-15%) in human peripheral blood and >85% of $CD3^{-ve}$ NK cells. As expected, no specificity was shown for T-cells, B-cells or monocytes (Harris *et al.*, 1991). The 5C6 antibody identifies a 34kDa type III membrane protein (Shen *et al.*, 2002) expressed on the surface of NCC's called NCCRP-1 (non-specific cytotoxic cell receptor protein-1). Expression of this molecule is proportional to cytotoxicity; cross-linking of NCCRP-1 with 5C6 results in the amplification of cell signalling and subsequent enhanced killing activity. RT-PCR has established the presence of NCCRP-1 mRNA in two T-cells lines, but not in B-cells (Jaso-Friedmann *et al.*, 1997).

Unimmunised channel catfish PBL populations also contain effector cells distinct from NCC's. These cells are termed PBL-E cells and are capable of spontaneous cytolytic activity towards allogeneic (Yoshida *et al.*, 1995) and virally-infected cells (Hogan *et al.*, 1996; Shen *et al.*, 2002). PBL-E's are distinct from NCC's as they do not bind with the 5C6 monoclonal antibody (Stuge *et al.*, 1995; Hogan *et al.*, 1996). Transmission electron microscopy has shown these effectors to be agranular and approximately 4μm in diameter (Hogan *et al.*, 1999). In 1995, Yoshida et al reported that PBL-E's lack surface Ig and are therefore not B-cells or macrophages. It remained to be seen however, whether the effector cells were NK cells or T-cells. Possible evidence for the latter originated from studies into the monoclonal antibody 1H5, which detects an integrin-like molecule similar to mammalian LFA-1 on the surface of PBL-E's. Binding of this antibody to PBL-E's results in the inhibition of effector functions. As α-LFA-1 antibodies inhibit mammalian CTL cytotoxicity, this may suggest the possibility of PBL-E's belonging to a T-cell lineage (Yoshida *et al.*, 1995). However, as 1H5 reacts positively with the majority of PBL's and only inhibits cytotoxicity after a considerable length of time, the conclusion that PBL-E's are the mammalian CTL equivalent cannot be drawn (Yoshida *et al.*, 1995). Anti-fish-T-cell antibodies would be required to clarify these controversies (Horton *et al.*, 1998b). More recently, flow cytometric analysis has established heterogeneous expression of Ig molecules on the surface of cells from $\alpha\beta TCR^{-ve}$ PBL-E clones, the enigma being that these cells do not possess Igμ or L chains. This is suggestive that the IgM

molecules visualised by flow cytometry are IgM molecules present in culture serum which have adsorbed onto the surface of the PBL-E population (Shen *et al.*, 2002).

The killing capacity of catfish PBL-E's is enhanced following culture in mixed-lymphocyte populations, which may be due to the up-regulation of FasL or the activation of cytotoxic granule secretion (Hogan *et al.*, 1999). ADCC mechanisms are not involved as the anti-catfish Ig antibody, 9E1 does not inhibit cytotoxicity (Yoshida *et al.*, 1995). PBL-E's lyse their targets by inducing apoptosis; DNA fragmentation occurs within 1 hour of co-culture (Hogan *et al.*, 1999). Introduction of Ca^{2+} chelating agents such as EGTA and fixation of the effector cells results in a failure to destroy target cells, implying that peripheral blood effectors may induce apoptosis by use of secretory-based rather than ligand-based mechanisms. It should be taken into consideration however, that fixation of cells may disrupt surface ligands (Hogan *et al.*, 1999).

NK-like activity has also been discovered in earthworms. Flow cytometric analysis has established two populations of lymphocytes within the coelomic fluid, small NK-like coelomocytes and large phagocytic coelomocytes (Cooper *et al.*, 1996). On lysis of the target cells, the phagocytic population form a barrier to enclose and contain the cell debris, effectively engulfing it. The NK-like cells have also shown cytolytic activity towards the human K562 tumour cell line. Cytotoxicity is thought to be mediated by two monomeric glycoproteins of 40 and 45kDa located within the coelomic fluid of the earthworm (Cooper *et al.*, 1996).

The discovery of NK-like activity in such a range of lower vertebrates, and even in invertebrates, suggests that the NK cell system evolved at an early stage in immune system development and most probably preceded the development of T-cell lineages (Harris *et al.*, 1991).

1.2.2 *Xenopus* model system

The comparative model *Xenopus laevis* offers an ideal opportunity to investigate the evolution of the immune system and to provide fundamental information regarding the biology of various lymphoid populations and their receptors. Although the last known common ancestor of *Xenopus* and mammals dates back 350 million years (Shum *et al.*, 1996), many similarities between *Xenopus* and mammalian immune systems remain, such as expression of MHC class-I and class-II proteins and TCR and Ig gene re-arrangements. With regard to the latter, *Xenopus* possess three Ig heavy chain classes, namely IgM, IgY (structurally and functionally similar to mammalian IgG) and IgX (a possible forerunner of mammalian IgA). *Xenopus* also displays Ig light chain diversity (Hsu and Du Pasquier, 1984a).

Xenopus MHC (termed the XLA system, reviewed in (Horton *et al.*, 1996b) encodes proteins similar in structure to mammalian MHC antigens (Flajnik *et al.*, 1984). The XLA genes are located on the same region of a single chromosome (reviewed in Salter-Cid *et al.*, 1998), indicating further similarities to the mammalian MHC system. *Xenopus* MHC-Ia genes are polymorphic (≈20 alleles) and the proteins they encode comprise a 40-44kDa glycosylated heavy chain, non-covalently associated with the 13kDa light chain β2m (Flajnik *et al.*, 1984). *Xenopus* MHC proteins are encoded for by one gene locus (as opposed to three in humans and two in mice) (Horton and Ratcliffe, 1998a).

Xenopus class Ib molecules are monomorphic, bear homologies to the HSP70 family (molecules which act as chaperones in protein folding and intracellular transport, (Horton and Ratcliffe, 1998a) and are encoded for by genes outside the MHC (Salter-Cid *et al.*, 1998). They tend to be expressed on epithelial surfaces and may be the first line of defence against pathogens. Although the *Xenopus* class Ib sequence is dissimilar to that of mammalian class Ib, expression resembles that of mammalian MIC proteins (Salter-Cid *et al.*, 1998).

Xenopus class II molecules are polymorphic (≈30 alleles) transmembrane glycoproteins of 30-35kDa (Kaufman *et al.*, 1985) comprised of α and β chains, the latter sharing 50% homology with mammalian class-IIβ chains. Both humans and *Xenopus* have three class-IIβ gene loci. The expression pattern is limited in comparison to class I; class II is present only on adult thymocytes, B-cells, T-cells and some antigen-presenting cells (APC's) (Horton and Ratcliffe, 1998a).

Free-living *Xenopus* larvae are susceptible to pathogens and must therefore be immunocompetent. Tadpoles have the ability to produce specific antibodies and reject MHC-disparate skin grafts (Flajnik and Du Pasquier, 1990a). However, one major distinction between larval and adult immune systems is MHC expression. *Xenopus* MHC class-I is first expressed late in larval life (stage 55-56 at 34 days of age) in low levels in the spleen (Flajnik and Du Pasquier, 1990a; Rollins-Smith *et al.*, 1997). Previous to this, MHC-I is not detectable on cell surfaces (Flajnik *et al.*, 1991), although class Ia transcripts have been discovered in tadpole intestine and gills. Class Ib mRNA is not detectable in tadpoles (Salter-Cid *et al.*, 1998). Following metamorphosis (stage 65-66), class-I expression is dramatically upregulated (Rollins-Smith *et al.*, 1997), although this is not a metamorphosis-dependent event. Tadpoles blocked from developing for significant periods of time still begin to express class-I (Flajnik and Du Pasquier, 1990a) albeit at low levels (Rollins-Smith *et al.*, 1997). Similarly, tadpoles forced to metamorphose prematurely do not express class-I until some time after metamorphosis (Rollins-Smith *et al.*, 1997). The reason behind the absence of MHC class-I in larvae may be the prevention of autoimmunity (Salter-Cid *et al.*, 1998). Should larval cells express MHC class-I, during metamorphosis, those MHC proteins could present new "adult-specific" antigens. Larval effector cells would be able to recognise the new antigens as foreign and the cells expressing them would be lysed by T_c cells that are known to exist prior to metamorphosis (Flajnik and Du Pasquier, 1990a).

The lack of class-Ia and Ib in tadpoles indicates that class-I proteins are not essential for larval development or immunocompetence. It is therefore possible that class-II proteins play a central role in larval immunity. Class II distribution in *Xenopus* varies with development in a metamorphosis-dependent manner (Flajnik

and Du Pasquier, 1990a). At stage 48 (day 7), the thymic epithelium begins to express class-II proteins at low levels, expression increasing with time (Du Pasquier and Flajnik, 1990). In general, class II expression gradually increases throughout larval life, with stabilisation of class II coinciding with the immune system becoming competent (stage 51-52). B-cells, APC's, 50% of splenocytes and epithelial areas of gut, skin, pharynx and gills express class II in larvae, although larval thymocytes remain negative (Du Pasquier and Flajnik, 1990). Lack of class-II^{+ve} T-cells in the tadpole may suggest the absence of appropriate adult-specific cytokines necessary for class II expression. In support of this, it has been established that larval thymocytes are unable to produce T-cell growth factors, although they are capable of responding to such factors (Rollins-Smith *et al.*, 1984).

The search for candidate NK cells has been greatly aided by the study of early-thymectomized (Tx) *Xenopus*. Thymectomy involves the complete removal (by microcautery) of the thymus gland (the site of T-cell development) when *Xenopus* larvae are 5-7 days old. This procedure effectively eradicates T-cells in the adult (Horton *et al.*, 1998c), and reveals thymus-dependent and -independent features of the *Xenopus* immune system (Manning and Turpen, 1982). Interestingly, the Tx animals survive well in the absence of T-cells, suggesting the importance of thymic-independent components in their immune system.

Although a variety of α-*Xenopus* monoclonal antibodies have been available for some time to aid study of the *Xenopus* immune system (*Table 1.3)*, the generation of putative α-*Xenopus* NK monoclonal antibodies has been a very recent development (Horton *et al.*, 2000). These α-NK antibodies were generated from mice immunised with splenocyte populations from thymectomised animals, which had been immunomagnetically depleted of B-cells and thrombocytes. Screening of supernatants against *Xenopus* splenocytes was carried out by flow cytometry and identified three monoclonal antibodies (mAb's), 1F8, 4D4 and 1G5.

1.3 Purpose of thesis

- The initial aim of this thesis is to search for and characterise candidate NK cells within various *Xenopus* lymphoid organs, using recently generated α-NK monoclonal antibodies (*Table 1.3*).

- The phenotypic and functional properties of immunomagnetically purified NK cells will be assessed and the issue of whether *Xenopus* NK cells kill by inducing apoptosis in tumour target cells will be addressed.

- Another aim of this thesis is to characterise the *Xenopus* NK antigen identified by mAb 1F8 and investigate whether this antigen can be induced on T-cells *in vitro*, to probe the question of whether NKT-like cells exist in this amphibian.

- The ontogenetic development of *Xenopus* NK cells will also be investigated, using phenotypic and functional studies.

- Finally, the expression of β2-microglobulin in adult and larval tissues is examined, as study of this invariant component of MHC class-I may aid future studies on the ontogeny of immunity in *Xenopus*.

Table 1.1: A summary of NK markers and their shared specificities.

Marker	Shared specificity
CD56	minority of T-cells
CD16	minority of T-cells, granulocytes, some macrophages
CD2	all T-cells
CD11b	granulocytes, monocytes, some T-cells
CD38	activated T-cells, plasma cells, haemopoietic precursors
CD7	all T-cells
CD8	cytotoxic T-cells
CD57	some T-cells
IL2Rβ	activated T-cells
CD94	some T-cells
2B4	some cytotoxic T-cells, monocytes and basophils
NK1.1	lineage specific
Asialo-GM1	lineage specific

(Adapted from Lydyard and Grossi, 1998)

Table 1.2: Members of the Bcl-2 family

Promoters	Reference	Inhibitors	Reference
Bax	(Oltavi *et al.*, 1993)	Bcl-2	(Hengartner *et al.*, 1992)
Bad	(Yang *et al.*, 1995)	Mcl-1	(Kozopas *et al.*, 1993)
Bid	(Raff, 1998)	CED-9	(Hengartner and Horvitz, 1994b)
Bak	(Chittenden *et al.*, 1995)	Bcl-W	(White, 1996)
Bcl-xS	(Boise *et al.*, 1993)	Bcl-xL	(Boise *et al.*, 1993)

Table 1.3: Anti-*Xenopus* antibodies used in this Thesis

Antibody	Isotype	Antigen	Specificity	Reference
2B1	IgG_1	CD5 homologue (71-82kDa)	T-cells, mitogen-activated B-cells	(Jurgens *et al.*, 1995)
AM22	IgM	Putative CD8α (30kDa)	Cytotoxic T-cells	(Flajnik *et al.*, 1990b)
F17	IgM	Putative CD8β (30kDa)	Cytotoxic T-cells	(Ibrahim *et al.*, 1991)
D12-2	IgG_1	Putative γδTCR (56kDa)	Minority of thymocytes, splenocytes	(Ibrahim *et al.*, 1991)
XT-1	IgG_2	XTLA-1 (120kDa)	T-cell subset	(Nagata, 1986)
X71	IgG	CTX (55kDa)	Cortical thymocytes, thymic tumour cell lines	(Chretien *et al.*, 1996)
8E4	IgG	IgM (25kDa and 75kDa)	B-cells, macrophages	(Langeberg *et al.*, 1987)
D8	IgG_3	IgM (25kDa and 75kDa)	B-cells, macrophages	(Jurgens, *et al.*, 1995)
10A9	IgG	IgM (25kDa and 75kDa)	B-cells	(Du Pasquier and Hsu,1983)
1F8	IgG_2	Putative NK antigen	NK cells	(Horton *et al.*, 2000)
4D4	IgG_2	Putative NK antigen	NK cells	(Horton *et al.*, 2000)
1G5	IgG_2	Putative NK antigen	NK cells	(Horton *et al.*, 2000)

Fig. 1.1: MHC protein interactions with T-cell subsets

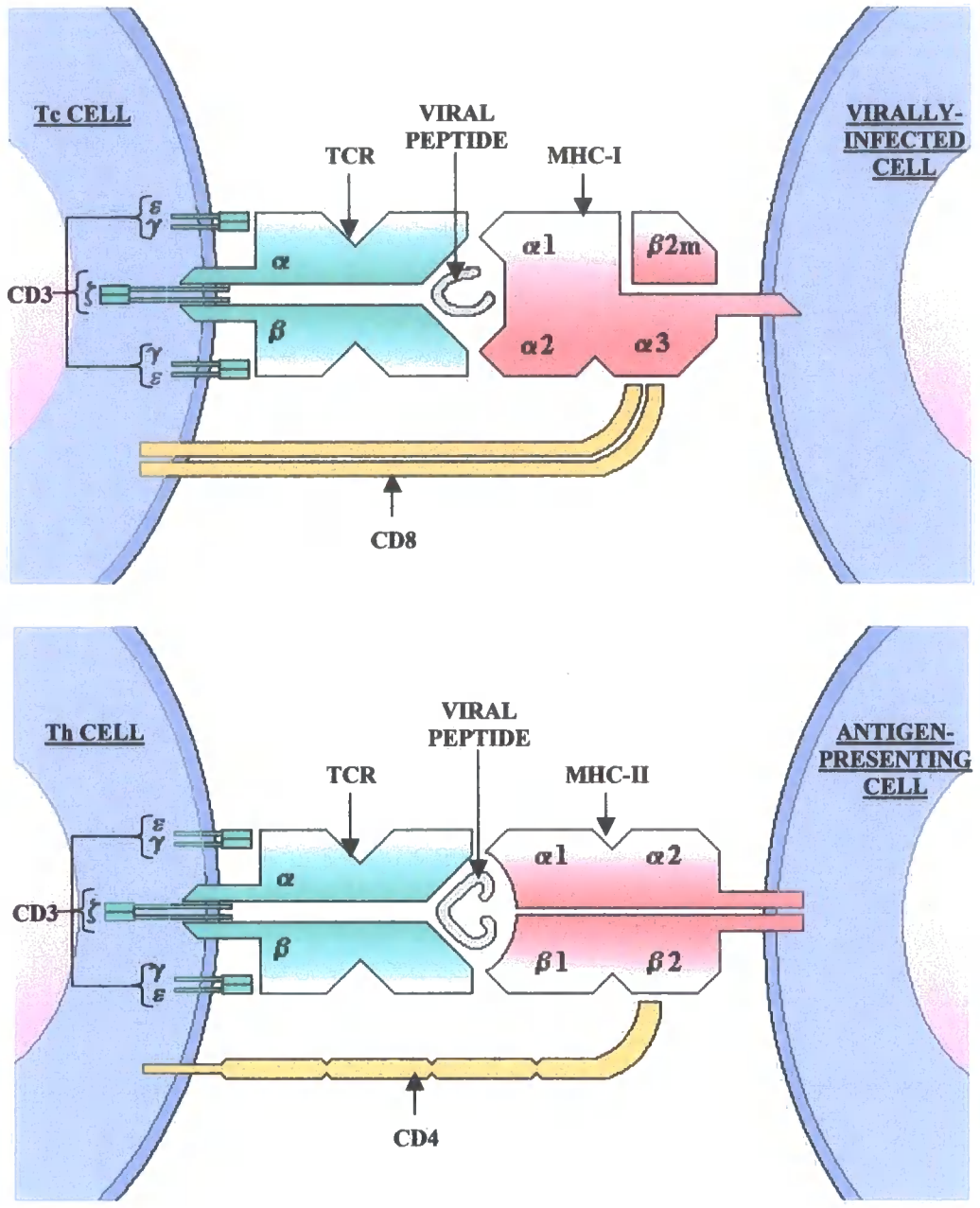
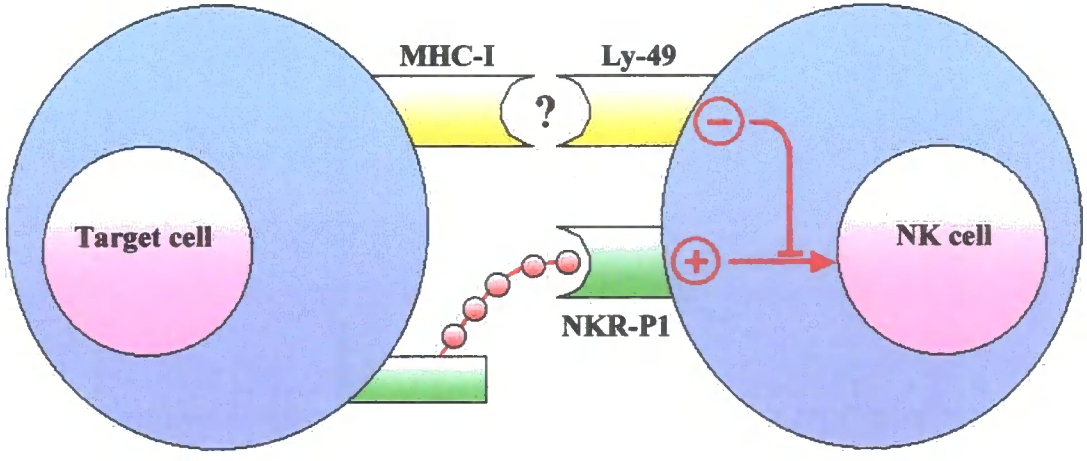


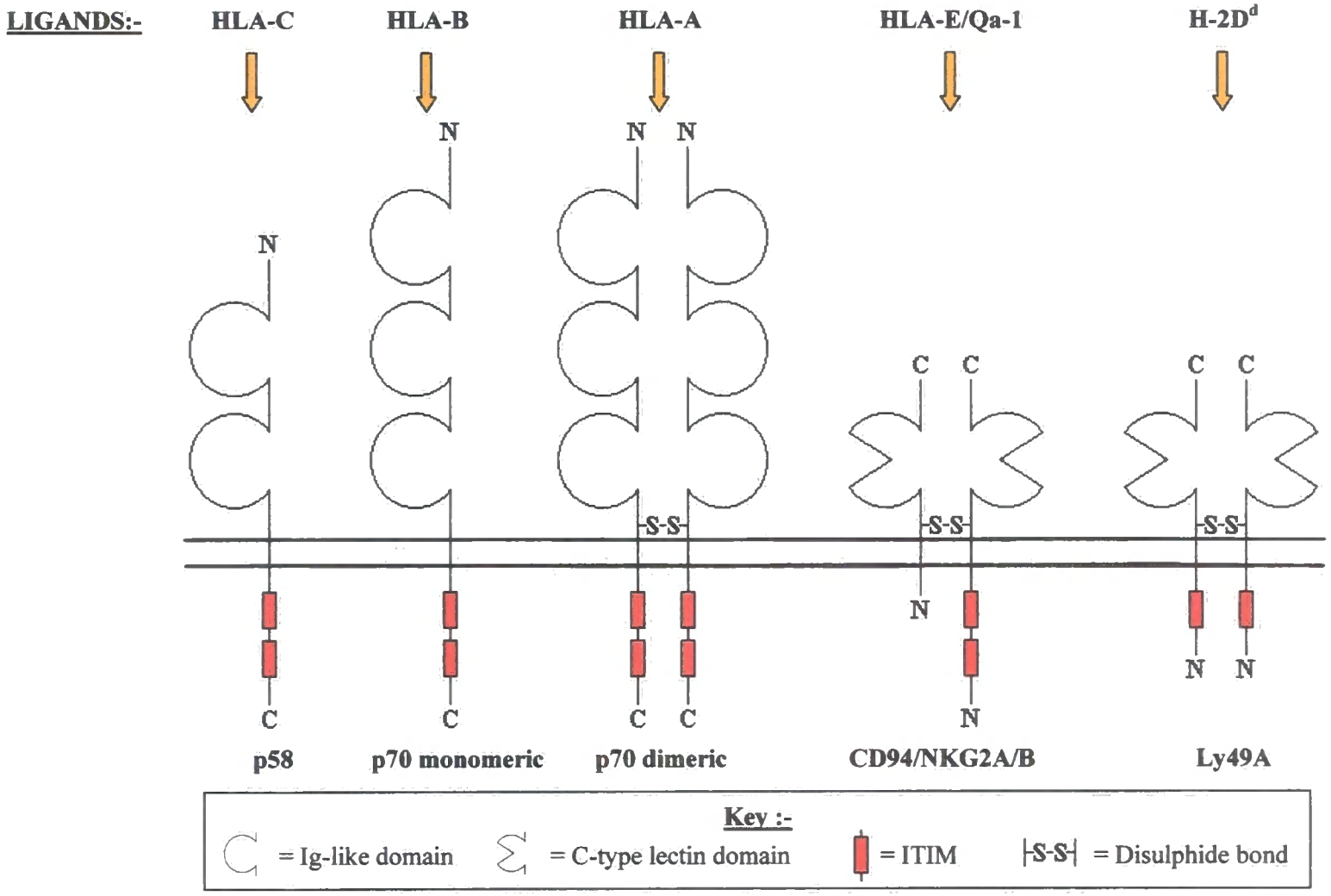
Fig. 1.2: The two receptor hypothesis of natural killing



Key: -OOOO- = carbohydrate

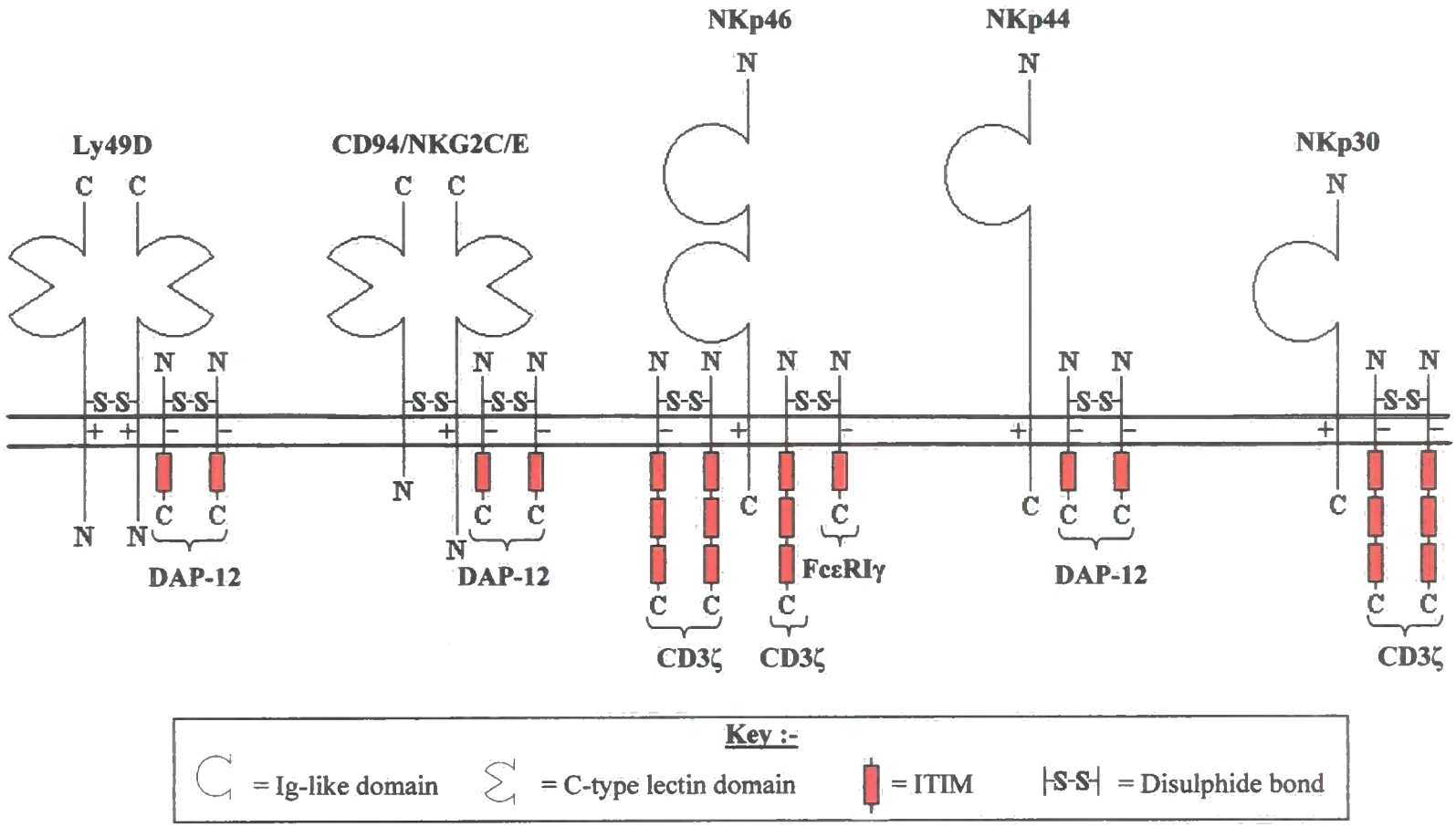
(Adapted from Yokoyama, 1995)

Fig. 1.3: NK cell inhibitory receptors



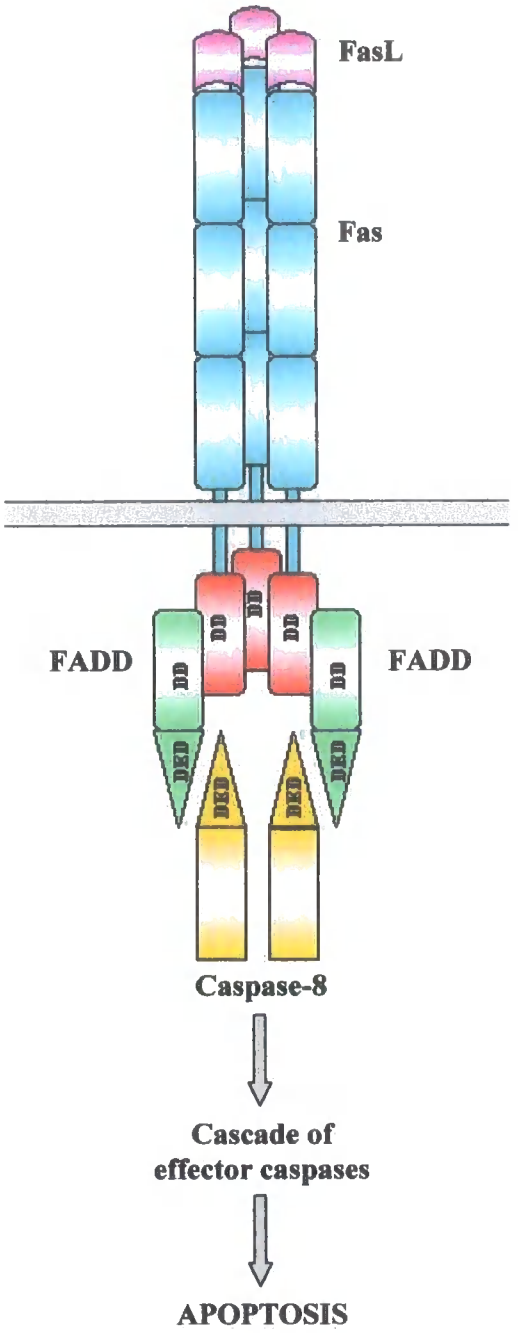
(Adapted from Brooks, 1998; Ugolini and Vivier, 2000)

Fig. 1.4: NK cell activatory receptors



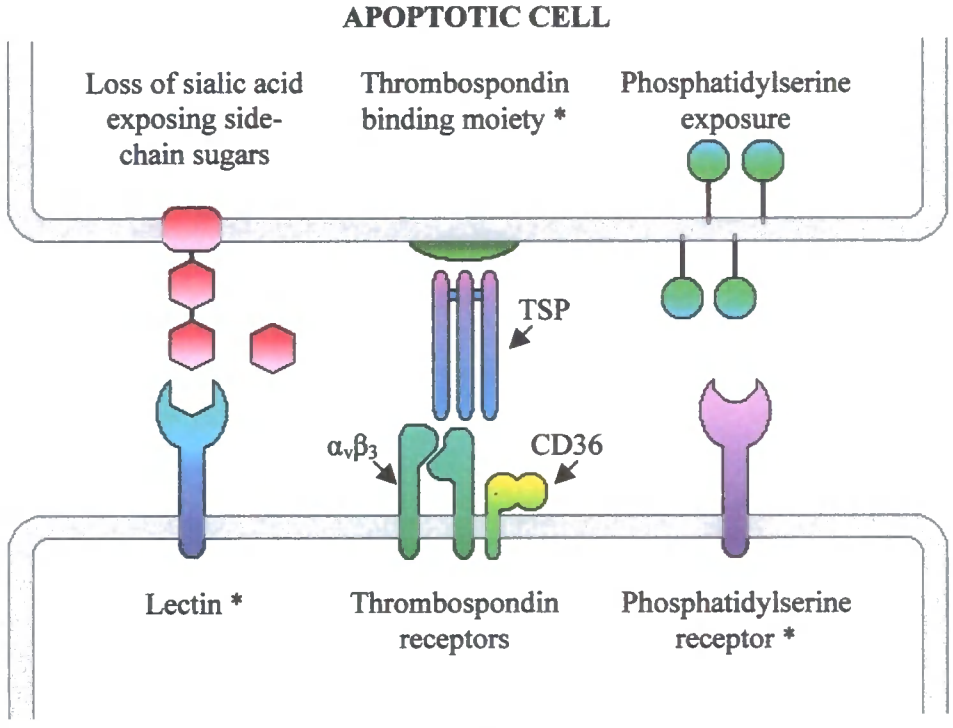
(Adapted from Brooks, 1998; Moretta *et al.*, 2000)

Fig. 1.5: Apoptosis signalling by Fas



Key: DD = death domain; DED = death effector domain.
(Adapted from Ashkenazi and Dixit, 1998)

Fig. 1.6: The three mechanisms of phagocytic recognition of apoptotic cells



Key: TSP = thrombospondin; * = not yet characterised.
(Adapted from Savill *et al.*, 1993)

CHAPTER 2

Lymphoid tissue distribution of mAb-defined NK cells in control and thymectomised *Xenopus laevis*

2.1 Introduction

The aim of this chapter is to explore the phenotype and tissue distribution of putative NK cells in *Xenopus* using the recently generated anti-*Xenopus* NK mAb's, 1F8, 4D4 and 1G5 (Horton *et al.*, 2000). The lymphoid organs to be investigated are the thymus, spleen, liver and intestine. The distribution of cells expressing the putative NK marker is compared with proportions of lymphocytes expressing previously defined T- and B-cell markers. The effect of thymectomy on lymphoid populations is examined in detail. Studies regarding T- and B-cell proportions in Tx animals has previously been examined by Gravenor (1996), whose PhD studies also explored the major lymphoid tissues in *Xenopus* (thymus, spleen, liver and intestine).

In *Xenopus*, the thymus, a primary lymphoid organ, develops dorsally from the 2nd pharyngeal pouch approximately 3 days after fertilization (Manning and Horton, 1982) and coincides with the commencement of the circulation of blood in the external gills (Nieuwkoop and Faber, 1967). Lymphoid cells originating from the embryonic lateral plate mesoderm (Turpen *et al.*, 1982) migrate to the thymus at approximately day 4 (Tochinai, 1980). By day 7, these cells have colonized the epithelial thymus bud (Horton *et al.*, 1996b). During metamorphosis (between 38-57 days post-fertilisation, (Turpen and Smith, 1989), the thymus migrates to the ear region and undergoes temporary lymphocyte depletion (Du Pasquier, 1982b). A new wave of stem cells then enter the thymus and undergo T-cell development whilst exposed to adult-specific antigens essential to the education of the T-cells (Horton, *et al.*, 1996b). The thymus is comprised of an outer cortex and a central medulla and houses dendritic cells, macrophages, cysts and granular cells, in addition to its lymphocyte and epithelial cell populations.

The spleen is a secondary lymphoid organ (reviewed in Plytycz and Bigaj, 1983), first detectable at stage 50 (15 days of age) (Du Pasquier and Flajnik, 1990), and originates from a mesenchymal condensation in close proximity to the pancreatic rudiment. Functioning to trap antigen, the spleen is a globular structure and is divided into two regions, the red pulp, rich in lymphocytes, erythrocytes and macrophages, and the

white pulp, in which the lymphocytes (mainly B-cells) are located in a concentric fashion around the central arteriole (Horton and Ratcliffe, 1998a). The red and white pulp are separated by a boundary layer, T-cells being concentrated in the region just outside the boundary.

Both liver and intestine are, in mammals, considered to be sites for possible extrathymic T-cell development. Rag-1 gene transcripts have been discovered within the intraepithelial lymphocytes (IEL's) of murine small intestine (Guy-Grand *et al.*, 1991) and TCR molecules are detectable on liver lymphocytes prior to thymus development (Poggi *et al.*, 1993), such findings heavily implicating these organs as sites for extrathymic development. In *Xenopus*, lymphocytes are located just beneath the capsule of the liver, and within the epithelium and lamina propria of the small intestine (Gravenor, 1996; Horton and Ratcliffe, 1998a).

In this Chapter, single colour flow cytometric analysis, together with immunohistochemistry are used to investigate the presence of putative NK antigens on the surface of various lymphoid populations. Findings with α-NK mAb's on control and Tx frogs are compared with α-T-cell and α-B-cell mAb's and these latter mAb's are used in dual colour flow cytometry to probe the phenotype of putative NK cells. The effect of *in vitro* culture, on NK markers in particular, is also examined in this chapter. In this respect, it has previously been shown in our laboratory that lymphocytes from control (thymus intact) *Xenopus* are able to display NK-like killing following *in vitro* maintenance in normal culture medium, whereas lymphocytes from early-thymectomised (Tx) animals require medium supplemented with T-cell-derived growth factors (referred to throughout as growth-factor-rich medium or GFM) in order to promote NK cytotoxicity. Such T-cell-derived factors are generated by culturing control splenocytes with stimulating agents such as phytohaemagglutinin (PHA) or concanavalin-A. After 24 hours, the cell supernatants are removed and on partial purification, are found to contain a 16kDa protein with biochemical and functional similarities to mammalian IL-2 (Watkins and Cohen, 1987) (*Fig. 2.1*).

2.2 Methods

Further details of reagents are given in the appendices.

2.2.1 Extraction of lymphocytes

Outbred *Xenopus laevis* were anaesthetised in aminobenzoic acid ethyl ester (MS222) (Sigma) and swabbed in 70% alcohol before dissection of the required organs, which were placed into separate sterile petri dishes containing 2-3ml cold amphibian strength Hank's balanced salt solution HBSS (Gibco) (supplemented with 1%FCS (HBSS/1%FCS).

2.2.1.1 Preparation of lymphoid cells from spleen, liver and thymus

Spleen, liver and thymus were gently teased apart in cold HBSS/1%FCS using tungsten needles. Cell suspensions were pipetted into sterile 5ml centrifuge tubes (Greiner) and placed on ice for 1-2 minutes to allow for larger particles to settle. Supernatant cell suspensions were transferred to a fresh tube and cells pelleted by centrifugation at 300g, 4°C for 10 minutes. Spleen and liver pellets were resuspended in 2ml HBSS/1%FCS and layered over 3ml Ficoll-paque (Pharmacia). Centrifugation at 250g, 20°C for 4.5 minutes produced a white (lymphoid-enriched) band of cells above the Ficoll, which was transferred to a fresh centrifuge tube and washed 3 times in HBSS/1%FCS to remove Ficoll. Thymocytes were washed in HBSS/1%FCS without Ficoll-enrichment.

2.2.1.2 Preparation of lymphoid cells from small intestine

This method for isolating cells from the small intestine was adapted from a murine IEL isolation protocol (Mosley and Klein, 1992).

The small intestine was placed into a dry petri dish and gentle pressure applied along its length to remove faecal matter. The intestine was cut open longitudinally, and then transversely into 2-3 pieces and placed into a 15ml centrifuge tube. 10ml cold CMF (Ca^{2+}/Mg^{2+} free) medium (see appendix 1) was added and the tube inverted several times. The gut pieces were allowed to settle and the supernatant discarded, this procedure being repeated several times. The intestine was placed into a siliconised flask containing 30ml CMF/EDTA/DTT medium (see appendix 1) and continuously stirred for 1 hour at room temperature with occasional vortexing to facilitate the release of intra-epithelial lymphocytes (IEL's). The supernatant cell suspension was transferred to a 50ml centrifuge tube (Greiner), which was placed on ice. 30ml CMF medium (at room temperature) was added to the gut pieces which were incubated for a further hour, again at room temperature with constant stirring and occasional vortexing. The supernatants from each incubation were pooled and centrifuged at 300g, 4°C for 10 minutes. The cell pellet was resuspended in 6ml HBSS/1%FCS and passed through a sterile pre-wetted nylon wool (Fisher) column. The column was flushed through with 20ml HBSS/1%FCS and the eluate centrifuged at 300g, 4°C for 10 minutes. The cell pellet was resuspended in 2ml HBSS/1%FCS and centrifuged over Ficoll as described in section 2.2.1.1.

2.2.2 Culturing of lymphocytes

Cells were centrifuged at 300g, 4°C for 10 minutes and resuspended in B_3B_7 medium (see appendix 1) prior to transfer to a 24-well plate (Greiner) at a concentration of approximately 1.5 x 10^6 cells/well for incubation at 27°C, 5% CO_2. When culturing lymphocytes from a thymectomised animal, growth factor-rich medium (see section 2.2.3) containing T-cell-derived growth factors was routinely added at a concentration of 1:4.

2.2.3 Generation of growth factor-rich medium

Spleen cells from control *Xenopus* were prepared for culture (see section 2.2.1.1) and pipetted into a 24-well plate (Greiner) at a concentration of approximately 3 x 10^6 cells/well. Concanavalin A (Sigma) was added to an in-well concentration of 2.5μg/ml and the cells incubated at 27°C, 5%CO_2 for 24 hours. The plates were centrifuged at 300g, 4°C for 10 minutes and the supernatant transferred to a fresh centrifuge tube for the addition of α-methyl mannoside (Sigma) (1:10), which binds to any residual Con A. The supernatant was flushed through a 0.2μm filter (Gelman) and the growth factor-rich medium (GFM) stored at 4°C.

2.2.4 Flow cytometry

Cells required for flow cytometric analysis were washed and resuspended in FACS medium (see appendix 1) at a concentration of 1 x 10^6 cells/ml. 200μl of cell suspension (2 x 10^5 cells) was pipetted into each well of a 96-well plate (Greiner), centrifuged at 300g, 4°C for 10 minutes and the supernatant discarded. 50μl of the required primary α-*Xenopus* monoclonal antibody was added to the cells at the appropriate concentration and the plate incubated on ice for 20 minutes. Following 2 washes with 150-200μl FACS medium, 50μl secondary FITC antibody (rabbit anti-mouse Ig Fab_2 fraction, FITC conjugated - DAKO) adsorbed with 1:20 *Xenopus* serum was then added to the cells (1:20) for a 20 minute incubation on ice in the dark. If single staining only was required, the cells were then washed twice with FACS medium and transferred in 500 μl aliquots to 5ml centrifuge tubes (Greiner) for flow cytometric analysis. If double staining was required, the cells were washed twice in FACS medium containing mouse serum (Sigma) (1:100) prior to a 20 minute incubation on ice in the dark with 50μl of the required PE-conjugated anti-*Xenopus* mAb's. The cells were washed twice with FACS medium and transferred to 5ml centrifuge tubes for flow cytometric analysis. 10μl of 0.25mg/ml propidium iodide (Sigma) was added to each sample to assess viability of cell populations. 5-10,000 viable cells were analysed using a Coulter XL flow cytometer. Markers were set to

exclude 98% of cells stained with control primary mAb CD3 (anti-chicken CD3) or mouse Ig-PE.

2.2.5 Preparation of frozen sections

Organs were removed as described above and placed into separate petri dishes containing 2-3ml cold HBSS/1%FCS. A small amount of OCT compound (Tissue-Tek®) was poured into aluminium foil boats and the organ to be sectioned was placed into the boat in the desired orientation. OCT was poured on top of the organ and the boat lowered gradually into liquid nitrogen until completely frozen. The aluminium foil was then removed from the block of tissue, which was mounted onto a chuck and placed onto the cryostat. Sections of thickness 6-8μm were collected onto poly-l-lysine (Sigma) coated slides and fixed in either acetone for 2 minutes (at room temperature), or methanol for 10 minutes (at -20°C). The slides were then allowed to air dry for several minutes and stored at -80°C until required for staining.

2.2.6 Immuno-peroxidase staining

Slides required for staining were incubated at room temperature for 30 minutes in 1-2ml blocking buffer (see appendix 1) prior to careful drying and incubation for 30 minutes with 50μl of the required primary mouse mAb. (All antibodies used were diluted to the appropriate concentration using wash buffer (see appendix 1)). The slides were washed 3 times in wash buffer, carefully dried and incubated for 30 minutes with 50μl of the secondary antibody (biotinylated anti-mouse IgG) (Vector laboratories) at a concentration of 1:130. The slides were again washed 3 times in wash buffer and then incubated for 20 minutes with 1-2ml 30% H_2O_2 (Sigma) (diluted to 1:100 using methanol) to remove endogenous peroxidase. Following another wash step, 150μl of ABC solution (made up 30 minutes before use, see appendix 1) (Vector laboratories) was pipetted onto each slide and left to incubate for 30 minutes. The slides were then washed and incubated with 150μl of 3,3-diaminobenzidine (DAB) substrate (see appendix 1) for approximately 10 minutes, or until a brown precipitate was seen to form. Distilled water was used to wash the slides thoroughly, which were

then carefully dried, and counter stained in methyl green stain (Vector laboratories) for approximately 10 minutes. Excess stain was removed by washing in distilled water. The slides were dehydrated through 2 changes of isopropanol, followed by two changes of xylene and mounted in DPX for examination by a Nikon optiphot microscope.

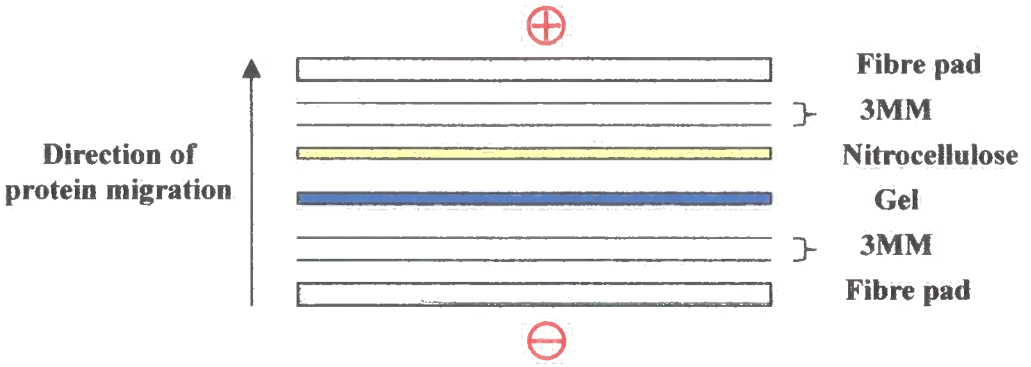
2.2.7 Immunofluorescence

The slides required for staining were incubated at room temperature for 30 minutes in 1-2ml blocking buffer (see appendix 1) prior to careful drying and incubation for 30 minutes with 50µl of the required primary mouse mAb. (All antibodies used were diluted to the appropriate concentration using wash buffer (see appendix 1)). The slides were washed 3 times in staining buffer, carefully dried and incubated for 30 minutes with 50µl of the secondary antibody (rabbit anti-mouse Ig-FITC) (Sigma) at a concentration of 1:20. Again, the slides were washed 3 times in wash buffer, carefully dried and mounted in PBS/glycerol (Citifluor) containing anti-fadant for examination under a Nikon optiphot fluorescence microscope.

2.2.8 Western blotting

The lymphocytes required for Western blotting were lysed on ice for 30 minutes in NP-40 protein lysis buffer (see appendix 2) containing 1%NP-40 and protease inhibitors and then centrifuged at 15,000rpm for 3 minutes to pellet cytoskeletal debris. 1/5 volume of 5x denaturing SDS loading buffer was added to the lysate and boiled for 3 minutes. Proteins were separated according to size by SDS-PAGE using Mini-Protean II gel apparatus (BioRad). Samples were run at 200v down a minigel of 10% acrylamide separating gel, 4% stacking gel (see appendix 2), together with pre-stained markers of 66kDa and 87kDa. The gel was then removed and washed in Towbin transfer buffer (see appendix 2) for 10-15 minutes to remove SDS. Transfer of proteins to nitrocellulose (Schleicher and Schuell) was carried out using the Trans-Blot® Electrophoretic Transfer cell (BioRad). The gel and nitrocellulose were sandwiched between 4 sheets of 3MM paper (Whatman) and two fibrepads (BioRad)

as shown in the diagram below. Each component of the blot was soaked in Towbin transfer buffer prior to use.



Protein transfer to the nitrocellulose membrane was performed in Towbin transfer buffer overnight at 4°C at 30V (limited to 2mA). The nitrocellulose was removed and stained with Ponceau S stain (see appendix 2) to check transfer of proteins. The membrane was destained in 5% acetic acid and washed in Tris-buffered saline (TBS) (see appendix 2) for 20 minutes. Blocking solution (see appendix 2) was then added for 2 hours at room temperature and was then replaced with blocking solution containing 50% primary antibody supernatant. The nitrocellulose was incubated at room temperature for a further 2 hours and was washed 3 times with blocking solution prior to the addition of the secondary antibody (goat anti-mouse IgG-HRP) (Sigma) at 1:20,000 diluted in blocking buffer for 2 hours at room temperature. The nitrocellulose was washed extensively in TBS prior to detection of labeled proteins. Immunodetection was carried out in the dark using chemiluminescent solutions 1 and 2 (see appendix 2), which were combined immediately prior to 1 minute incubation with the nitrocellulose. The nitrocellulose was removed from the chemiluminescent solutions, blotted to remove excess, covered in cling film and exposed to X-ray film (Fujifilm) for the required exposure time.

2.3 Results

2.3.1 Surface expression of lymphocyte markers by *ex vivo* and *in vitro*-cultured lymphocytes

2.3.1.1 Single colour flow cytometry

a) *Ex vivo*

(Tables 2.1/2.2, Figs 2.2/2.3)

Qualitative staining patterns of lymphocytes in thymus, spleen, liver and intestine in control and Tx frogs, using 2B1 mAb (anti-T-cell), D8 mAb (anti-B-cell) and 1F8 mAb (anti-NK) are shown in *Fig. 2.2.* This figure reveals that thymectomy significantly lowers CD5 expression (2B1 mAb) in all peripheral lymphoid tissues, causes elevation of IgM^{+ve} cell proportions (in spleen and intestine) and results in an increase in percentages of $1F8^{+ve}$ putative NK cells.

T-cells : In control animals there was a high proportion of $2B1^{+ve}$ (CD5) T-cells in spleen, liver and intestine, the highest percentage being found within the intestine (71%) (Quantitative staining patterns shown in *Table 2.1* and *Fig. 2.3*). Levels of $AM22^{+ve}$ (putative CD8α) and $F17^{+ve}$ (putativeCD8β) cells in spleen, liver and intestine were generally comparable (approximately 25%), although rather higher proportions (36%) of putative $CD8\alpha^{+ve}$ cells were present in intestine. The majority of thymocytes (80%) stained positively for the XTLA-1 antigen (data not shown) and approximately 60% stained for CD8 and CD5. D12-2, the putative α-γδTCR monoclonal antibody identified low percentages of cells in spleen and liver (4% and 9% respectively), negligible amounts in the thymus (3%) but relatively high levels in the intestine (28%).

In thymectomised animals, CD5 levels decreased significantly (60→12% in the spleen, 51→22% in the liver and 71→33% in the intestine); similar observations were

made with regard to putative CD8β^{+ve} cells, levels falling to just 3% in the spleen. The effect of thymectomy on putative CD8α^{+ve} cells was far less, there being a slight decline in numbers of these cells in spleen, but not in liver or intestine. D12-2^{+ve} cells increased in proportion in Tx spleen and liver to 13% and 21% respectively, but actually decreased in intestine following thymectomy.

B-cells : IgM^{+ve} B-cells identified by the D8 monoclonal antibody were present in similar proportions in the control spleen (29%) and liver (20%), with slightly lower percentages recorded in the intestine (13%). Negligible levels (4%) of B-cells were found in the thymus. Following thymectomy, the proportion of B-cells increased substantially to 53%, 34% and 25% in spleen, liver and intestine respectively.

NK cells : Three putative anti-NK monoclonal antibodies, 1F8, 4D4 and 1G5 were used. 1G5 consistently identified slightly lower percentages of positive cells in comparison with 1F8 and 4D4. Putative NK cells were negligible in the thymus and were at low levels in the spleen (5%1F8, 5%4D4, 4%1G5) and liver (8%1F8, 7%4D4, 6%1G5) of control *Xenopus*. In contrast, the intestine proved to be a good source for putative NK cells (27%1F8, 20%4D4, 12%1G5). In thymectomised animals, cell proportions identified by the three putative anti-NK mAb's increased significantly, e.g. 1F8 stained 16% cells in Tx spleen, 17% in Tx liver and 36% in Tx intestine. The mean intensity of fluorochrome associated with the NK antibodies was also assessed (*Table 2.4*) and it was observed that 1F8^{+ve} candidate NK cells from Tx spleen generally showed increased fluorescence intensity when compared to the same population of cells in control spleen (from 2.6 in control animals to 4.8 in Tx animals). Increased brightness of fluorescence following thymectomy in *ex vivo* splenocytes was not readily apparent when 4D4 and 1G5 were used.

b) Following *in vitro* culture for 48 hours

T-cells : *(Tables 2.3 and 2.4)* 48 hour *in vitro* culture of control *Xenopus* lymphocyte populations had no effect on the proportions of CD5^{+ve}, D12-2^{+ve} and putative CD8α^{+ve} lymphocytes, although levels of putative CD8β^{+ve} cells in the liver and intestine increased from 23%→29% and 22%→35% respectively.

48 hour culture of Tx spleen, liver and intestinal lymphocytes in GFM resulted in an increase in percentages of $CD5^{+ve}$ cells (12→17% in the spleen, 22→38% in the liver and 33→51% in the intestine). D12-2^{+ve} cells also increased in proportion in the liver (21→39%) and intestine (22→35%), but not in the spleen.

B-cells : B-cell levels from both control and Tx animals were not affected by *in vitro* culture.

NK cells : Levels of putative NK-cells in control animals were affected minimally by *in vitro* culture, even when cultured in GFM (data not shown). Cells from Tx animals showed a small increase in $1F8^{+ve}$ proportion after 48 hour culture in GFM (16→18% in the spleen and 36→42% in the intestine), whereas the putative $1F8^{+ve}$ NK cells of the liver showed more significant increases in percentage (17→31%) (Similar data also with 4D4 and 1G5 mAb's). Mean fluorochrome intensity of NK cells from control spleen showed no increase after culture, whereas an increase in intensity of NK markers was consistently detectable after splenocytes from Tx animals were cultured in GFM (4.8→6.0 1F8, 3.0→6.2 4D4, 1.9→6.4 1G5) (*Table 2.4*).

2.3.1.2 Dual colour flow cytometry

The following two co-staining profiles were examined in an attempt to learn more about the cell surface epitopes expressed by $1F8^{+ve}$ cells: 1F8-FITC/2B1-PE and 1F8-FITC/D12-2-PE. The PE mAb's were directly conjugated, whereas 1F8 is visualized indirectly by anti-mouse Ig-FITC-conjugated antibody.

a) *Ex vivo*

As *Fig. 2.4* illustrates, candidate NK cells of spleen and liver did not co-stain with the α-CD5 antibody 2B1. A distinct population of $1F8^{+ve}$ cells within the intestine (11%) stained positively for CD5, although this fluorescence intensity was low. These cells were therefore termed $CD5^{lo}$. $1F8^{+ve}$ splenocytes from Tx frogs were $CD5^{-ve}$, whereas nearly 50% liver and intestinal $1F8^{+ve}$ lymphocytes from Tx frogs expressed $CD5^{lo}$.

Most $1F8^{+ve}$ cells of control spleen and liver co-stained with the putative α-γδTCR antibody D12-2, but only one-third intestinal $1F8^{+ve}$ Tx lymphocytes were D12-2^{+ve}. Dual staining ($1F8^{+ve}$/D12-2^{+ve}) was consistently high in Tx animals, virtually all splenic and hepatic NK cells being D12-2^{+ve}. An additional experiment revealed that D12-2^{+ve} cells predominantly co-stained for CD5 in spleen, liver and intestine taken *ex vivo* from controls *(Fig. 2.5)*.

b) Following *in vitro* culture for 48 hours

Fig. 2.4 demonstrates that 48 hour *in vitro* culture of splenocytes and gut lymphocytes from both control and Tx animals generally had minimal effect on levels of $1F8^{+ve}$ cells co-staining with 2B1 or D12-2. However, 48 hour culture of Tx hepatic lymphocytes in GFM resulted in a significant increase of 1F8/2B1 and 1F8/D12-2 co-staining, although fluorescence intensity of CD5 was low. 1F8 fluorescence intensity on Tx cells was consistently brighter following 48 hours culture.

2.3.2 Distribution of lymphocyte populations: Immunohistological analyses

As shown in *Fig. 2.6*, both putative $CD8\alpha^{+ve}$ ($AM22^{+ve}$) lymphocytes and IgM^{+ve} B-cells are, in the main, located within the lamina propria of the intestine, although a minority of B-cells are found scattered throughout the epithelium. Putative $\gamma\delta TCR^{+ve}$ cells (D12-2^{+ve}) and candidate NK cells ($1F8^{+ve}$) are predominantly present in the basal layer of the epithelium. In Tx animals, an increase in proportion of candidate NK cells is apparent, together with a vast reduction (but not elimination) of putative $CD8\alpha^{+ve}$ lymphocyte levels.

The spleen and thymus repeatedly proved refractory to staining with the α-*Xenopus* NK cell monoclonal antibodies, although these organs stained with α-CD8α mAb and α-IgM mAb as established elsewhere (Horton and Ratcliffe, 1998a; Horton *et al.*, 1998c). The liver was not examined histologically.

2.3.3 Preliminary molecular characterisation of NK cells

As *Fig. 2.7* illustrates, B_3B_7 tumour cell and control splenocyte lysates probed with the XT-1 antibody (identifying a subset of T-cells), revealed a protein of approximately 120kDa. Staining with 1F8 and 4D4 was faint compared to that of 1G5 (data not shown), which was therefore used routinely in Western blotting to probe for NK cell antigens. 1G5 reacted well with lysates of cells from Tx *Xenopus* intestine, identifying two proteins of approximately 66-75kDa. The band at approximately 75kDa was also present in control and Tx spleen lysates, both of which also contained a protein at approximately 85kDa. Lysates of Tx spleen revealed four protein bands in all specific to 1G5 mAb, ranging from 66-85kDa. Blots were also probed with secondary antibody only as a control. No protein bands were observed in these control lanes.

2.4 Discussion

Single colour flow cytometry has investigated the proportions of lymphocytes in thymus, spleen, liver and intestine of control and Tx *Xenopus* that stain with known α-T-cell mAb's (α-CD5 and α-CD8 reagents), a putative α-γδTCR mAb, an α-B cell mAb (α-IgM) and recently generated mAb's considered to be α-NK cell reagents. This work therefore expands on the earlier studies of Gravenor (1996) who examined the effect of thymectomy on lymphocyte staining with the T-cell and B-cell-specific reagents. Findings presented here are generally in agreement with Gravenor's work. For example, the proportion of $CD5^{+ve}$ cells is shown in both studies to be reduced to low levels in the spleen following thymectomy, whereas liver and intestinal lymphocytes still contain appreciable proportions of $CD5^{+ve}$ cells following thymic ablation, although these cells were $CD5^{lo}$ compared with the brighter CD5 staining seen on the T cells of control frogs.

Of the two α-CD8 mAb's, $F17^{+ve}$ cells (putative $CD8\beta^{+ve}$), are substantially lowered (liver and intestine) or removed (spleen) by early thymectomy, whereas $AM22^{+ve}$ cells (putative $CD8\alpha^{+ve}$) are far less thymus-dependent. In both the present study and in Gravenor's work, liver and especially intestine from Tx frogs had substantial percentages of $AM22^{+ve}$ cells. $AM22^{+ve}$ splenocytes were virtually absent from Tx frogs in Gravenor's study, but a mean level of 15% was seen in the present experiments. The difference may be explained in part by the substantial standard error (7.6) seen in the present study, some Tx animals having virtually no $AM22^{+ve}$ splenocytes, others having appreciable levels of this CD8 marker. Perhaps the latter frogs were incompletely thymectomised? The AM22 staining seen in Tx frogs tends to be $AM22^{lo}$, compared to the brighter AM22 staining seen on control T-lymphocytes.

IgM^{+ve} B-cells were routinely increased in proportion in all three lymphoid tissues following thymectomy, in agreement with Gravenor's findings. Also in agreement between the two studies is the flow cytometric data recorded with the putative γδTCR mAb D12-2. Thus levels of $D12\text{-}2^{+ve}$ lymphocytes increase 2-3-fold in spleen and

liver following thymectomy, whereas proportions stay approximately the same in the intestine, which contains the highest levels of D12-2^{+ve} cells.

Use of three α-NK mAb's has been possible in this Thesis. The present studies indicate that the proportions of putative NK cells are consistently negligible in thymus and are increased in all three peripheral lymphoid organs (spleen, liver and intestine) following thymectomy, the highest levels being seen in the intestine. 48 hour culture of Tx cells in GFM (associated with appearance of cytolytic behaviour of splenocytes from Tx frogs) resulted in higher proportions of NK lymphoid cells, especially in the liver. The brightness of this fluorescence on splenocytes of Tx frogs is also increased. 48hr culture in medium alone (or in GFM, not shown) had no apparent effect on control lymphocytes, in terms of increasing NK cell proportions or brightness of staining with the α-NK mAb's.

Dual staining studies on Tx frogs have revealed that 1F8^{+ve} putative NK cells from spleen are CD5^{-ve}, but frequently D12-2^{+ve} cells, whereas NK cells in liver frequently express CD5^{lo} (especially after 48hr culture in GFM) and are predominantly D12-2^{+ve}. Intestinal NK cells are ≈50% 1F8^{+ve}/CD5^{+ve}, 50% 1F8^{+ve}/CD5^{-ve}, and between a third (*ex vivo*) and a half (48hr culture) are D12-2^{+ve}. The expression of CD5^{lo} (seen constitutively on liver and intestinal NK cells) also occurs on splenic NK cells following their isolation by immunomagnetic sorting with 1F8 mAb (see Chapter 3). This might suggest that CD5^{lo} represents an activation marker on NK cells. In this respect it is known that CD5^{lo} also appears on B cells following their *in vitro* stimulation by PMA and calcium ionophore (Jurgens *et al.*, 1995) (see also Chapter 5).

Co-expression of 1F8 antigen and D12-2 antigen is of particular interest. The D12-2 antigen is a 56kDa protein that is known (Ibrahim *et al.*, 1991) to be expressed on a minor subset of CD5 T-cells and this has been confirmed here. It was conjectured that D12-2 might represent the γδTCR (Ibrahim *et al.*, 1991). The question of whether 1F8^{+ve}/D12^{+ve}, seen distinctly in Tx frogs, represent γδT-cells or indicates a special subset of NK cells remains uncertain. In mice, γδTCR^{+ve} IEL's appear to represent thymus-independent cells (Lefrancois, 1991; Lin *et al.*, 1995), so the

development of D12-2^{+ve} cells in Tx animals could represent thymus-independent γδT-cells. However, since mammalian γδTCR^{+ve} intestinal T-cells are mostly CD5^{-ve} (Lefrancois, 1991), the expression of CD5 by control *Xenopus* D12-2^{+ve} intestinal lymphocytes might be used to argue that these cells are <u>not</u> γδT-cells. Clearly, reagents specific for γδTCR's are required to resolve this issue. We do know, however, that 1F8^{+ve} cells purified from the Tx spleen fail to express TCRVβ transcripts (Rau *et al.*, 2002), indicating that 1F8^{+ve} (D12-2^{+ve} or D12-2^{-ve}) cells are a lymphocyte population distinct from αβT-cells.

The persistence of AM22^{lo} cells, but not F17^{+ve} cells following thymectomy seen by Gravenor is confirmed here. These AM22^{lo} cells may well represent NK cells as 1F8 purified splenocytes have been identified as predominantly AM22^{+ve} (Horton *et al.*, 2000). AM22 is believed to identify the CD8α chain and so it is feasible that NK cells may express CD8αα dimers, as has also been found for avian NK cells (Gobel, 1996).

The immunohistological data presented here on the *Xenopus* intestine reveal the similar intra-epithelial location of 1F8^{+ve} and D12-2^{+ve} lymphocytes, and provides visual confirmation that thymectomy increases the incidence of cells expressing the putative NK marker. The basal layer of *Xenopus* intestinal epithelium proves to be an excellent source of candidate NK cells. This is not surprising, since approximately 50% avian IEL's belong to the NK cell subset (Gobel, 1996). Furthermore, >25% murine (Tagliabue *et al.*, 1982) and rabbit (Rudzik and Bienenstock, 1974) IEL's and >50% guinea pig IEL's (Arnaud-Battandier *et al.*, 1978) are large granular lymphocytes. The location of putative NK cells in the *Xenopus* spleen was not possible, because the α-NK mAb's disappointingly failed to stain frozen sections of this tissue.

Flow cytometry revealed that the 1G5 α-NK mAb consistently identifies a slightly lower percentage of cells in comparison with 1F8 and 4D4, the latter two mAb's having almost identical staining patterns. Dual colour flow cytometry indicates (Horton *et al.*, unpublished) that 1G5 targets a different epitope of the same candidate NK surface antigen identified by 1F8 and 4D4. The Western blotting experiments

described here have in fact made use of the 1G5 mAb to provide preliminary evidence that the molecular weight of the NK antigen is between 66-85 kDa, although it is the 75kDa protein which is common to splenocytes and IEL's. The array of bands identified by 1G5 may represent different degrees of glycosylation of the same cell surface protein. Recent Western blot studies by Minter (2000) in this laboratory showed that the 1G5 mAb identified a doublet at 72-74kDa in Tx *Xenopus* spleen, whereas the mAb's 1F8 and 4D4 (used at what was judged by flow cytometry to be the same concentration as the 1G5 mAb) failed to stain the same Tx splenocyte lysates. In contrast, Minter showed that all three α-NK mAb's stained Tx gut lysates, although 1G5 showed distinctly stronger staining patterns than the other two mAb's. Interestingly he revealed that the D12-2 mAb stained Tx spleen and gut lysates in comparable fashion to the 1G5 mAb, revealing a doublet at 72-74kDa.

The work in this chapter provides clear evidence that B-cells and candidate NK cells are found in higher proportions in Tx animals compared to control animals of the same age. However, there is no absolute increase in B-cell and NK cell numbers in spleen since splenocyte numbers are reduced by approximately two thirds following thymectomy (Tagliabue *et al.*, 1982). Information on comparison of absolute lymphocyte numbers in liver and intestine of control and Tx frogs has proved difficult to obtain due to the varied lymphocyte yield achieved in each separation. Since Tx *Xenopus* fail to develop *bona fide* T-cells (Horton *et al.*, 1998c) these frogs must rely heavily on NK cells, B-cells and macrophages for immunological defence. However, this cellular immune armoury cannot provide sufficient defence against certain pathogens, such as nematode parasites (*Capillaria*) which are the cause of 'flaky skin' frequently found in old (>2 years) Tx *Xenopus*, a condition which can be treated by reimplantation of the thymus gland (Cohen's laboratory, Rochester, NY). T-cells are also known to play a crucial role in *in vivo* α-tumour immunity (Robert *et al.*, 1997b), although a role for a non-T-cell population was also indicated in their experiments.

In the next Chapter the phenotype and function of lymphoid cells purified by immunomagnetic sorting using the putative α-NK mAb 1F8 will be addressed. The ability of such purified NK cells to destroy tumour target cells *in vitro* will be examined and the mechanism of tumour cell cytotoxicity explored.

Table 2.1: Percentage of mAb-defined lymphocytes *ex vivo* from control and Tx *Xenopus* as determined by flow cytometry

		CONTROL			Tx		
Antigen	**mAb**	**Spleen**	**Liver**	**Intestine**	**Spleen**	**Liver**	**Intestine**
CD5	**2B1**	60 ± 2.1	51 ± 3.6	71 ± 3.2	12 ± 1.6	22 ± 6.2	33 ± 5.9
CD8α?	**AM22**	22 ± 2.8	26 ± 3.5	36 ± 6.0	15 ± 7.6	38 ± 3.0	35 ± 13
CD8β?	**F17**	22 ± 1.4	23 ± 2.6	22 ± 4.6	3 ± 1.2	12 ± 2.0	14 ± 5.4
γδTCR?	**D12**	4 ± 0.33	9 ± 0.86	28 ± 5.7	13 ± 2.9	21 ± 7.2	22 ± 3.1
IgM	**D8**	29 ± 2.9	20 ± 7.1	13 ± 2.7	53 ± 6.0	34 ± 5.0	25 ± 4.3
NK?	**1F8**	5 ± 0.38	8 ± 0.67	27 ± 3.4	16 ± 1.6	17 ± 4.7	36 ± 5.8
NK?	**4D4**	5 ± 0.27	7 ± 0.87	20 ± 2.3	14 ± 1.5	16 ± 4.7	33 ± 6.3
NK?	**1G5**	4 ± 0.39	6 ± 0.76	12 ± 2.4	10 ± 1.1	10 ± 3.6	23 ± 4.6

Mean % ± standard error where number of adult *Xenopus* analysed ≥5. Control cells were cultured in medium alone, Tx cells in GFM-supplemented medium. "?" = "putative".

Table 2.2: Percentage of mAb-defined lymphocytes in ex vivo *Xenopus* thymus as determined by flow cytometry

Antigen	**mAb**	**Thymus**
CD5	**2B1**	64 ± 7.0
CD8α?	**AM22**	62 ± 3.2
γδTCR?	**D12**	3 ± 0
IgM	**D8**	4 ± 0
NK?	**1F8**	3 ± 0.42
NK?	**4D4**	4 ± 0.37
NK?	**1G5**	4 ± 0

Mean % ± standard error where number of adult *Xenopus* analysed ≥3. "?" = "putative".

Table 2.3: Percentage of mAb-defined lymphocytes in lymphoid tissues of control and Tx *Xenopus ex vivo* and after 48 hr culture as determined by flow cytometry

		CONTROL (0hr)			CONTROL (48hr)		
Antigen	**mAb**	**Spleen**	**Liver**	**Intestine**	**Spleen**	**Liver**	**Intestine**
CD5	**2B1**	60 ± 2.1	51 ± 3.6	71 ± 3.2	63 ± 2.3	57 ± 1.4	71 ± 7.0
CD8α?	**AM22**	22 ± 2.8	26 ± 3.5	36 ± 6.0	25 ± 2.9	27 ± 4.8	
CD8β?	**F17**	22 ± 1.4	23 ± 2.6	22 ± 4.6	20 ± 3.8	29 ± 0.50	35 ± 1.0
γδTCR?	**D12**	4 ± 0.33	9 ± 0.86	28 ± 5.7	5 ± 0.56	10 ± 0.70	29 ± 1.5
IgM	**D8**	29 ± 2.9	20 ± 7.1	13 ± 2.7	28 ± 3.2	20 ± 2.3	14 ± 1.0
NK?	**1F8**	5 ± 0.38	8 ± 0.67	27 ± 3.4	5 ± 2.44	9 ± 3.0	25 ± 3.5
NK?	**4D4**	5 ± 0.27	7 ± 0.87	20 ± 2.3	5 ± 2.34	8 ± 2.68	24 ± 5.0
NK?	**1G5**	4 ± 0.39	6 ± 0.76	12 ± 2.4	4 ± 2.38	8 ± 2.66	18 ± 2.50

		Tx (0hr)			Tx (48hr)		
Antigen	**mAb**	**Spleen**	**Liver**	**Intestine**	**Spleen**	**Liver**	**Intestine**
CD5	**2B1**	12 ± 1.6	22 ± 6.2	33 ± 5.9	17 ± 4.8	38 ± 2.4	51 ± 6.9
CD8α?	**AM22**	15 ± 7.6	38 ± 3.0	35 ± 13	9 ± 3.5	33 ± 7.5	
CD8β?	**F17**	3 ± 1.2	12 ± 2.0	14 ± 5.4	2 ± 1.2	8 ± 0.50	7 ± 0.50
γδTCR?	**D12**	13 ± 2.9	21 ± 7.2	22 ± 3.1	13 ± 3.1	39 ± 2.7	35 ± 4.1
IgM	**D8**	53 ± 6.0	34 ± 5.0	25 ± 4.3	57 ± 9.7	35 ± 4.0	33 ± 0
NK?	**1F8**	16 ± 1.6	17 ± 4.7	36 ± 5.8	18 ± 2.7	31 ± 2.9	42 ± 2.6
NK?	**4D4**	14 ± 1.5	16 ± 4.7	33 ± 6.3	16 ± 2.1	29 ± 4.0	35 ± 2.3
NK?	**1G5**	10 ± 1.1	10 ± 3.6	23 ± 4.6	14 ± 2.4	30 ± 6.5	22 ± 3.5

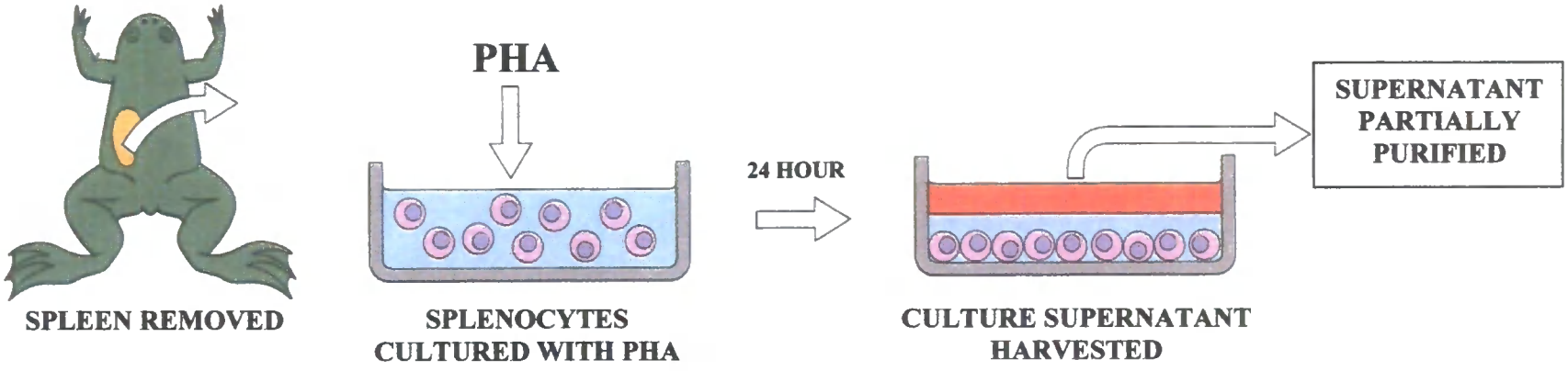
Mean % ± standard error where number of adult *Xenopus* analysed ≥3. Control cells were cultured in medium alone, Tx cells in GFM-supplemented medium. "?" = "putative".

Table 2.4: The effect of thymectomy and *in vitro* culture on the mean fluorescence intensity of anti-NK mAb-staining on splenocytes

		MEAN FLUORESCENCE INTENSITY			
		CONTROL		Tx	
Antigen	**mAb**	**0hr**	**48hr -GFM**	**0hr**	**48hr +GFM**
NK?	**1F8**	2.6 ± 0.26	2.8 ± 0.21	4.8 ± 0.80	6.0 ± 1.68
NK?	**4D4**	2.2 ± 0.025	3.2 ± 0.52	3.0 ± 0.005	6.2 ± 0.2
NK?	**1G5**	2.2 ± 0.48	3.2 ± 0.29	1.9 ± 0	6.4 ± 0.54

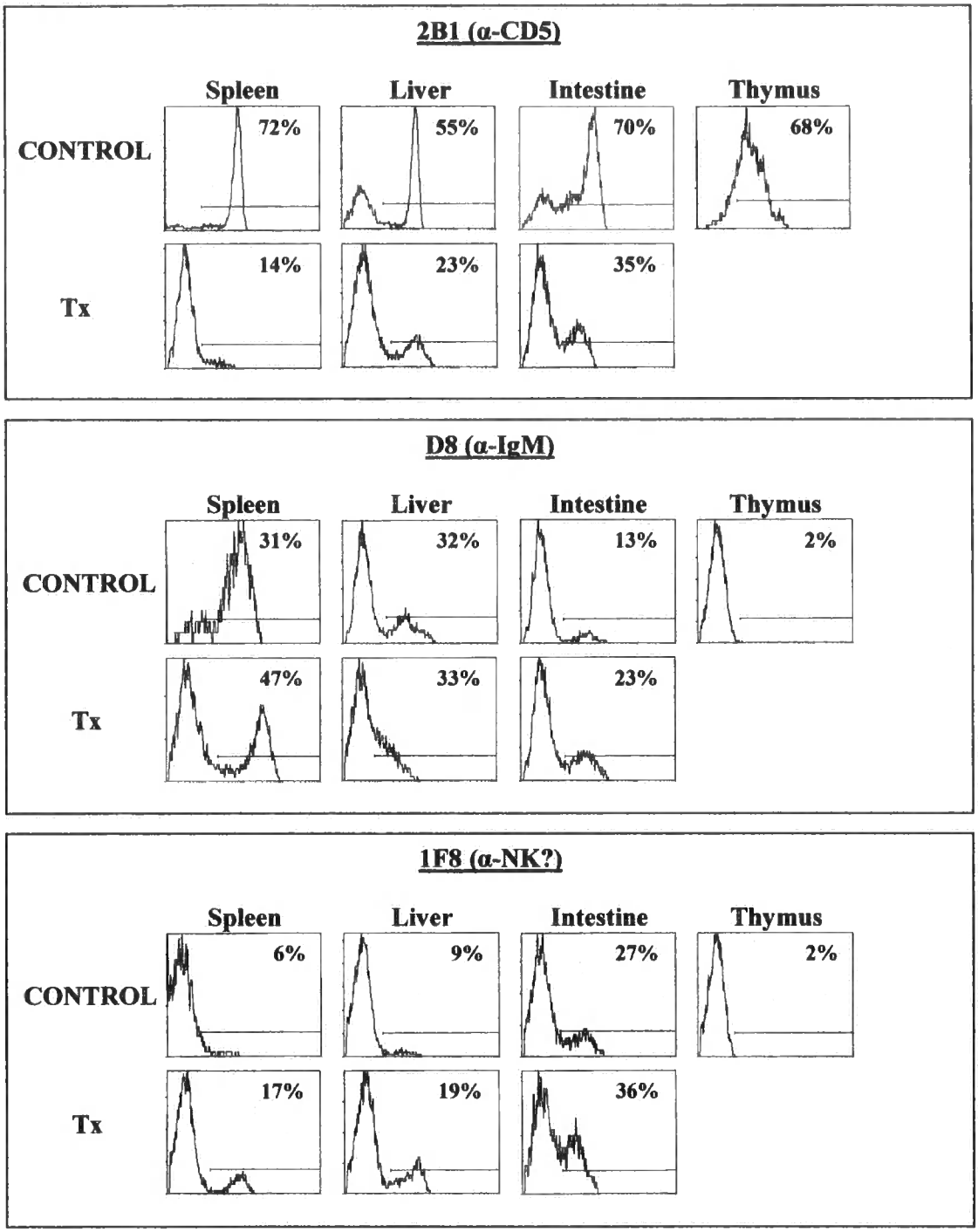
Mean % ± standard error where number of adult *Xenopus* analysed ≥3. Control cells were cultured in medium alone, Tx cells in GFM-supplemented medium. "?" = "putative".

Fig. 2.1: Production of growth factor-rich medium



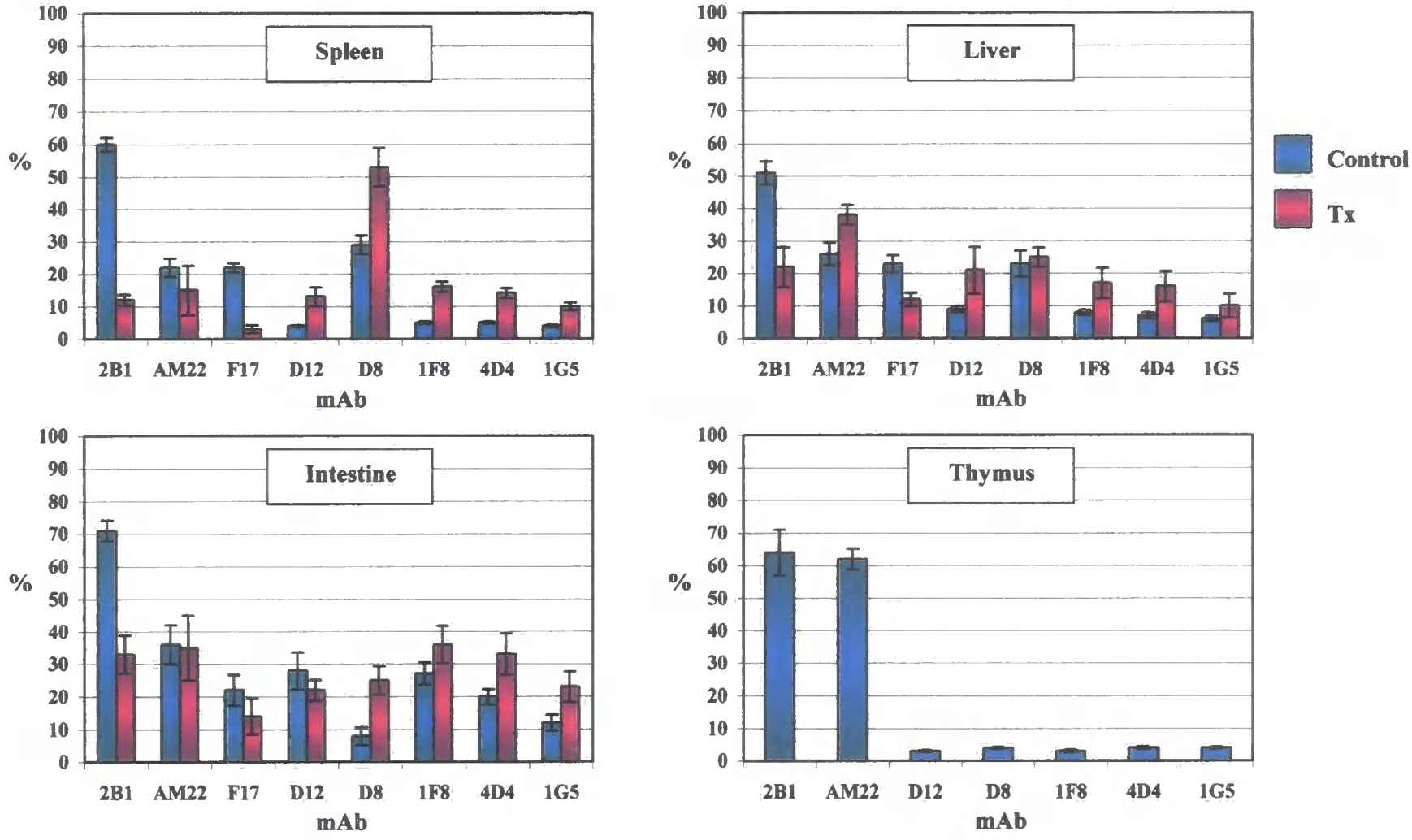
(Adapted from Horton and Ratcliffe, 1998a)

Fig. 2.2: Single colour flow cytometric data to demonstrate mAb-staining patterns of various lymphocyte populations in lymphoid tissues of control and Tx *Xenopus*



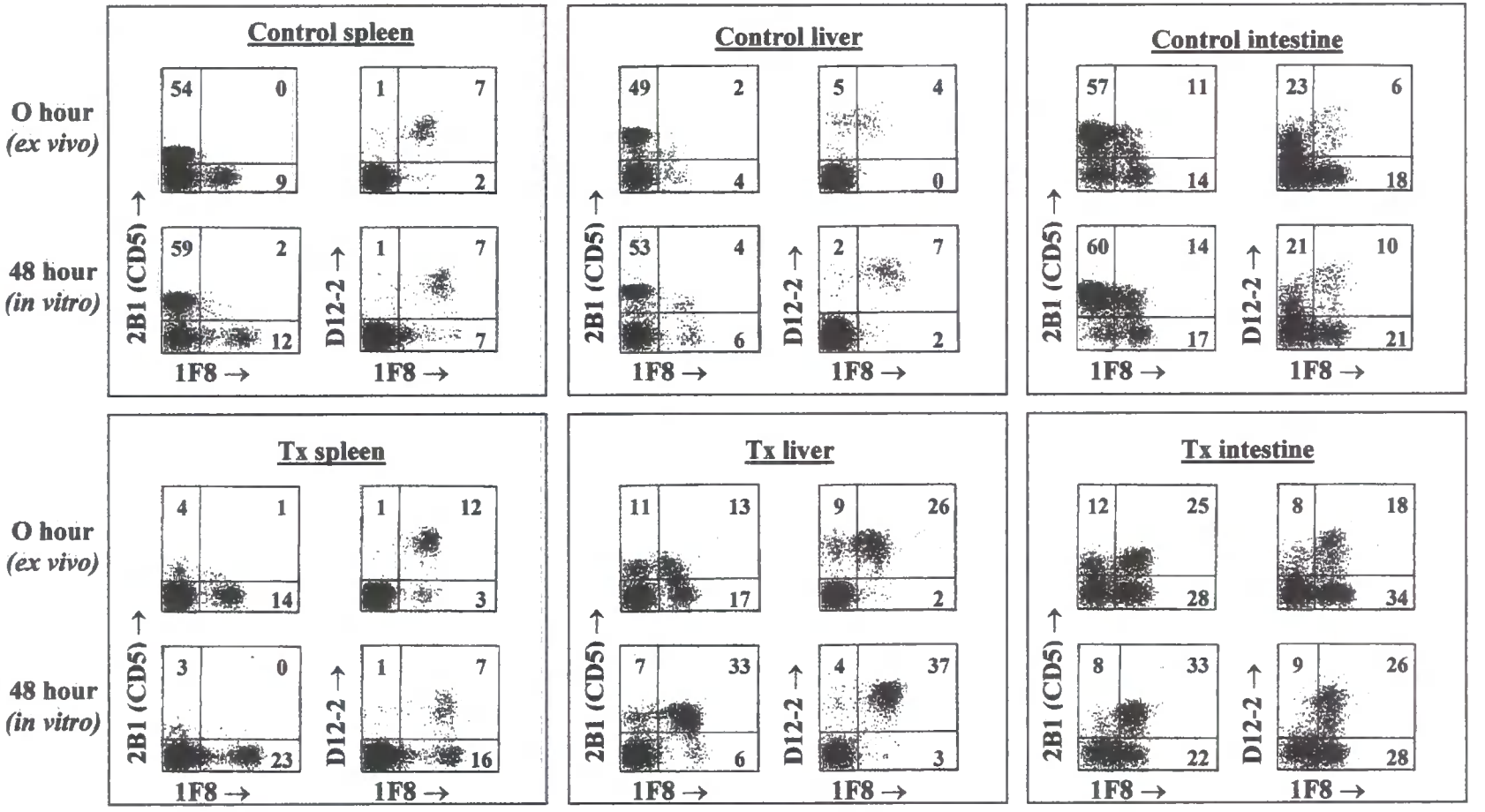
Typical data is shown representing ≥5 data sets; markers were set to exclude 98% cells stained with control reagents. "?" = "putative".

Fig. 2.3: The proportions of various lymphocyte populations in lymphoid tissues of control and Tx *Xenopus*



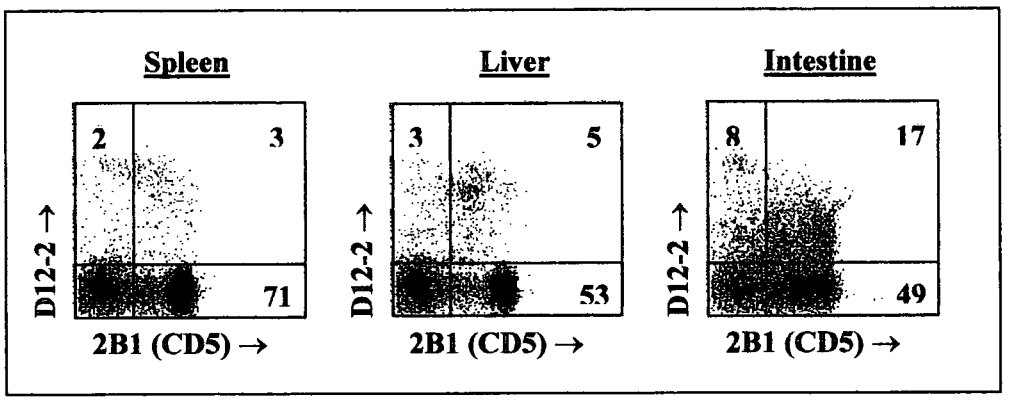
Graphs display mean percentage of antigen expression ± standard error where number of adult *Xenopus* analysed ≥5. Control cells were cultured in medium alone, Tx cells in GFM-supplemented medium.

Fig. 2.4: Dual colour flow cytometric analysis to show the co-staining properties of 1F8^{+ve} NK-like cells in spleen, liver and intestine from control and Tx *Xenopus ex vivo* (0 hour) and after 48 hours culture



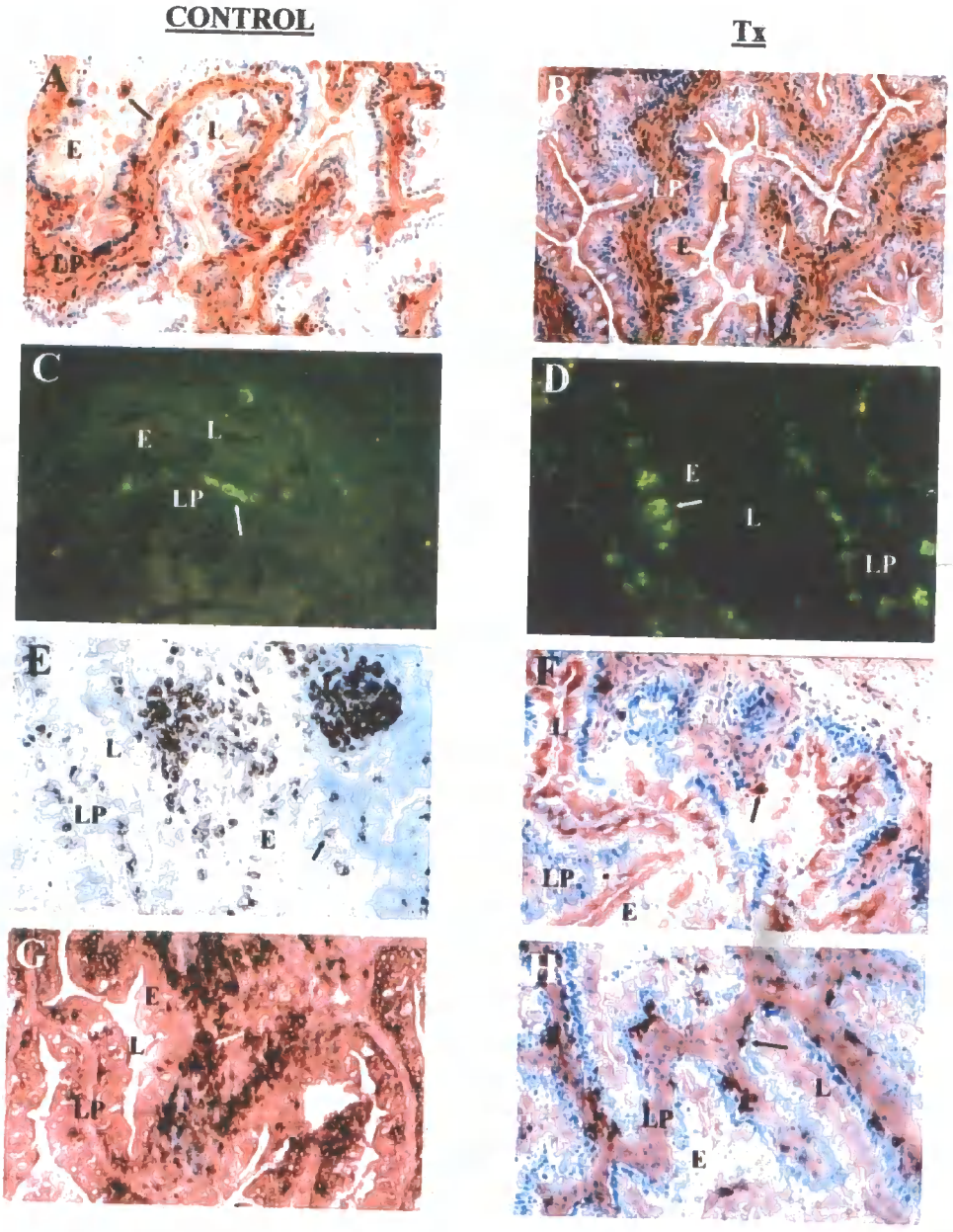
Typical data is shown representing ≥5 data sets; quadrants were set to exclude 98% cells stained with control reagents

Fig. 2.5: Dual colour flow cytometric analysis to characterise 2B1/D12-2 co-staining of lymphocytes of spleen, liver and intestine from control *Xenopus ex vivo*



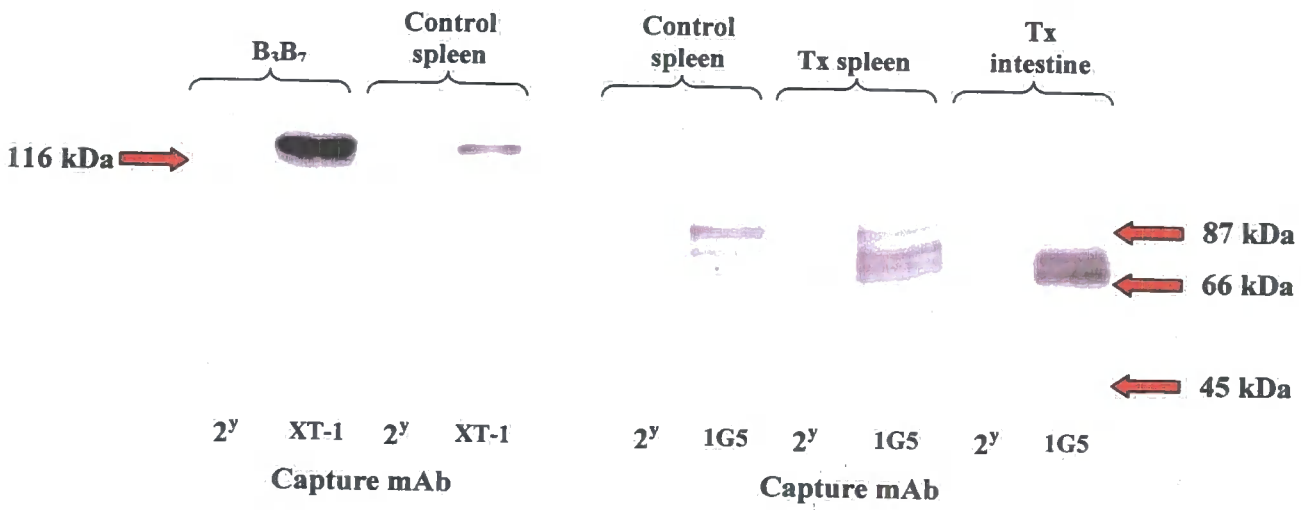
Typical data is shown representing ≥5 data sets; quadrants were set to exclude 98% cells stained with control reagents

Fig. 2.6: Frozen sections of control and Tx intestine showing immunoperoxidase and immunofluorescent staining using mAb's 1F8, AM22, 8E4 and D12-2



Cryostat sections (6-8um thick) of control and Tx intestine were stained using either immunoperoxidase **(A, B, E-H)** or immunofluorescence **(C, D)**. Frames show $1F8^{+ve}$ candidate NK cells **(A-D)**, $AM22^{+ve}$ putative $CD8\alpha^{+ve}$ cells **(E, F)**, $8E4^{+ve}$ B-cells **(G)** and $D12\text{-}2^{+ve}$ putative γδT-cells **(H)**. Sections incubated with CT3 control mAb failed to stain (data not shown). Scale: x125 **(A, B, E-H)**, x250 **(C, D)**. Key: **E** – epithelium, **LP** – lamina propria, **L** – lumen, **Arrow** indicates positively stained cell

Fig. 2.7: Western blots showing proteins detected by monoclonal antibodies XT-1 (α-T-cell) and 1G5 (α-NK cells) in lysates from B_3B_7 tumour cells, and spleen and intestine of control and Tx *Xenopus*



Each lane represents the lysate from $2.5x10^6$ cells. Blots were also probed with secondary antibody only (2^y)

CHAPTER 3

Phenotypic and functional characterisation of mAb-purified NK cells

3.1 Introduction

Previous functional studies on unsorted *Xenopus* splenocytes has suggested that NK-like cells exist in this amphibian. Specifically, splenocytes from control and Tx *Xenopus* adults display *in vitro* cytotoxicity against MHC-I deficient allogeneic thymus tumour target cells (Robert *et al.*, 1994; Horton *et al.*, 1998b), but not against MHC-I^{+ve} lymphoblasts (Horton *et al.*, 1996a). This cytotoxicity is enhanced in animals which have had prior injections of allogeneic tumour cells (Horton *et al.*, 1998b). Splenocyte populations from control and Tx animals frequently require *in vitro* culture in order to develop their cytotoxic potential, and Tx populations require *in vitro* supplementation with T-cell derived growth factors if they are to display cytolytic activity (Haynes and Cohen, 1993; Horton *et al.*, 1998b). Immunomagnetic sorting has enabled the cytotoxic potential towards tumour targets (MHC-I deficient) by various purified lymphocyte populations to be assessed. Purified B-cells from T-cell-deficient Tx splenocyte populations show no cytotoxic activity, even after culture in GFM, whereas the remaining non-T-non-B-cell fraction exhibits high levels of tumour target killing, implying the existence of an NK-like population (Horton *et al.*, 1998b).

In this chapter, flow cytometry and immunomagnetic sorting of cells labelled by the putative α-NK mAb 1F8 (see Chapter 2) are used to probe whether $1F8^{+ve}$ cells do indeed represent the NK population. The cytotoxic potential of NK-enriched lymphoid populations towards MHC-deficient tumour targets will be determined by use of two different label-release killing assays. Assays of cytotoxicity usually involve radioactive labelling of the cell with ^{51}Cr or ^{125}I to monitor the release of cytoplasmic molecules following disruption of the plasma membrane. However, relying upon the knowledge that most cells undergoing apoptosis will fragment their DNA, the "JAM" assay developed by Matzinger (1991), actually measures the amount of DNA retained by intact, healthy cells, providing a safer, faster, more economical and more sensitive method of measuring apoptosis induced by cytotoxic cells. The JAM technique for monitoring target cell cytotoxicity will be compared here with the ^{51}Cr-release assay method.

Further confirmation that NK cells kill by inducing apoptosis of target cells are addressed by examination of target cells for evidence of apoptotic changes. The target cells employed for cytotoxicity assays are the MHC-I/II-deficient (Robert *et al.*, 1994) B_3B_7 cells, a *Xenopus* thymus tumour cell line derived from the MHC-homozygous family *ff* (Du Pasquier and Robert, 1992; Robert *et al.*, 1994). In order to assess apoptosis of the B_3B_7 target cells following co-culture with effector cells, it is first necessary to isolate them from the effector/target mixture. This is achieved by immunomagnetic sorting using the monoclonal antibody X71 (Chretien *et al.*, 1996), which interacts with the novel IgSF member, CTX (cortical thymocyte-specific antigen of *Xenopus*, Robert *et al.*, 1997a; Robert and Cohen, 1998a). This molecule was first identified in *Xenopus* and is expressed homogeneously by the B_3B_7 cell line in addition to a high proportion of *Xenopus* cortical thymocytes, the amphibian equivalent of mammalian double positive ($CD4^+CD8^+$) immature thymocytes (Robert and Cohen, 1998a). CTX is not expressed by any peripheral lymphocyte population. It is a monomeric type-I transmembrane glycoprotein of 55kDa (Chretien *et al.*, 1996) and is comprised of two Ig domains (constant and variable), a transmembrane domain and a conserved cytoplasmic domain of 70 amino acids. It is developmentally regulated and shows similarities to both cell adhesion molecules and antigen-specific receptors (Chretien *et al.*, 1996). As CTX is expressed on such immature thymocyte populations, a role in thymocyte differentiation has been proposed (Robert and Cohen, 1998a). The possible involvement in control of the cell cycle has also been implied due to the discovery that CTX crosslinking results in the accumulation of B_3B_7 cells in the G2/M phase (Robert *et al.*, 1997a).

Two methods are used to assess target cell apoptosis. The first involves detection of apoptosis-specific protein (ASP). ASP was first identified in human Burkitt lymphoma cells and in adenovirus-transformed human and rat embryo cells through cross-reactivity with a polyclonal antibody raised against a synthetic peptide corresponding to a proportion of the *c-jun* sequence (a component of the AP-1 transcription factor and implicated in the regulation of apoptosis, Grand *et al.*, 1995). This polyclonal antibody has been shown to identify apoptosis-specific proteins in *Xenopus* (Horton *et al.*, 1998d) and also in a variety of mammalian and avian model

systems (Horton, JD and Jahoda, CAB, unpublished observations from the laboratory). The ASP system has proved to be an ideal immunohistological method for detecting programmed cell death in *Xenopus* (Horton *et al.*, 1998d).

The second method involves detection of phosphatidylserine (PS) molecules which are translocated from the inner leaflet of the plasma membrane to the surface of the cell once the apoptotic pathway has been initiated. These PS molecules can be detected by use of FITC-conjugated annexin-V proteins, which have a high affinity for phosphatidylserine. Although annexin-V-FITC was generated for use with mammalian apoptotic cells, we and others (Nera *et al.*, 2000) have established that adult *Xenopus* apoptotic splenocytes and thymocytes express PS, possibly by similar translocation mechanism, and may therefore may also be detected by annexin-V-FITC. Externalisation of PS also occurs following the onset of necrosis, and it is therefore necessary to assess membrane integrity in order to distinguish between the intact apoptotic cells and those whose plasma membranes have become "leaky" due to necrosis. This can be achieved by simultaneously performing a dye exclusion test with propidium iodide (PI), which labels DNA of non-permeablised cell populations and therefore discriminates between apoptotic and necrotic populations (Vermes *et al.*, 1995).

3.2 Methods

Further details of reagents are given in the appendices.

3.2.1 Extraction and preparation of lymphocytes

Lymphocytes from spleen, liver, intestine and thymus were extracted and prepared as described in section 2.2.1.

3.2.2 Culturing of lymphocytes

Lymphocytes were cultured as described in section 2.2.2.

3.2.3 Generation of growth factor-rich medium

Growth factor-rich medium was generated as described in section 2.2.3.

3.2.4 Flow cytometry

Flow cytometric analysis was carried out as described in section 2.2.4.

3.2.5 Cytospins

Cells to be investigated were extracted from the animal as previously described (see section 2.2.1) and centrifuged at 300g, 4°C for 10 minutes. Pellets were resuspended in APBS (see appendix 1) supplemented with 40%FCS (APBS/40%FCS) at a concentration of 1 x 10^6 cells/ml and stored on ice until required. Slides were cleaned with 70% alcohol, loaded into the cytospin (Shandon Southern) and pre-wetted with 10-15µl APBS/40%FCS. 100µl of cell suspension (i.e. 1 x 10^5 cells) was pipetted into each centrifuge chamber and spun at 600rpm for 5 minutes. The slides were carefully

removed and allowed to air dry for several minutes before fixing in either acetone for 5 minutes (at room temperature) or methanol for 10 minutes (at -20°C); the slides were stored at -80°C until required.

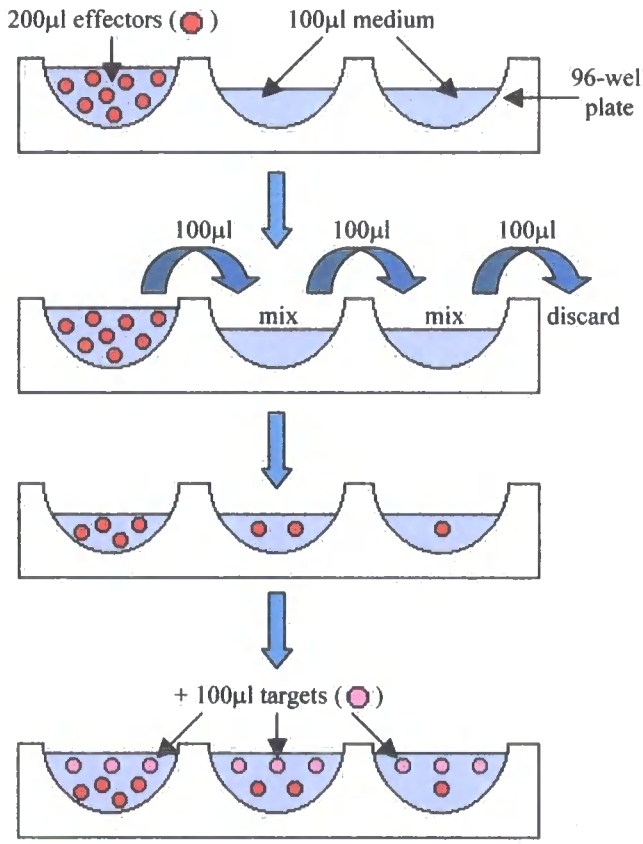
3.2.6 Immunomagnetic sorting

The cells required for separation were washed in amphibian strength HBSS supplemented with 1%FCS (HBSS/1%FCS) prior to incubating for 20 minutes on ice with 600µl of the required mouse mAb supernatant. (NB when separating $>5x10^6$ cells, the suspension was divided into 2 for this incubation and subsequent wash steps). 1ml HBSS/1%FCS was then added to the cells which were centrifuged at 300g, 4°C for 10 minutes. This wash step was then repeated using HBSS/1%FCS containing goat serum (Sigma) (at 1:100). The supernatant was removed and the cells resuspended in 80µl HBSS/1%FCS. 20µl goat anti-mouse IgG (or IgM if appropriate) MACS microbeads (Miltenyi Biotec) were added to the suspension and incubated for 15 minutes at 4°C. The cells were then washed twice and resuspended in 500µl HBSS/1%FCS. The magnetic unit (Miltenyi Biotec) was assembled and a column pre-wetted with 500µl HBSS/1%FCS. The 500µl cell suspension was pipetted into the magnetised column (Miltenyi Biotec), followed by a further 500µl HBSS/1%FCS. This eluate, containing the "non-adherent" cells, was collected. 500µl HBSS/1%FCS was then flushed through the column and the eluate discarded. The column was then removed from the magnet, 1ml HBSS/1%FCS added, and the "adherent" cells flushed through using the plunger. To ensure the efficiency of the separation, a small aliquot from each population was removed for incubation with FITC-conjugated goat anti-mouse IgG (DAKO) and checked for presence of surface-bound mouse mAb using a Coulter XL flow cytometer.

3.2.7 Cytotoxicity assays

3.2.7.1 ^{51}Cr-release cytotoxicity assays

$1x10^6$/ml B_3B_7 tumour target cells were labelled overnight with 100μCi/ml $Na_2[^{51}Cr]O_4$ (Amersham, 350-600mCi/mgCr) at 27°C, 5% CO_2. The cells were then washed three times at 300g, 4°C for 10 minutes in B_3B_7 medium (see appendix 1) and adjusted to $5x10^5$ cells/ml. The lymphocyte population to be used as effectors were pipetted in serial dilution into a 96-well plate (Greiner), each well containing 100μl effector cells. 100μl of the ^{51}Cr-labelled B_3B_7 cells ($5x10^4$ cells) was then added into each well, therefore generating samples of differing effector to target (E:T) ratios.



The plate was centrifuged at 300g, 4°C for 2 minutes to bring the effector and target cells together and placed in a 27°C, 5% CO_2 incubator for 6 hours. Three wells were

set aside for minimum ^{51}Cr release (100µl B_3B_7 cells cultured in medium alone) and three for maximum ^{51}Cr release (100µl B_3B_7 cells with 100µl ddH_20 freeze/thawed three times). After the 6 hour incubation, the cells in each well and tube were pipetted gently to ensure an even distribution of ^{51}Cr, and then centrifuged at 300g, 4°C for 10 minutes. 100µl supernatant from each well was transferred to scintillation vials and prepared for counting by the addition of 3ml Ecoscint (National Diagnostics). The samples were shaken and amount of radioactivity established using a Packard Tri-carb analyser. The percentage of killing was calculated as follows :-

(E-C) / (M-C) x 100 where E = experimental value (B_3B_7 cells cultured for 6 hours with effectors)
C = minimum release value
M = maximum release value

3.2.7.2 JAM assays

This method was adapted from the JAM assay described by Matzinger (1991).

$1x10^6$/ml B_3B_7 tumour target cells were labelled overnight with 5µCi/ml ^3HTdR (specific activity = 5Ci/mmol) at 27°C, 5% CO_2, prior to washing, as above. The lymphocyte population to be used as effectors were pipetted in serial dilution into a 96-well plate (Greiner), and $1x10^4$ B_3B_7 target cells were added to each well, generating samples of differing effector to target (E:T) ratios. ^3H counts from $1x10^4$ B_3B_7 targets alone were monitored at 0 hour and again at 6 hours to obtain total ^3HT incorporated and natural ^3HT loss at 6 hours respectively. Additionally, red blood cells (RBC's) at appropriate effector to target ratios were also placed in culture with B_3B_7 cells to act as a control to calculate the % DNA loss caused by a "non-cytotoxic" population. The plate was centrifuged at 300g, 4°C for 2 minutes to bring the cells together and placed into a 27°C, 5% CO_2 incubator for 6 hours. Following incubation, the cells were aspirated onto filter paper (Whatman) using a cell harvester (Skatron), the fragmented DNA of dead cells being washed through and discarded. Filter papers were dried, transferred to scintillation vials and prepared for counting by the addition of 3ml Ecoscint (National Diagnostics). The amount of radioactivity was

established using a Packard Tri-carb analyser. The radioactivity remaining represents the intact DNA left behind on the filter and corresponds to the number of living cells in the assay. % DNA loss was calculated as follows :-

(T-E)-(T-C) / T-(T-C) x 100 where E = experimental value (B_3B_7 cells cultured for 6 hours with effectors)
T = counts from B_3B_7 cells cultured alone at 0 hour
C = counts from B_3B_7 cells cultured for 6 hours with medium

3.2.8 Apoptosis assays

3.2.8.1 Annexin-V-FITC

$0.5x10^6$ B_3B_7 tumour target cells were washed, resuspended in 500µl B_3B_7 medium and transferred to a 5ml centrifuge tube (Greiner). Effector cells were added to the target cells at the appropriate concentration generating the required effector to target (E:T) ratio. The tube was centrifuged at 300g, 4°C for 2 minutes to bring the cells together and placed into a 27°C, 5% CO_2 incubator for 6 hours. A control tube was set up in which B_3B_7 cells were cultured alone in medium. Following incubation, the α-CTX monoclonal antibody, X71 was employed to immunomagnetically isolate the B_3B_7 target cells (see section 3.2.6). (The B_3B_7 population cultured alone was also immunomagnetically sorted using X71). The target cells were then centrifuged at 300g, 4°C for 10 minutes and resuspended in 150µl amphibian-strength binding buffer (Sigma). 3µl Annexin-V-FITC (Sigma) was added and the tubes incubated for 10 minutes in the dark. Samples were washed twice and resuspended in 500µl FACS medium (see appendix 1). 10µl of 0.25mg/ml propidium iodide (Sigma) was added to each sample immediately prior to flow cytometric analysis to discriminate necrotic from apoptotic populations.

3.2.8.2 ASP-staining

$0.5x10^6$ B_3B_7 tumour target cells were washed, resuspended in 500µl B_3B_7 medium (see appendix 1) and transferred to a 5ml centrifuge tube (Greiner). Effector cells were added to the target cells at the appropriate concentration generating the required effector to target (E:T) ratio. The tube was centrifuged at 300g, 4°C for 2 minutes to bring the cells together and placed into a 27°C, 5% CO_2 incubator for 6 hours. A control tube was set up in which B_3B_7 cells were cultured alone in medium. Following incubation, the α-CTX monoclonal antibody, X71 was employed to immunomagnetically isolate the B_3B_7 target cells (see section 3.2.6). (The B_3B_7 population cultured alone was also immunomagnetically "sorted" using X71). Cytospins of the target cells were then prepared as described in section 3.2.5. The slides were incubated at room temperature for 30 minutes in 1-2ml blocking buffer (see appendix 1) prior to careful drying and incubation for 30 minutes with 50µl of rabbit α-ASP antibody (SC-45 α-c-jun/Ap-1 – Santa Cruz Biotechnology), diluted to a concentration of 1:70 using diluting buffer (see appendix 1). The slides were washed 3 times in wash buffer (see appendix 1), carefully dried, and incubated for 45 minutes with 50µl of the secondary antibody (goat-anti rabbit IgG FITC) (Sigma) at a concentration of 1:70 (diluted using wash buffer). Again, the slides were washed 3 times in wash buffer prior to incubation for 2 minutes with propidium iodide (Sigma) (0.25mg/ml, diluted 1:80 using wash buffer). The slides were then washed again briefly, carefully dried, and mounted in PBS/glycerol (Citifluor) for examination under a Nikon optiphot fluorescence microscope.

3.2.9 Wright-Giemsa staining

Cytospins were prepared as described in section 3.2.5. Wright-Giemsa stain (see appendix 1) was pipetted onto the slide and incubated for 5-10 minutes. Slides were washed twice in distilled water, dehydrated through 2 changes of isopropanol followed by two changes of xylene and mounted in DPX for examination by a Nikon optiphot microscope.

3.3 Results

3.3.1 Morphology of $1F8^{+ve}$ cells

Fig. 3.1 shows typical data of MACS immunomagnetic sorting of 1F8 lymphocytes from Tx *Xenopus* spleen. Following the separation procedure, the 1F8-enriched population routinely contains ≈90% $1F8^{+ve}$ cells, the 1F8-depleted population containing <4% $1F8^{+ve}$ cells. Enrichment from control animals is routinely less successful, on average yielding ≈70-80% $1F8^{+ve}$ cells (data not shown).

The 1F8-enriched population in *Fig. 3.2*, isolated by immunomagnetic sorting, are large granular lymphocytes (12μm) with noticeable pseudopodia, whereas the 1F8-depleted population contains a diverse array of cells such as basophils, thrombocytes, macrophages and erythrocytes.

3.3.2 Dual colour flow cytometric analysis of 1F8-enriched splenocyte populations

Co-staining of 1F8-enriched splenocytes from Tx *Xenopus* with various mAb's is illustrated in *Fig. 3.3*. Some 24% $1F8^{+ve}$ cells co-express CD5, although fluorescence intensity is low compared with T-cells (see Chapter 2). These cells are therefore termed $CD5^{lo}$. $1F8^{+ve}$ cells do not express surface IgM (as determined by D8) or putative CD8β (as determined by F17), whereas the majority (83%) co-stain with the putative α-γδTCR monoclonal antibody, D12-2. Approximately 50% purified $1F8^{+ve}$ splenocytes express AM22 (putative α-CD8α), although 1F8/AM22 staining is not usually seen on unpurified splenocytes (data not shown).

3.3.3 Cytotoxicity assays

3.3.3.1 Chromium-release assays

Splenocytes from control (thymus intact) *Xenopus* were examined first *(Fig. 3.4)*. Unsorted splenocytes cause significant ^{51}Cr-release from the B_3B_7 target cells (25% at 30:1), 1F8-depleted cells displayed negligible cytotoxicity (1% at 30:1), whereas the 1F8-enriched population contained effective killers (35% killing at 15:1). Thymocytes display no cytotoxicity, the level of killing of B_3B_7 targets remaining at 0% throughout the effector to target ratios tested.

In a second series of experiments, the cytotoxic potential of lymphocytes from Tx spleen, liver and intestine was compared *(Fig. 3.5)*. The level of killing displayed by unsorted splenocytes from Tx animals (42% at 30:1) was higher than that shown by control splenocytes. Unsorted liver lymphocytes from Tx *Xenopus* were also effective killers, causing 35% killing at 30:1. As extraction of IEL's is accompanied by large numbers of epithelial cells, it was not possible to determine accurate E:T ratios using unsorted IEL populations. The need for T-cell-derived factors (GFM) in Tx cells cultures was emphasized by the drop in cytotoxicity of unsorted splenocytes and hepatic lymphocytes (to 13% and 2% respectively at 30:1) when cultured in GFM-free medium. $1F8^{+ve}$ cells from all three organs proved to be extremely cytotoxic towards the MHC-I-deficient B_3B_7 tumour target cells, (50% spleen, 46% liver, 38% intestine at 15:1). 1F8-depleted populations of Tx spleen and liver cells failed to cause ^{51}Cr-release (6% and 0% respectively at 30:1). $1F8^{-ve}$ IEL's however showed a small level of killing (17% at 30:1).

3.3.3.2 JAM assays

As *Fig. 3.6* illustrates, unsorted control splenocytes cultured in T-cell factor-free medium effected moderate DNA loss (25% at 30:1); a similar level was obtained (23%) when the same cells were cultured in GFM. Unsorted Tx splenocytes (routinely cultured in GFM) caused higher DNA loss (37% at 30:1) in comparison

with unsorted control splenocytes. The $1F8^{+ve}$ Tx population was extremely effective, causing 52% DNA loss at 30:1, whilst Tx $1F8^{-ve}$ lymphocytes were poor at causing DNA fragmentation (10% DNA loss at 30:1). Thymocytes did not cause DNA loss from B_3B_7 target cells, even at the highest E:T ratio of 30:1.

3.3.4 Apoptosis assays

3.3.4.1 Annexin/PI

Fig. 3.7 is a diagrammatic representation of flow cytometric data of tumour cells stained with annexin-V-FITC and PI. Area 4 of the flow cytometric trace represents apoptosing target cells which are externally expressing PS (annexin-V-$FITC^{+ve}$), but whose membranes are intact thereby preventing PI staining. Following further co-culture with an effector population, these target cells begin to lose membrane integrity, resulting in PI staining; the target cells are now located in area 2 of the flow cytometric trace and are termed secondary necrotic. The target cells located in area 1 are those whose membranes have become permeable through physical damage to the cell (necrosis) and are thereby PI^{+ve}. These cells have not been killed by a specific mechanism (i.e. apoptosis) and are therefore annexin-V^{-ve}. Viable cells staining negative for both annexin and PI are located in area 3.

As *Fig. 3.8* illustrates, B_3B_7 tumour target cells when cultured alone and treated with the X71 mAb, showed no signs of annexin-V binding (2%), with only 2% of target cells located in gate B. Following 6 hour co-culture with unseparated control splenocytes and unseparated Tx splenocytes (the latter cultured in GFM) at effector:target ratios of 5:1, 8% of X71-separated target cells falling in gate A (intact cells) were externally expressing PS in each case. Only 1% of target cells cultured with unseparated Tx splenocytes cultured without GFM stained positive with annexin-V-FITC. *Fig. 3.9* demonstrates that 1F8-depleted and 2B1-enriched populations had little effect on the target cells in gate A at the same E:T ratio, (only 1% and 2% were annexin-V^{+ve} respectively). The 1F8-enriched population proved to be extremely

effective at 5:1 and caused 32% of the intact target population to become stained positive for annexin-V-FITC.

3.3.4.2 ASP

Similar results were obtained from ASP detection studies (*Table 3.1* and *Fig. 3.10).* Target cells (treated with X71 mAb) cultured in medium alone showed minimal apoptosis, only 1% staining positive for ASP. The 1F8-depleted population caused a negligible levels (3%) of target cells to become ASP^{+ve} following 6 hour co-culture (at an E:T ratio of 5:1), whereas the 1F8-enriched populations were extremely effective and induced ASP expression in 30% of the target cells (also at 5:1).

3.4 Discussion

Immunomagnetic sorting has proved to be an effective technique for the isolation and subsequent phenotypic and functional analysis of *Xenopus* $1F8^{+ve}$ lymphocytes.

3.4.1 Phenotype of $1F8^{+ve}$ cells

Histological data shows candidate *Xenopus* NK cells to be large lymphocytes, 12μm in diameter with distinct pseudopodia. Although unsorted $1F8^{+ve}$ splenocytes do not constitutively express the pan T-cell marker CD5 (*Fig. 2.4*), 1F8-enriched cells show a degree of 2B1(α-CD5) co-staining, albeit of low intensity ($CD5^{lo}$). $1F8^{+ve}$ splenocytes do not express surface IgM (and are therefore not of B-cell lineage) and routinely fail to stain with F17, the putative α-CD8β mAb). On purification, over half become $AM22^{lo}$ (the putative α-CD8α mAb) (data not shown), which is perhaps not surprising given that subpopulations of both avian and mammalian NK cells are reported to be $CD8\alpha^{+ve}CD8\beta^{-ve}$ (Gobel *et al.*, 1994).

Despite low level CD5 and putative CD8α expression, recent experimental evidence strongly supports the notion that *Xenopus* $1F8^{+ve}$ cells are a population distinct from T-cells. Rau et al (2002) have shown that there is no transcription of TCRβV regions in $1F8^{+ve}$ (or $1F8^{-ve}$) cells from Tx frogs, whereas such transcripts are regularly found in $CD5^{+ve}$, but not $CD5^{-ve}$ cells from control frogs. TCRβ constant region mRNA can be found at very low levels following thymectomy, but two log decades more cells are required to obtain positive results in comparison to studies on control cells (Horton *et al.*, 1998c).

3.4.2 Cytotoxic potential of $1F8^{+ve}$ cells

As shown by both ^{51}Cr-release and JAM assays, unsorted control splenocytes display a significant level of cytotoxicity towards MHC-deficient tumour target cells following 6 hours co-culture. Culture of control splenocytes in GFM fails to enhance

cytotoxic potential, presumably as T-cells are already present in the culture and further supplementation with T-cell-derived factors is not required. Both assays affirmed unsorted Tx splenocytes to be effective killers, more so than their counterparts from control animals. Tx cells however, need to be cultured in GFM, a requirement which is emphasized with the observation that the killing capacity of both Tx spleen and liver lymphoid populations fall dramatically when these cells are cultured in medium alone, without T-cell-derived growth factor supplements (as noted previously, Horton *et al.*, 1998b). GFM-dependency for cytotoxicity is suggestive that the killing observed with Tx cells is lymphokine-activated killing (LAK). Unfortunately, as IEL preparations are routinely and unavoidably contaminated with intestinal epithelial cells, unsorted IEL's could not be used as an effector population. Significant cytotoxicity would nevertheless be expected from unsorted *Xenopus* IEL's given that cytolytic activity by intestinal granular lymphoid populations is observed in other models such as the mouse, (Tagliabue *et al.*, 1981; Petit *et al.*, 1985), man (Chiba *et al.*, 1981; MacDermott *et al.*, 1986) and guinea pig (reviewed in Tagliabue *et al.*, 1982). It would also have been of interest to investigate the cytotoxic potential of *Xenopus* lamina propria lymphocytes (LPL's) as this subset has been shown to be cytotoxic in mice (Tagliabue *et al.*, 1982). However, isolation of these cells involves the use of trypsin, which can strip cells of surface markers (Dr. M. Bailey, University of Bristol, pers. comm.), a fact which may explain the lack of LPL cytotoxicity in the guinea pig.

In order to determine the cytotoxicity of specific lymphocyte populations, immunomagnetic sorting was employed to generate 1F8-depleted and 1F8-enriched Tx populations. The 1F8-enriched populations contain the candidate NK cells, whereas the 1F8-depleted populations include B-cells, macrophages, erythrocytes and thrombocytes. As both populations are derived from Tx animals and therefore lack T-cells, each was routinely cultured in GFM. Both ^{51}Cr-release and DNA loss assays show that in spleen, liver and intestine, the 1F8-enriched population contains a high proportion of effectors able to kill B_3B_7 targets. As these cells are derived from Tx animals, killing cannot be attributed to cytotoxic T-cells. Incubation of 1F8-enriched cells with the 1F8 mAb either 1 hour prior to or during the 6 hour co-culture period, does not block or enhance the killing activity of $1F8^{+ve}$ cells (data not shown)

implying the 1F8 antigen is not directly involved in killing. Previous studies have shown that cytotoxicity attributed to $1F8^{+ve}$ cells appears to be restricted to MHC-deficient cells; 1F8-enriched populations of *Xenopus* routinely fail to lyse allogenic $MHC\text{-}I^{+ve}$ splenic lymphoblasts (Horton *et al.*, 1996a).

Both JAM and ^{51}Cr-release assays establish that the 1F8-depleted population (containing B-cells, macrophages, erythrocytes etc) in spleen and liver are not cytotoxic towards the B_3B_7 target cells. This concurs with previous studies which show that B-cell-enriched populations display no cytotoxicity towards tumour target cells, with or without T-cell-derived factors supplementing the culture medium (Horton *et al.*, 1998b). Unlike 1F8-depleted populations of spleen and liver, $1F8^{-ve}$ cells of the intestine do display some cytotoxicity towards the target cells. This is most probably attributable to the presence of contaminating large epithelial cells within the $1F8^{-ve}$ population. Co-culture with cells of this size would be detrimental to the health of the target cells and it is therefore probable that ^{51}Cr-release from B_3B_7 cells is not a result of specific cytotoxic activity, but merely due to less than optimal culture conditions.

Mechanism of killing

Having shown that $1F8^{+ve}$ cells display spontaneous cytolytic activity towards allogeneic MHC-deficient tumour target cells, it was of interest to determine the mechanism by which this occurred. Whether $1F8^{+ve}$ cells destroy their targets by inducing apoptosis was assessed using annexin-V to detect externalization of PS, an early sign of apoptosis, and α-ASP antibodies to identify the presence of cytoplasmic apoptosis-specific proteins (ASP's).

ASP was first identified in mammalian cells through cross-reactivity with a polyclonal antibody raised against the nuclear 39kDa c-Jun protein (Rauscher *et al.*, 1988), which detects a 45kDa cytoplasmic protein, whose expression levels were found to increase dramatically following initiation of apoptosis (Grand *et al.*, 1995). Although up-regulated during apoptosis (Colotta *et al.*, 1992), *c-jun* is not thought to be over-expressed to the levels as detected by the α-c-Jun antibody (Grand *et al.*,

1995). Such size, location and expression discrepancies led to the conclusion that the polyclonal antibody was detecting a novel protein unrelated in any way to transcription factors, and which is specific to cells displaying the classic symptoms of apoptosis e.g. DNA fragmentation. Levels of ASP are dramatically increased immediately prior to an irreversible point in the apoptotic pathway. ASP expression then persists throughout the process, even when DNA can no longer be detected (Grand *et al.*, 1995). ASP expression is also inhibited by IFN-α (an ASP-downregulator, Milner *et al.*, 1993) and by the apoptosis-suppressor protein Bcl-2, which actually blocks ASP, implying that Bcl-2 acts on the apoptotic pathway prior to the onset of ASP expression (Grand *et al.*, 1995). The role of ASP during apoptosis has been difficult to determine precisely. It has been suggested that the protein may function as a protease, similar to human ICE, although a more likely role for ASP would be as a stabilizer protein, maintaining the cytoskeleton and membrane integrity of the apoptotic cell before it is phagocytosed. Evidence in support of this comes from immunofluorescence, which demonstrates a high degree of association between ASP and non-muscle-β-actin (Grand *et al.*, 1995). It is due to this association that ASP is so difficult to purify. The protein cannot be extracted by non-ionic detergents; biochemical isolation is only possible once the cytoskeleton has been solubilised by urea, SDS or guanidine hydrochloride (Grand *et al.*, 1995). Throughout evolution, apoptosis has remained a conserved process and it is therefore not surprising that the mammalian α-ASP antibody also identifies *Xenopus* ASP's.

As described previously in the Introduction, once the apoptotic pathway has been initiated, phosphatidylserine (PS) molecules are translocated from the inner leaflet of the plasma membrane to the surface of the cell, triggering recognition by macrophages, resulting in the subsequent removal of the dying cell (Fadok *et al.*, 1992b; Savill *et al.*, 1993; Castedo *et al.*, 1996). PS is therefore ubiquitously expressed on apoptotic cells and is a more reliable hallmark of apoptosis than characteristics such as DNA fragmentation (Zhang *et al.*, 1997). Annexin-V is a phospholipid binding protein (Van Heerde *et al.*, 1995), which, in the presence of calcium ions, has a specific and high affinity for PS (Pigault *et al.*, 1994; Trotter *et al.*, 1995; Vermes *et al.*, 1995). Therefore, when conjugated to fluorescein, annexin-

V enables flow cytometric analysis of cells in the early stages of apoptosis (Fadok *et al.*, 1992b; Dachary-Prigent *et al.*, 1993; Zhang *et al.*, 1997).

Both PS and ASP-detection methods show that B_3B_7 target cells (isolated using the X71 mAb) cultured alone in medium remain healthy and intact throughout the 6 hour culture period, with [2% cells staining positive for either ASP or annexin-V. Annexin-V assays reveal that unsorted control splenocytes and GFM-cultured Tx splenocytes cause low, but significant numbers of morphologically intact target cells in gate A to become apoptotic at effector:target ratios of 5:1, although again, the need for T-cell-derived factors in Tx cell cultures is apparent, as levels of apoptotic targets fall to negligible levels following 6 hour incubation with Tx cells cultured in medium alone. Both assays concur that the 1F8-depleted population from Tx frogs (containing B-cells, macrophages, erythrocytes etc) does not induce significant levels of apoptosis in target cells at 5:1, or indeed at higher E:T ratios (data not shown). $2B1^{+ve}$ T-cells also fail to induce apoptosis, although previous ^{51}Cr-release assays have shown that CD5-enriched populations may exhibit cytolytic activity against MHC-deficient target cells, but surprisingly, only when cultured in GFM (Horton *et al.*, 1998b). The 1F8-enriched population, presumed to contain candidate NK cells, is extremely effective at inducing target cell apoptosis, with approximately one third of the intact (gate A) target cell population becoming apoptotic in just 6 hours at 5:1. Following extended co-culture periods of 24 hours, a high proportion of the target cell population falling within gate A become both annexin-V^{+ve} and PI^{+ve} (data not shown) and are termed secondary necrotic *(Fig. 3.7)*. Similar results are obtained at even lower E:T ratios of 2:1 and even 1:1 as established by annexin-V detection assays (data not shown).

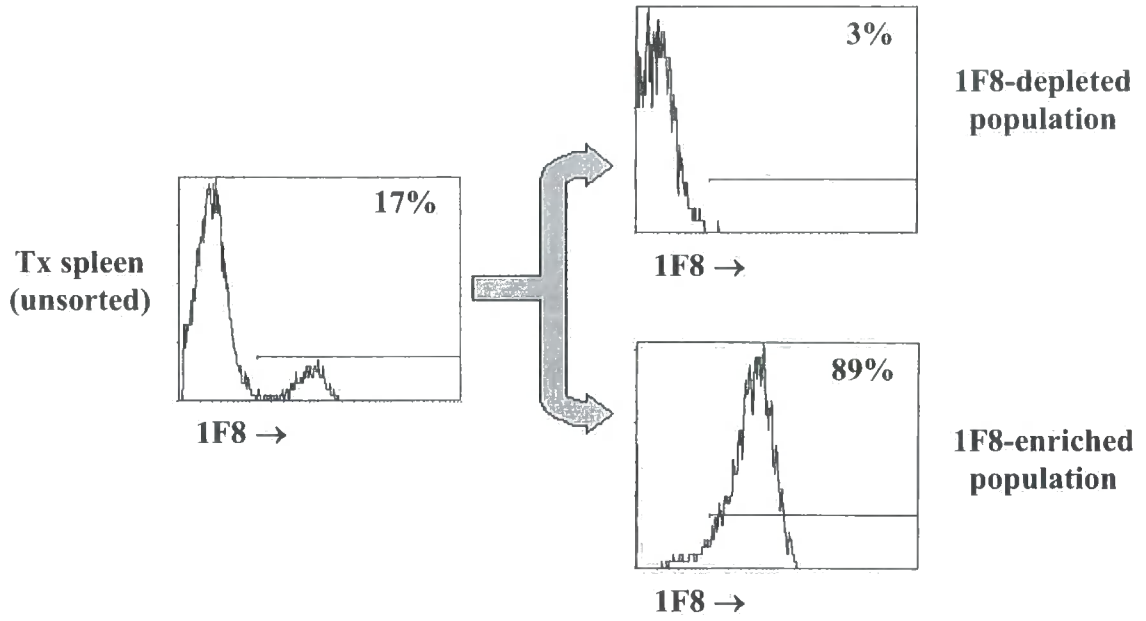
To summarize, the 1F8 mAb identifies a population of large lymphocytes, which lack surface IgM, TCRβV region mRNA and "normal" expression of CD5. These cells display spontaneous cytolytic activity towards MHC-deficient tumour target cells, by inducing target cell apoptosis. The conclusion is therefore drawn that 1F8 mAb identifies an NK-like population present in both control and Tx *Xenopus*.

Table 3.1: Percentage of B_3B_7 target cells staining positive for ASP following 6 hour culture in medium alone, with 1F8-depleted populations or with 1F8-enriched populations.

Target cells cultured with	% ASP^{+ve} cells
Medium	1 ± 0.29
$1F8^{-ve}$ cells	3 ± 1.2
$1F8^{+ve}$ cells	30 ± 4.1

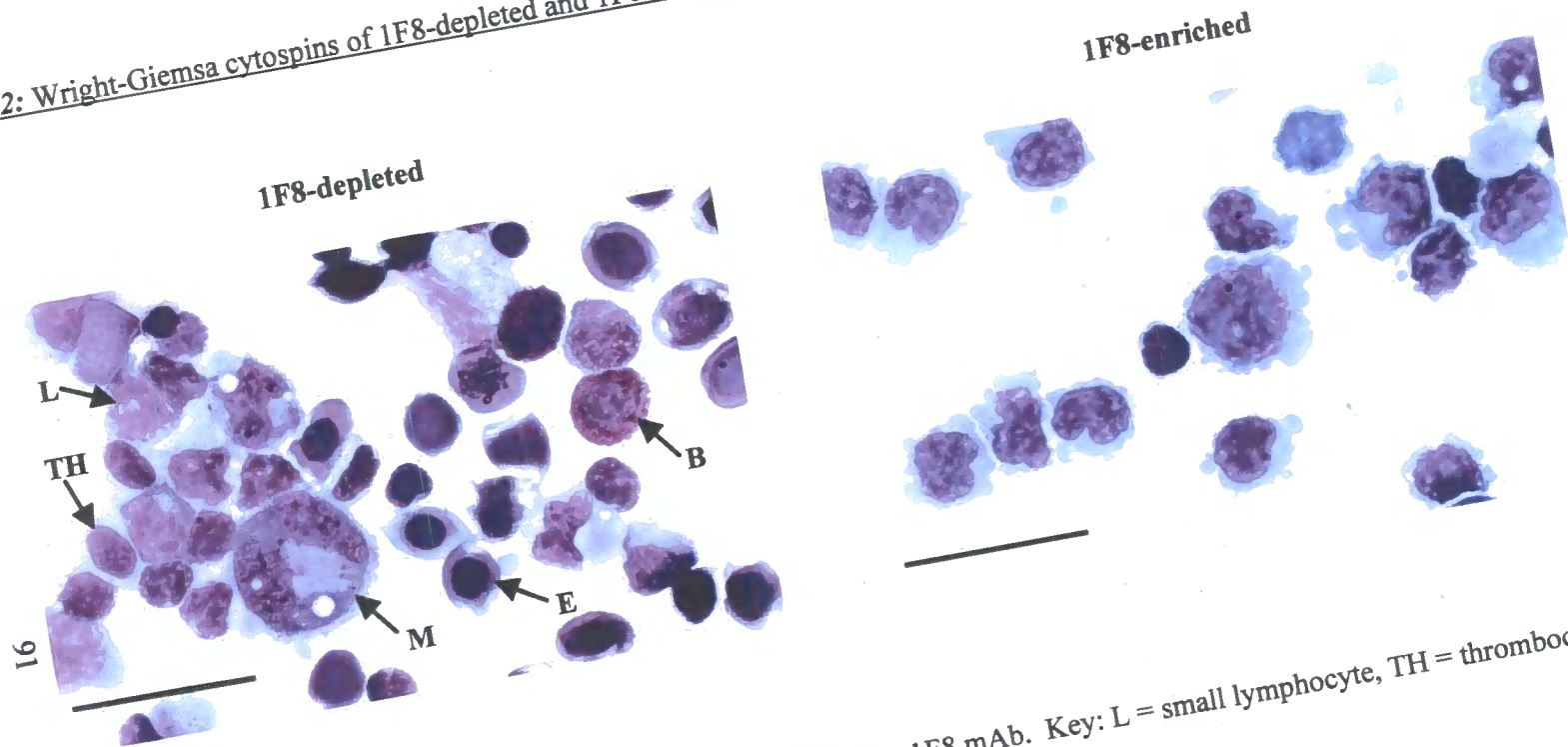
Mean % ± standard error where number of adult *Xenopus* analysed =3. All populations were cultured at E:T ratios of 5:1.

Fig 3.1: Single colour flow cytometric data showing MACS immunomagnetic isolation of splenic $1F8^{+ve}$ cells



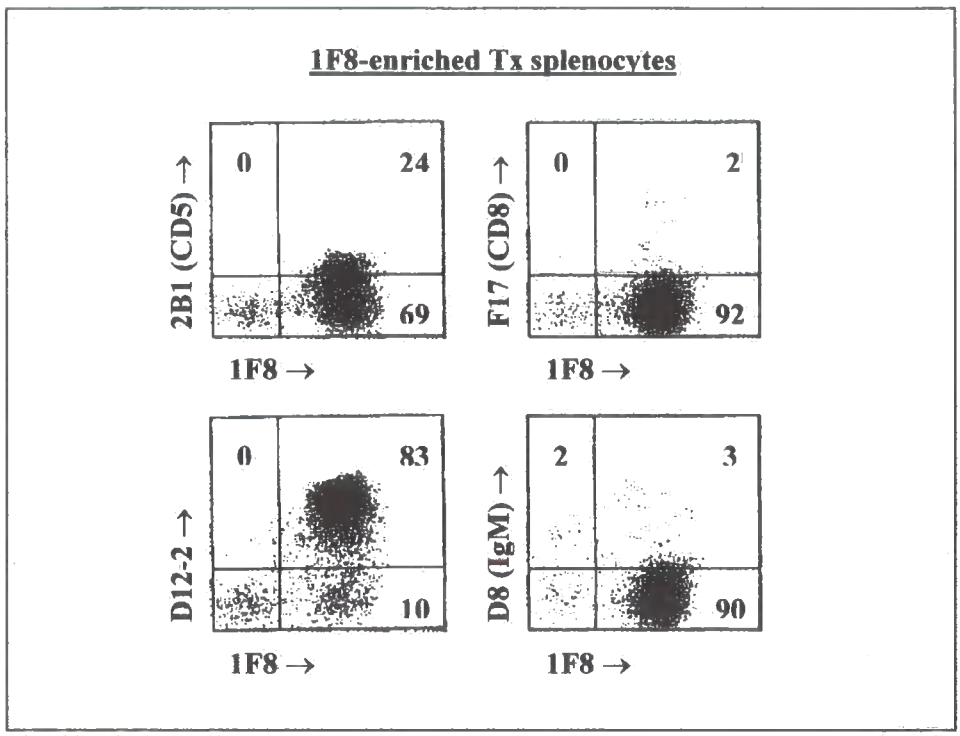
Typical data is shown representing ≥5 data sets; markers were set to exclude 98% cells stained with control reagents

Fig 3.2: Wright-Giemsa cytospins of 1F8-depleted and 1F8-enriched splenic lymphocytes



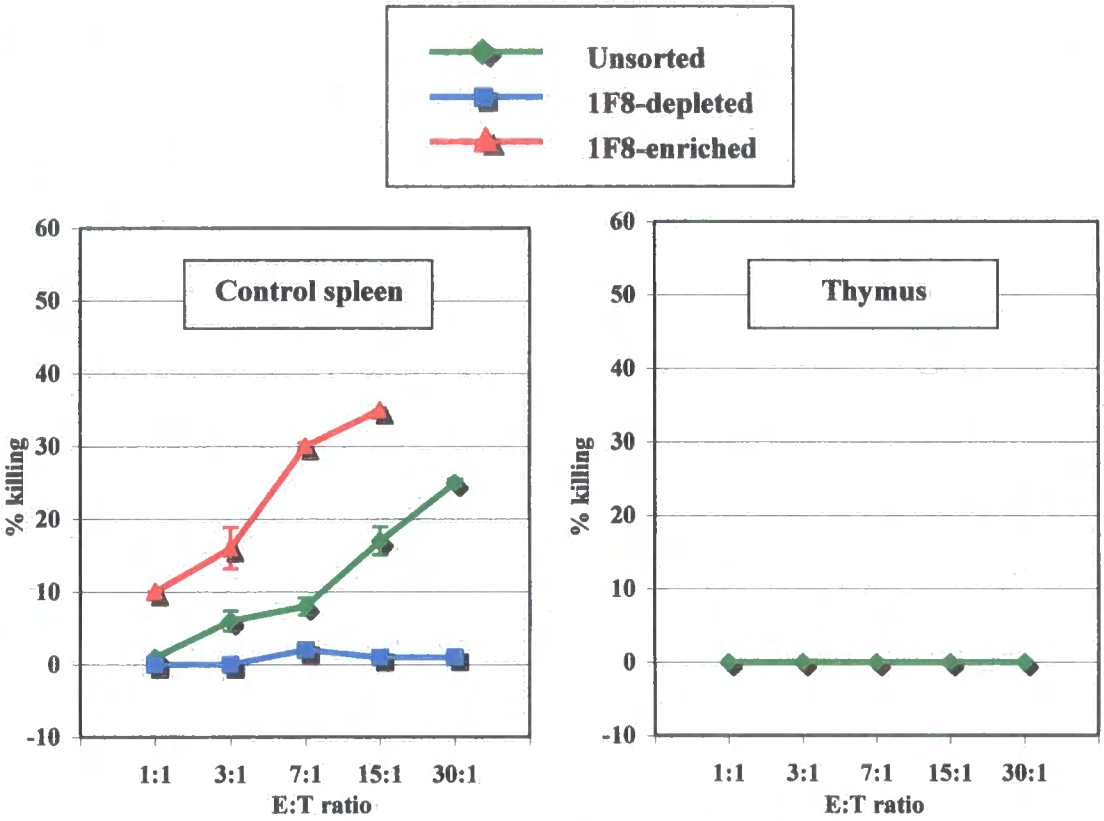
Cells were immunomagnetically sorted (see section 3.2.6) using the 1F8 mAb. Key: L = small lymphocyte, TH = thrombocyte, M = macrophage, B = basophil, E = erythrocyte. Bar = 25μm

Fig. 3.3: Dual colour flow cytometric analysis of 1F8-enriched Tx splenocytes of *Xenopus*



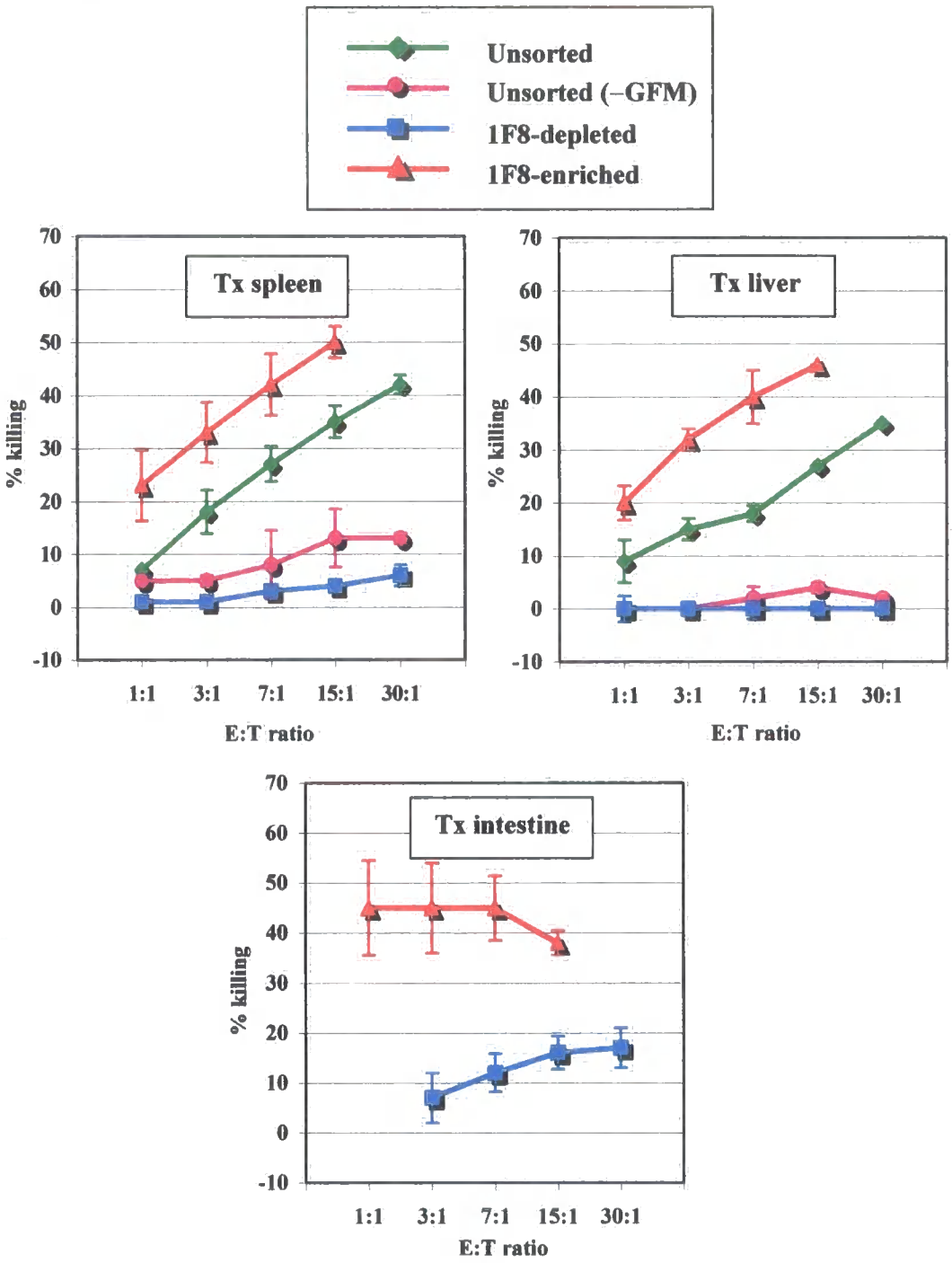
Typical data is shown representing ≥5 data sets; quadrants were set to exclude 98% cells stained with control reagents

Fig. 3.4: % specific ^{51}Cr-release from B_3B_7 target cells following 6 hours co-culture with splenocytes and thymocytes from control *Xenopus*



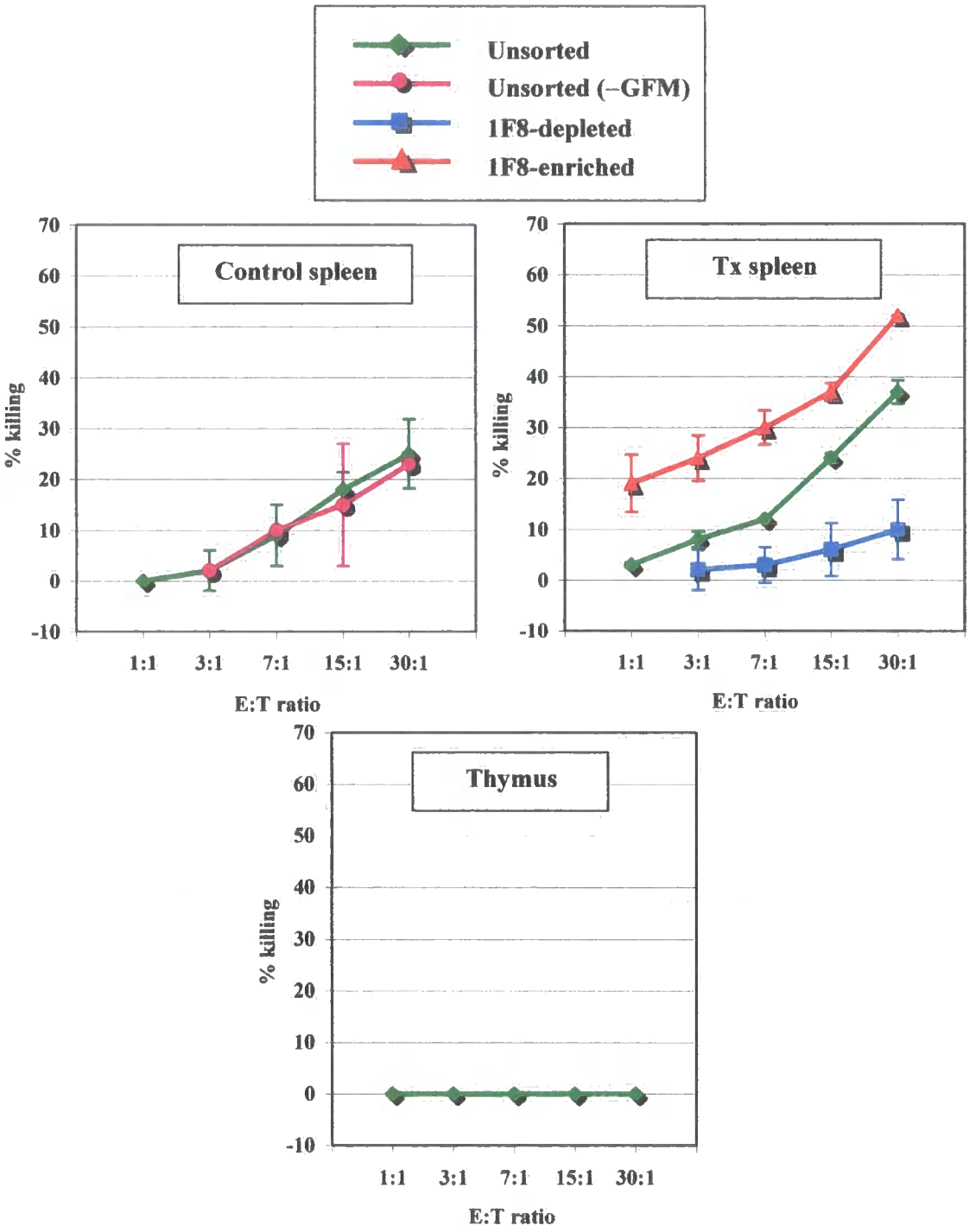
Values shown represent ≥5 data sets and show mean % killing ± standard error. All populations were cultured in GFM-free medium.

Fig. 3.5: % specific ^{51}Cr-release from B_3B_7 target cells following 6 hours co-culture with lymphocyte populations from spleen, liver and intestine of Tx *Xenopus*



Values shown represent ≥5 data sets and show mean % killing ± standard error. All Tx populations were cultured in GFM-supplemented media unless otherwise specified.

Fig. 3.6: % DNA loss from B_3B_7 target cells following 6 hours co-culture with lymphocyte populations from thymus and spleen of control and Tx *Xenopus*



Values shown represent ≥3 data sets and show mean % killing ± standard error. All control populations were cultured in medium alone and all Tx populations were cultured in GFM-supplemented media unless otherwise specified.

Fig. 3.7: Diagrammatic explanation of a flow cytometric trace showing cells stained with annexin-V-FITC and PI

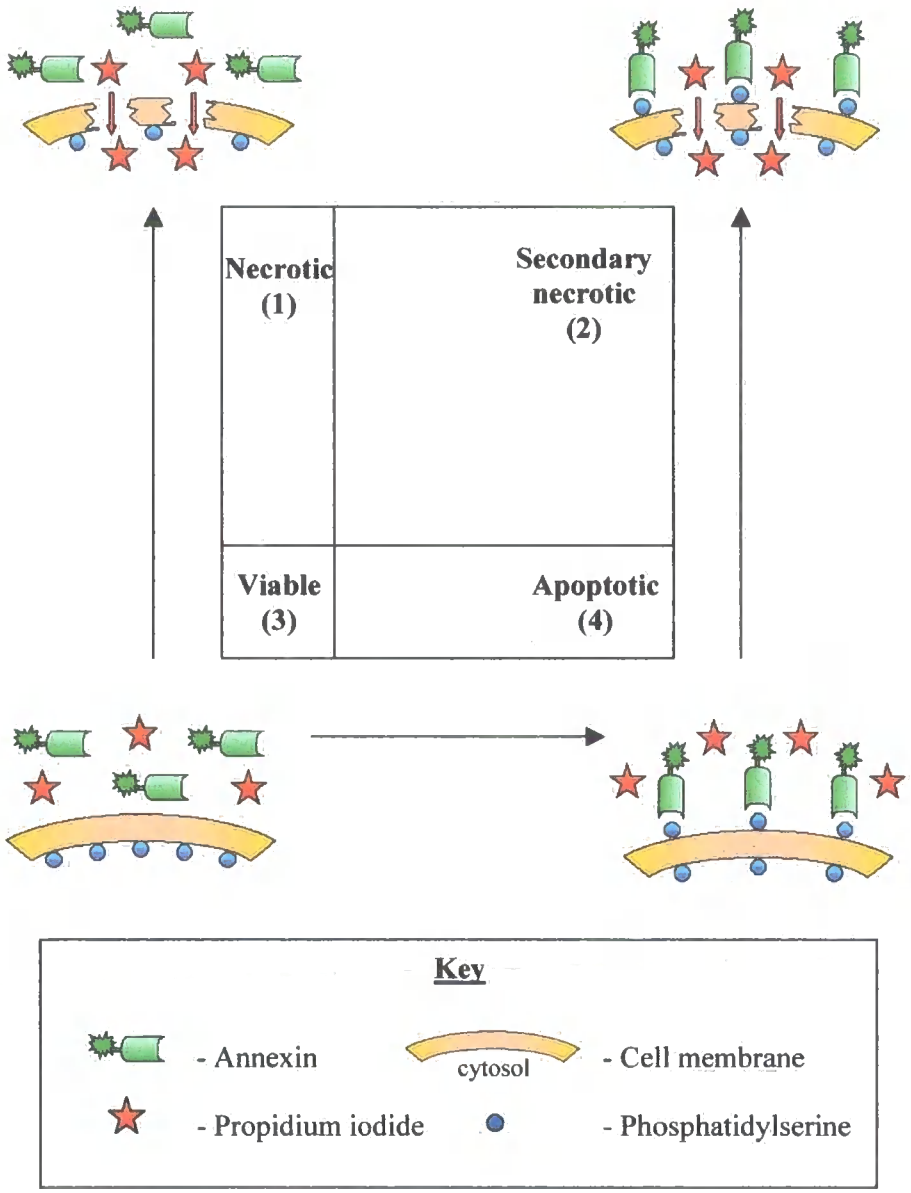
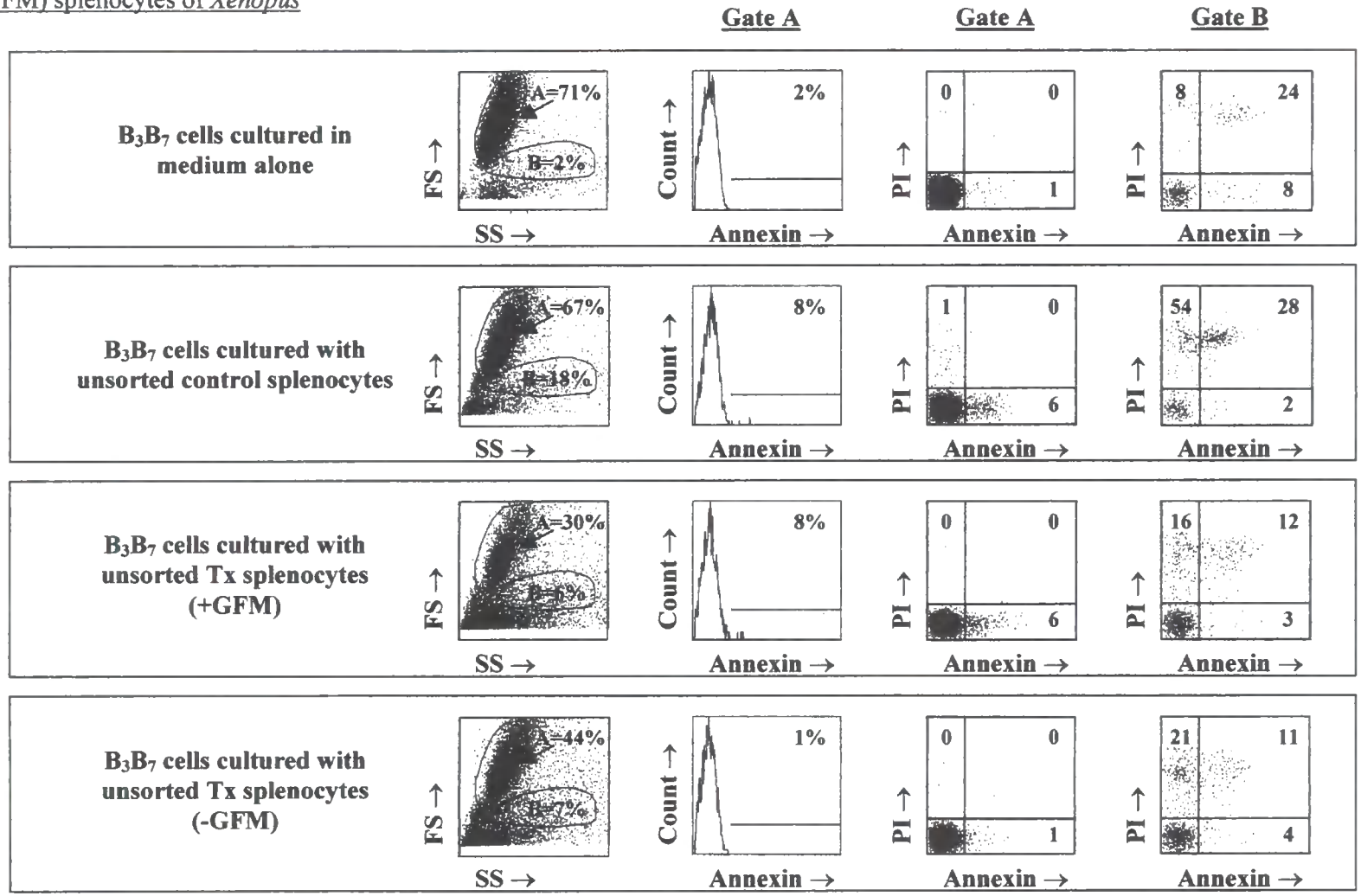
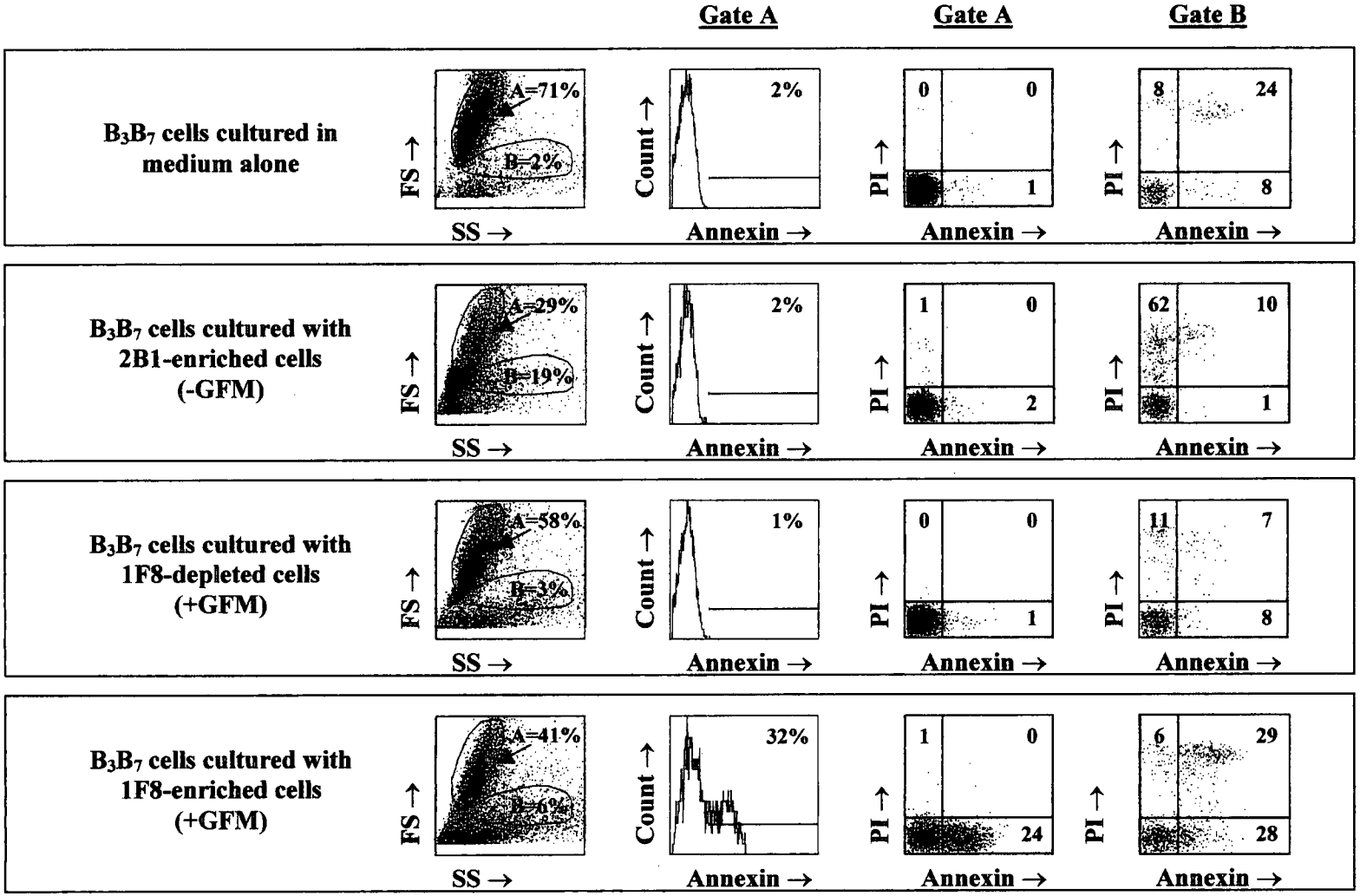


Fig. 3.8: Flow cytometric analysis of annexin-V/PI binding of B_3B_7 cells cultured for 6 hours either alone or with unsorted control or Tx (-/+GFM) splenocytes of *Xenopus*



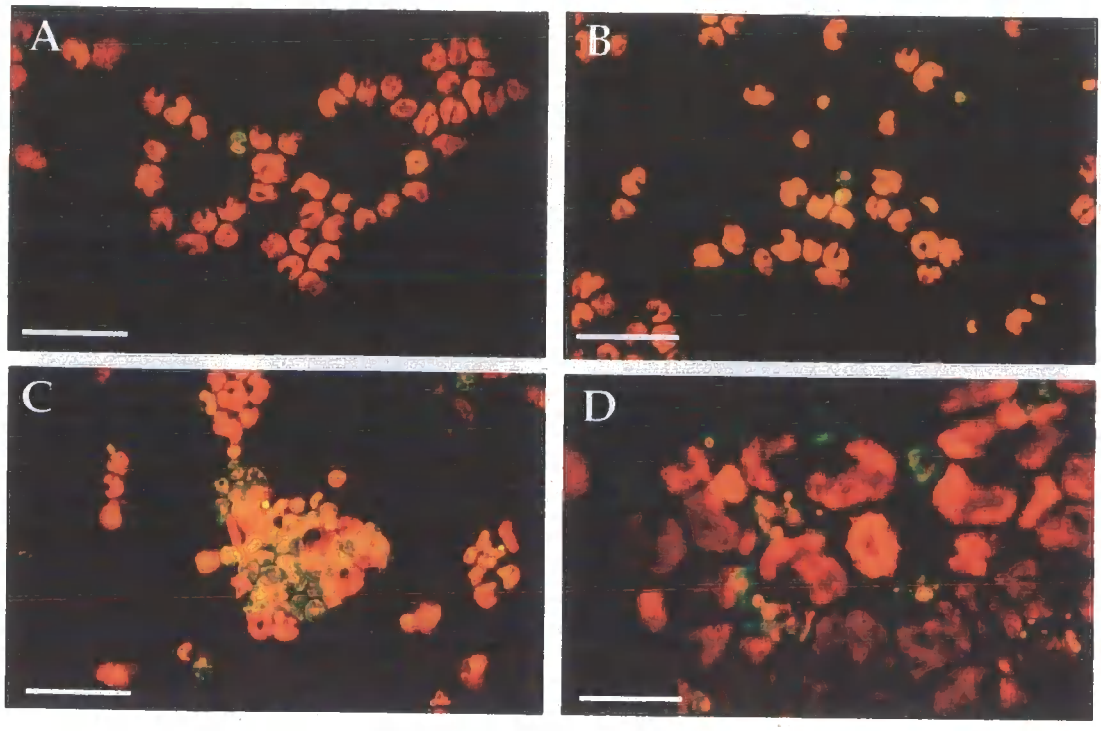
Typical data is shown representing ≥3 data sets; markers and quadrants were set to exclude 98% cells stained with control reagents. Gate A represents PI^{-ve} intact cells; gate B represents cells with lower FS/higher SS than intact population, many are PI^{+ve}. All effectors and targets were cultured at an E:T ratio of 5:1.

Fig. 3.9: Flow cytometric analysis of annexin-V/PI binding of B_3B_7 cells cultured for 6 hours either alone or with T-cell-enriched ($2B1^{+ve}$) cells from control *Xenopus*, or with NK-depleted ($1F8^{-ve}$) or NK-enriched ($1F8^{+ve}$) Tx splenocytes.



Typical data is shown representing ≥3 data sets; markers and quadrants were set to exclude 98% cells stained with control reagents. Gate A represents PI^{-ve} intact cells; gate B represents cells with lower FS/higher SS than intact population, many are PI^{+ve}. All effectors and targets were cultured at an E:T ratio of 5:1.

Fig. 3.10: Cytospins showing ASP staining of immunomagnetically sorted B_3B_7 tumour target cells following 6 hour culture with effector populations



B_3B_7 tumour target cells were co-cultured either alone **(A)**, with NK-depleted populations **(B)** or with NK-enriched populations **(C, D)**. Target cells were then stained with X71 mAb, immunomagnetically sorted and assessed for ASP expression (ASP^{+ve} cells fluoresce yellow/green. Cells were counter-stained with propidium iodide which labels nuclei orange/red. Bars (A-C) = 50μm, (D) = 20μm.

CHAPTER 4

NK cell ontogeny: Phenotypic and functional studies

4.1 Introduction

Xenopus provides an interesting model to explore the ontogeny of immunity, possessing larval and adult immune systems that are immunologically distinct (Horton, 1994).

T- and B-cells are established early in ontogeny, as shown in *Fig. 4.1.* The thymus anlage is seeded by a first wave of lymphoid progenitors beginning around 3-4 days of age (reviewed in Horton, 1994). T-cells, as evidenced by expression of the XTLA-1 antigen, are first detectable on thymocytes ate stage 48, at ≈7 days of age (Gravenor *et al.*, 1995). The expression of CD5 and CD8 is evident on thymocytes by day 12 (stage 49). T-cells expressing the pan T-cell marker CD5 and also a very few $CD8^{+ve}$ T-cells are first detectable in the spleen by day 12; splenic $XTLA1^{+ve}$ cells have been identified at stage 52 (day 18) (Gravenor *et al.*, 1995). Surface IgM^{+ve} B-cells are first found in larval liver and spleen at ≈12 days of age.

The ontogeny of MHC expression in *Xenopus* is particularly interesting. Class-II MHC expression is seen on a range of cell types from early larval life, these cells including B-lymphocytes and several tadpole epithelia (Flajnik and Du Pasquier, 1990a). In contrast, class-Ia transcripts are hardly detectable in most tissues until after metamorphosis and class-Ib transcripts are only found in the adult (Salter-Cid *et al.*, 1998). MHC class-Ia protein expression at the splenocyte surface from ≈5 weeks of age was identified by flow cytometry (Rollins-Smith *et al.*, 1997) *(Fig 4.1).* Universal expression of MHC class-Ia and class-Ib is restricted to adult *Xenopus.* MHC-II expression is also altered after metamorphosis, for example adult T-cells constitutively express class-II, in contrast to MHC II^{-ve} larval T-cells (Du Pasquier and Flajnik, 1990). The emergence of adult T-cells is the outcome of a second histogenesis of the thymus that occurs over metamorphosis; this histogenesis is evidenced by a period of temporary lymphocyte depletion followed by a second wave of thymocyte proliferation *(Fig 4.1).*

Lymphocyte functions have been studied in considerable depth in *Xenopus* larvae. B-cell function is detectable after stage 52 (Du Pasquier *et al.*, 1996), although there are noticeable differences between larval and adult Ig repertoire, which appear to be metamorphosis-dependent (Du Pasquier *et al.*, 1996). For example, the Ig heavy chain CDR3 in larvae is not diversified by N-residues (Schwager *et al.*, 1991) and IgY responses are observed only after metamorphosis (Horton *et al.*, 1996b). Poor T_h cell function in larvae may be the cause of lack of Ig class switching in tadpoles (Hsu and Du Pasquier, 1984b).

The cytotoxic potential of larval lymphocytes towards alloantigens and tumour cells has been investigated. Even young larvae with immune systems consisting of only 0.5×10^6 lymphocytes display T-cell-mediated alloimmune responses to foreign skin grafts (Horton *et al.*, 1996b). Due to lack of MHC class-I expression in larvae, it is probable that class-II-restricted killing by T-cells is relied upon for cytotoxic defence against pathogens (Horton *et al.*, 1989). Further indications of the cytotoxic capabilities of the larval immune system come from tumour transplantation studies. With regard to the latter, both B_3B_7 tumour cells and *ff* tumour cells (both of *ff* strain origin) grow in inbred *ff* larvae, but not in *ff* adults (Du Pasquier and Robert, 1992; Robert *et al.*, 1994; Robert *et al.*, 1995). Anti-tumour activity develops only after metamorphosis and appears to be a response directed against tumour-specific antigens (Robert and Cohen, 1998b). The inability of early-thymectomized *ff* adults to resist tumour growth indicates a crucial role of T-cells in tumour rejection (Robert *et al.*, 1997b; Robert and Cohen, 1998b). Presumably the tumour antigens can only be delivered effectively to T-cells by adult host cells expressing MHC-I proteins. In adults thymectomised at the onset of metamorphosis, although T-cell numbers are greatly reduced, α-tumour activity is still observed, suggesting that it is the first wave of T-cells to emanate from the early larval thymus that contains effective α-tumour cells (Robert and Cohen, 1998b).

The aim of this Chapter is to investigate the ontogeny of NK cells. It will be interesting to determine if larval NK cells can emerge at a time when expression of the presumed inhibitory ligand (MHC class-I) for these cells is minimal. NK cell ontogeny is probed through phenotypic studies exploring 1F8 antigen expression, and

through *in vitro* α-tumour cytotoxicity studies. The findings on NK cell development suggest that these lymphoid cells, in addition to T-cells, may play a crucial role in the ontogeny of α-tumour immunity.

The experiments in this Chapter were carried out in collaboration with Dr. J.D. Horton, Mrs. T.L. Horton and Mrs P. Ritchie, Horton *et al.,* 2002, in preparation.

4.2 Methods

Further details of reagents are given in the appendices.

4.2.1 Extraction of lymphocytes

Lymphocytes were extracted and prepared as described in section 2.2.1

4.2.2 Culturing of lymphocytes

Lymphocytes from adult and froglet spleen were cultured as described in section 2.2.2. Due to the low number of lymphocytes attainable from a single larval spleen, it was necessary to pool larval splenocytes to obtain sufficient cells for flow cytometric analysis and cytotoxicity assays. In order to prevent mixed lymphocyte reactions from occurring, cells from individual animals were cultured separately in flat-bottomed 96-well plates (Greiner) (at $1x10^6$/ml) and pooled only at the time of analysis/assay. All larval cells (both control and Tx) were cultured in medium supplemented with GFM (see section 2.2.3).

4.2.3 Generation of growth factor-rich medium

Growth factor-rich medium was generated as described in section 2.2.3

4.2.4 Flow cytometry

Flow cytometric analysis was carried out as described in section 2.2.4

4.2.5 Cytotoxicity assays

4.2.5.1 ^{51}Cr-release cytotoxicity assays

^{51}Cr-release cytotoxicity assays were carried out as described in section 3.2.7.1.

4.2.5.2 JAM assays

JAM assays were carried out as described in section 3.2.7.2, although the ^{3}HT labelling period for the B_3B_7 target cells was reduced from overnight to 3 hours to increase viability of the targets used in the assay.

4.3 Results

4.3.1 Phenotypic studies

Flow cytometry on 1-2 year-old adult (control and Tx) *Xenopus laevis* splenocytes cultured for 48 hr in GFM (a procedure that increases 1F8 expression and promotes NK-like killing (Horton *et al.*, 2000) confirmed that $1F8^{+ve}$ splenocytes lack the surface IgM of B-cells and the surface markers ($CD5^{hi}$ and CD8) characteristic of T-cells, but frequently express the 56kDa antigen recognized by mAb D12-2 (*Figs. 4.2 and 4.3*). A subset of $1F8^{+ve}/CD5^{lo}$ splenocytes was evident in some adult spleens (data not shown).

Flow cytometric analysis on pooled spleens from 5-week-old (stage 54/55) control and Tx *Xenopus laevis* larvae revealed no 1F8 staining of viable splenocytes following 48 hour culture in GFM (data not shown). Dual colour flow cytometric data on splenocytes pooled from 7-week larvae (stage 56-58) or from 3-4 month froglets, following 48 hour culture in GFM, is illustrated in *Figs. 4.2 and 4.3.* A few splenocytes expressing the 1F8 antigen (of lower fluorescence intensity ($1F8^{lo}$) than seen in adults) can be visualised for the first time in 7 week-old larvae especially in Tx tadpoles (*Fig 4.3*); such $1F8^{lo}$ cells are IgM^{-ve}, $CD5^{-ve}$ and $CD8^{-ve}$, but a few (3%) co-express the D12-2 antigen. The proportion of $1F8^{lo}$ cells remains unchanged in 3-4 month old froglets, where a few double-positive $1F8^{lo}/CD5^{+ve}$ cells were noted in addition to the $1F8^{lo}/D12\text{-}2^{+ve}$ population. $1F8^{+ve}$ cells were not found in the thymus at any stage of development.

4.3.2 Functional studies

The JAM assay proved to be more sensitive than ^{51}Cr release in monitoring killing of B_3B_7 targets following 6 hour co-culture with 1-2-year-old adult control and Tx splenocytes (*Fig 4.4, top left*). Consistent with findings from ^{51}Cr release, additional JAM assays on immunomagnetically-separated splenocytes identified the $1F8^{+ve}$

population as the one able to achieve target cell DNA fragmentation, whereas T- cells, B-cells and $1F8^{-ve}$ cells were unable to fragment B_3B_7 tumour targets (data not shown).

In our ontogenetic studies, JAM assays were initially carried out on 5 week (stage 54/55) and 7 week (stage 56/58) control and Tx *Xenopus laevis* larvae (*Fig 4.4, bottom*). Assays involved culture of splenocytes from individual larvae for 48 hours in GFM (to promote NK killing) prior to pooling of between 7-20 spleens for assay. No DNA fragmentation of B_3B_7 targets was observed in any larval experimental group (<10% specific killing even at 20:1 or 40:1 E:T ratio). This was true even for pooled spleens taken from control 7-week-old tadpoles injected with B_3B_7 cells 10 days prior to assay, in an attempt to elevate NK-like killing. Replicate experiments on additional outbred and *ff* strain *Xenopus* 7-week-old larvae confirmed their inability to kill B_3B_7 tumour targets *in vitro* (data not shown). Moreover, such cytotoxicity was still relatively poor (only ≈20% specific killing at the highest E:T ratios used) when splenocytes from individual 3-4-month-old froglets were tested (*Fig 4.4 top right*).

4.4 Discussion

In mammals, interactions between inhibitory receptors expressed by developing NK cells and MHC class-I proteins expressed by autologous cells are believed to play a crucial role in the education of NK cells and their development of self tolerance (Karre and Colonna, 1998). However, in humans it has recently been shown (Sivori *et al.*, 2002) that immature NK cells express activatory receptors before MHC-specific inhibitory receptors, implying that ligands other than MHC may in early ontogeny be crucial in NK inhibition. In this Chapter, the question of whether NK cells can develop in the MHC class-I-negative environment of larval *Xenopus* has been examined. Phenotypic (flow cytometric) studies revealed that cells expressing (low levels of) 1F8 antigen were just detectable in the spleen (especially in Tx tadpoles) late in larval life, at ≈7 weeks (stage 56/58). This is long after T- and B-cells have emerged in this organ during the second week of larval life (see Introduction). The surprisingly late appearance of NK cells in *Xenopus* comes ≈2 weeks after the ontogeny of MHC class-Ia expression but prior to the emergence of MHC class-Ib (Rollins-Smith *et al.*, 1997; Salter-Cid *et al.*, 1998). The proportion of splenocytes expressing 1F8 is slightly increased by 3 months of age, although there are only relatively few NK cells (≈4% in control froglets, ≈6% in Tx froglets) compared with the situation in adult *Xenopus* (≈12% in controls, 22% in Tx frogs). There were no $1F8^{+ve}$ cells present in the thymus at any stage of development.

The above phenotypic studies suggesting initial appearance of NK cells in late larval life is not precisely mirrored in the DNA fragmentation assays. Thus GFM-cultured splenocytes from control or Tx (5 and 7-week-old) tadpoles, even from B_3B_7-injected larvae, failed to achieve DNA fragmentation of B_3B_7 tumour targets. These findings using the JAM assay confirm and extend previous preliminary ^{51}Cr-release assay data (Horton *et al.*, 1998b), which showed 6-7 week control, *in vitro*-cultured, larval splenocytes failed to kill B_3B_7 targets. The very low percentage of $1F8^{lo}$ splenocytes detectable by flow cytometry in late larvae may be competent to kill, but the cytotoxicity is masked when unseparated splenocytes are assayed. Clearly 1F8-enrichment studies are needed, but this would necessitate very large numbers of (Tx)

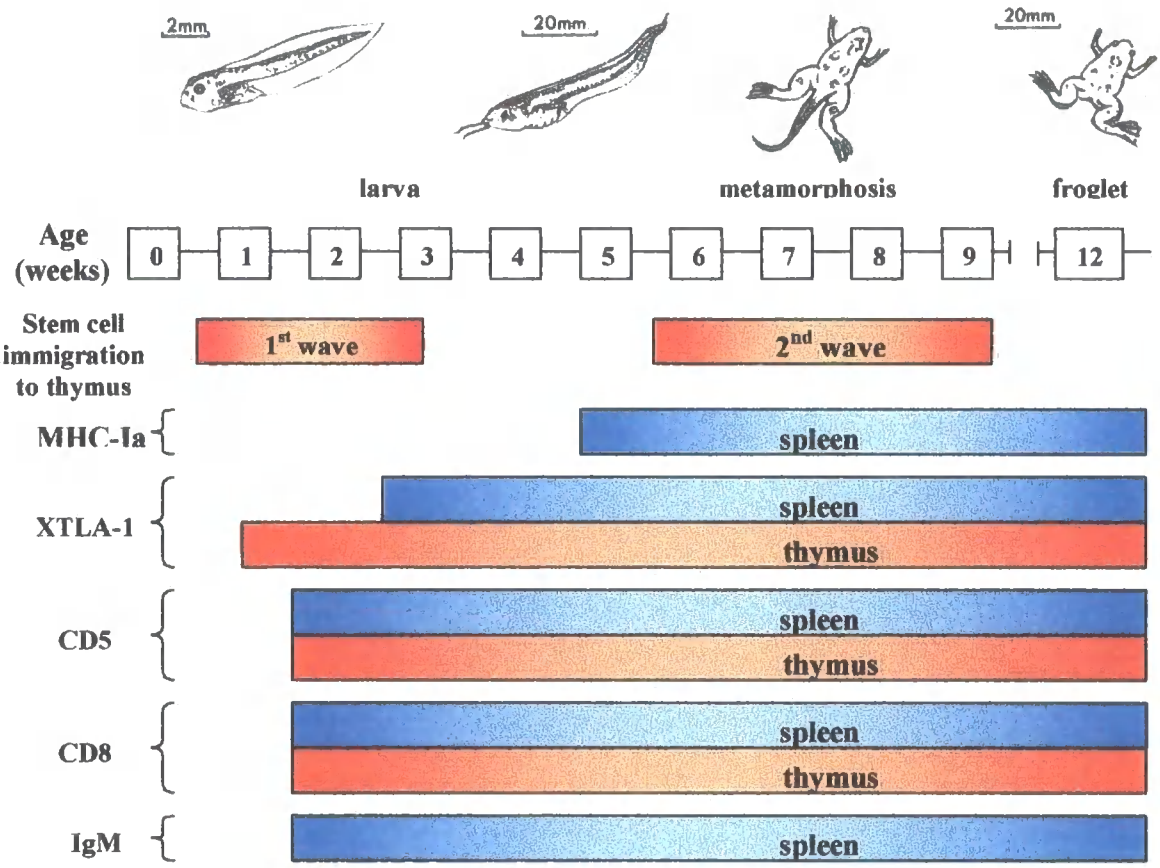
larvae to gather sufficient effector numbers. Significant, albeit low levels of B_3B_7 tumour killing by splenocytes (i.e. >10% level of killing that can be mediated in the JAM assay following 6 hour co-culture of tumour cells with 20-40-fold excess red blood cells (unpublished observations) are first seen only in 3-4 month-old froglets. Previous studies have shown that splenocytes from 6 month-old *Xenopus* (some 3.5 months post-metamorphosis) are almost as effective killers of B_3B_7 targets as splenocytes from year-old adults (Horton *et al.*, 1998b). It is conceivable that the poor cytotoxic potential displayed by larvae and 4 month-old froglets relates to the low intensity of 1F8 antigen expression. In humans only those NK cells expressing high levels of natural cytotoxicity receptors efficiently kill tumour cell lines (Moretta *et al.*, 2002).

The emergence of fully-effective *in vitro* killing of B_3B_7 tumour cells by NK cells at ≈6 months of age provides a new explanation for earlier findings on the ontogeny of *in vivo* alloimmune reactivity of X*enopus* towards injected tumour cells (Robert *et al.*, 1995). Thus *ff*$_2$ thymus lymphoid tumour cells injected into *ff* tadpoles and young post-metamorphic *ff* froglets up until nearly 4 months of age initiated tumour growth in the vast majority of cases, whereas when injected into 6 month and older frogs, tumours failed to develop. The development of this alloimmunity to *ff* tumour was originally considered to be most likely linked to the emergence of T-cell functions in the early post-metamorphic period, rather than to NK cell development (Robert *et al.*, 1995).

Although we know that $1F8^{+ve}$ cells are the lymphoid subset that kills tumour targets *in vitro* and that this killing is dependent on NK cell pre-culture in GFM (T-cell growth factor-rich culture medium), a role of NK cells in *in vivo* killing of tumours remains to be firmly established. Thus early-thymectomized *ff* strain *Xenopus laevis* adult frogs, with substantial numbers of NK cells in their lymphoid tissues, are susceptible to growth of both injected *ff*$_2$ thymus tumour cells (MHC-Ia^{+ve} cells from *ff* strain *Xenopus laevis*) (Robert *et al.*, 1997b) and LG-15-derived 15/0 tumour cells (Horton, TL and Horton, JD, unpublished), in contrast to thymus-intact *ff* frogs, in which such tumours fail to grow. This is consistent with the notion that T-cell-mediated tumour immunity plays a fundamental role in the vertebrate immune system

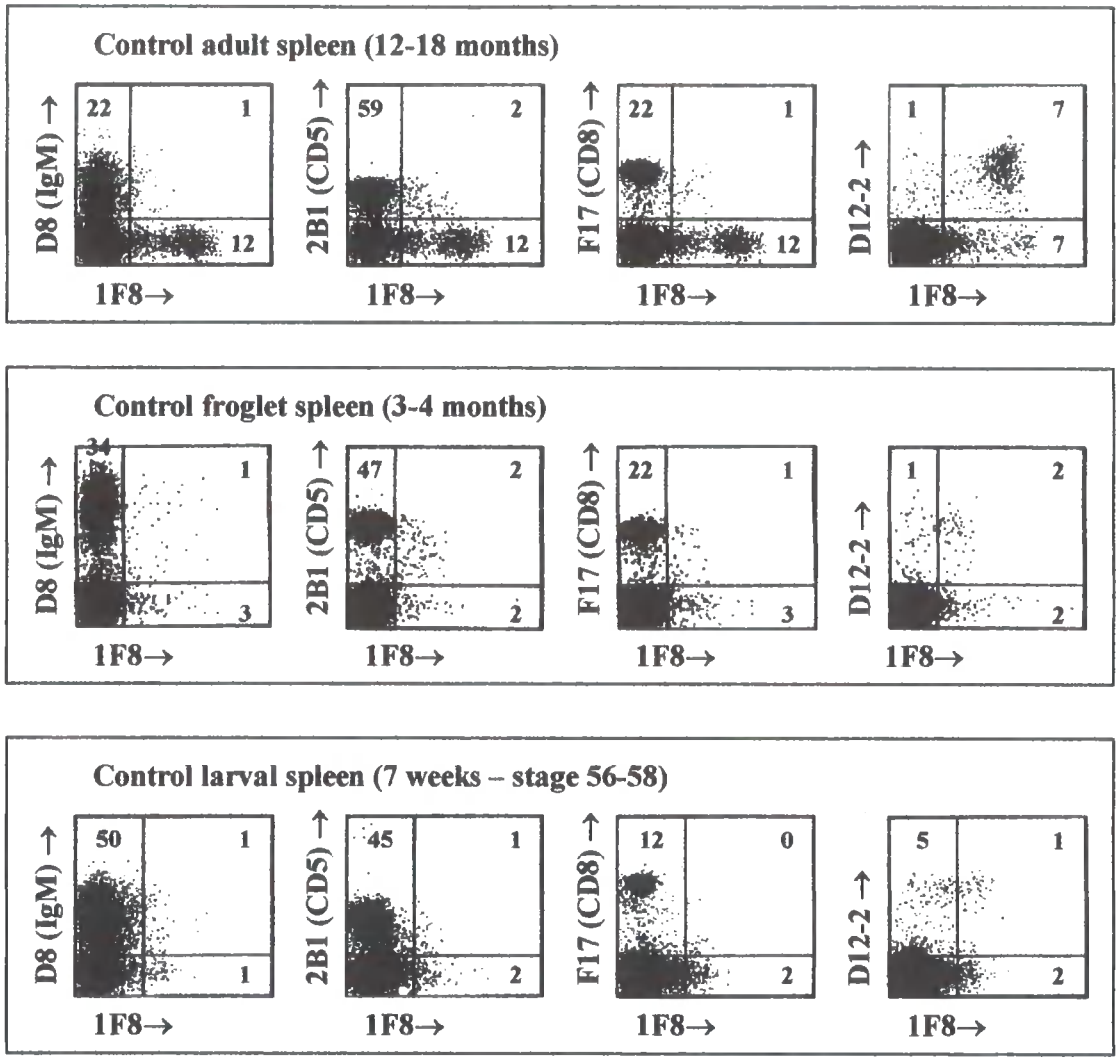
(Robert and Cohen, 1998b). Interesting questions remain, however, as to how T-cells would kill the MHC class Ia^{-ve} thymus tumour targets. Perhaps T-cells can visualise MHC class-Ib that thymus tumour cell lines synthesize (Robert *et al.*, 1994), or cytotoxic T-cells may recognize thymus tumour-derived gp96 heat shock protein that can elicit potent immunity towards tumour cells (Robert *et al.*, 2001a). Since injection of adult clonal *Xenopus* with α-CD8 mAb impairs the immune response against transplanted syngeneic MHC class I^{-ve} tumours, $CD8^{+ve}$ T-cells may be crucial effectors in MHC-unrestricted α-tumour responses (Rau *et al.*, 2001). However, NK cells may additionally play a crucial role in such tumour immunity, since we have recently shown that the 1F8 mAb injected into LG15 control *Xenopus* enhances the rapid growth of 15/0 tumour (Rau *et al.*, 2002). NK cells may only function in situations where T-cell-derived growth factors are plentiful (i.e. in controls), whereas the effectiveness of these cells in Tx frogs is compromised because such factors are absent.

Fig. 4.1: The expression of cell surface antigens in the spleen and thymus throughout the first three months of the life of *Xenopus laevis*



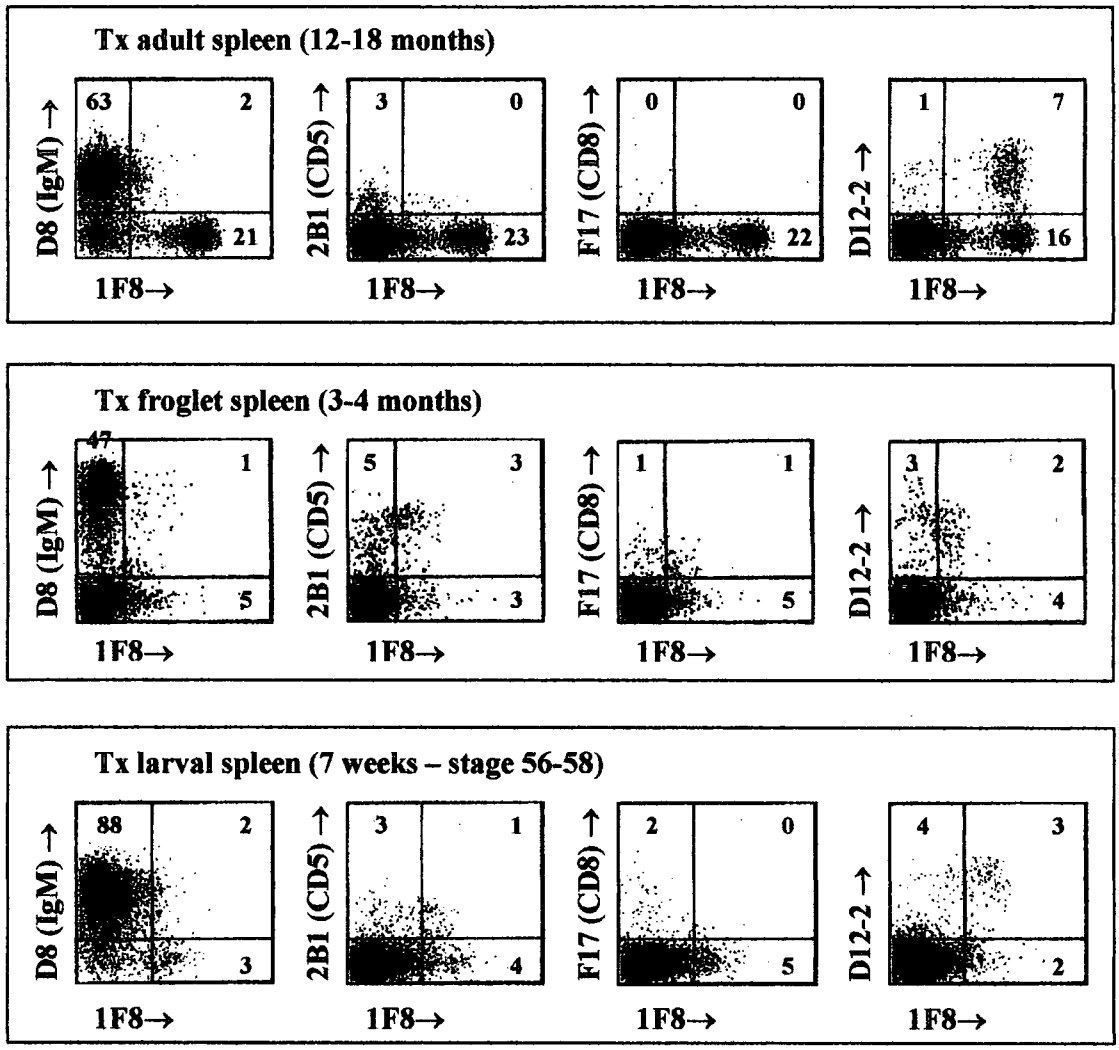
(adapted from Horton, 1994; Gravenor *et al.*, 1995; Salter-Cid *et al.*, 1998)

Fig. 4.2: Dual colour flow cytometric analysis on control splenocytes from adult, froglet and larval *Xenopus* following 48 hour culture in GFM



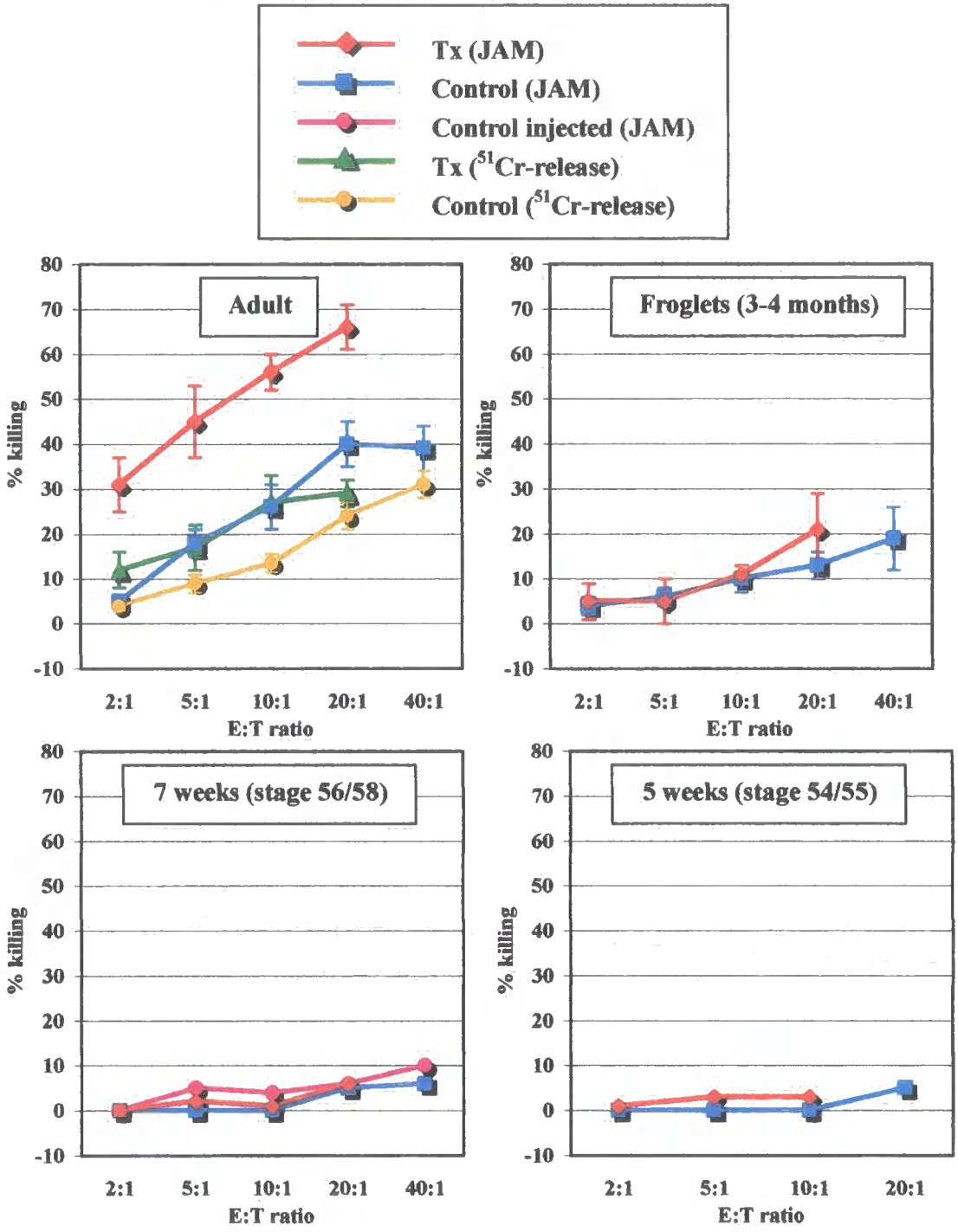
Typical data is shown representing ≥3 data sets; quadrants were set to exclude 98% cells stained with control reagents. Adult data shown here is from Chapter 2 and included for reference only.

Fig. 4.3: Dual colour flow cytometric analysis on Tx splenocytes from adult, froglet and larval *Xenopus* following 48 hour culture in GFM



Typical data is shown representing ≥3 data sets; quadrants were set to exclude 98% cells stained with control reagents. Adult data shown here is from Chapter 2 and included for reference only.

Fig. 4.4: % specific killing of tumour target cells following 6 hour co-culture with unsorted splenocytes from control and Tx *Xenopus laevis* of various stages of larval and adult development



Adult and froglet graphs: values shown represent 3 data sets and show mean % killing ± standard error. 5 week and 7 week graphs: values shown represent typical data set. Repeats were performed using separate pools of cells with similar results. All populations (both control and Tx) were cultured in GFM-supplemented media.

CHAPTER 5

NK antigen expression and characterisation following *in vitro* lymphocyte stimulation

5.1 Introduction

The initial aim of this Chapter is to investigate the effects of *in vitro* stimulation on the phenotype of adult and larval lymphocytes. Ligand-mediated T-cell activation is achieved following a series of biochemical events within the cell, which occur as a result of receptor/ligand association. The phosphorylation of ITAM's associated with the CD3/TCR signaling complex leads to phospholipase-C-γ(PLC-γ)-mediated hydrolysis of phosphatidylinositol biphosphate (PIP_2), generating the breakdown products 1,2-diacylglycerol (DAG) and inositol triphosphate (IP_3), the latter evoking a release of calcium from intracellular stores, accompanied by an influx of extracelluar calcium (Truneh *et al.*, 1985; Abbas *et al.*, 1991). Increases in DAG and intracellular calcium result in the activation of protein kinase C and calcineurin, which in turn leads to the activation of the cell (Abbas *et al.*, 1991), through triggering of several transcription factors *(Fig. 5.1)*.

Components of this response may be mimicked by mitogens such as the PKC activator phorbol 12-myristate 13-acetate (PMA), or calcium ionophores (e.g. A23187 and ionomycin), which induce calcium influx (Franklin *et al.*, 1994) and the activation of calcium-activated chloride channels (Yoshida and Plant, 1992). Although neither mitogens are capable of mimicking the full response alone, when applied together, they act synergistically to mimic T-cell activation (Truneh *et al.*, 1985; Abbas *et al.*, 1991).

The effects of such mitogens on lymphocyte populations of various vertebrate species have been examined. In mice, combinations of PMA and ionomycin evoke the differentiation of double positive immature thymocytes into single positive $CD4^{+ve}$ cells (Ohoka *et al.*, 1996; Takahama and Nakauchi, 1996) and thereby appear to mimic positive selection (Tanahashi *et al.*, 2001). Similar responses are seen with human thymocytes exposed to PMA and ionomycin (at 2ng/ml and 2μg/ml respectively), although in this case, it is CD4 which is downregulated. PMA alone can inhibit the differentiation of murine bone marrow precursor cells (cultured with IL-2) into mature NK cells, in addition to downregulating NK1.1 expression (Ayroldi

et al., 1993). It has previously been shown that PMA/ionomycin stimulation of *Xenopus* cortical thymocytes (thought to be the equivalent of mammalian double positive thymocytes) results in the generation of T-lymphoblasts, the downregulation of CTX (cortical thymocyte-specific antigen of *Xenopus*, Robert *et al.*, 1997a; Robert and Cohen, 1998a) and upregulation of CD5 and CD45 (Robert and Cohen, 1999; Robert *et al.*, 2001b).

This Chapter describes the effects of PMA and calcium ionophore on the phenotype of both adult and larval lymphocytes of *Xenopus* spleen and thymus. Changes in NK antigen expression are detected by flow cytometry. The effect of PMA/Ca^{2+} ionophore on the cytotoxic potential of lymphocyte populations towards tumour cells is also assessed. Finally, PMA/Ca^{2+} ionophore stimulation is used as a tool to generate 1F8 antigen expression on T-cells to allow further molecular characterisation of this NK-associated molecule through immunoprecipitation experiments. Evidence that NKT-cells exist at the amphibian level of evolution is discussed.

5.2 Methods

Further details of reagents are given in the appendices.

5.2.1 Extraction of lymphocytes

Lymphocytes were extracted as described in section 2.2.1

5.2.2 Culturing of lymphocytes

Lymphocytes were cultured as described in section 2.2.2.

5.2.2.1 Culturing of lymphocytes with PMA and calcium ionophore

Adult cells were transferred to a 24-well plate (Greiner) at a concentration of $2x10^6$/well and the appropriate concentrations of PMA (Sigma) and calcium ionophore (Sigma) were added. Following 24 hour culture, the cells were washed in B_3B_7 culture medium (see appendix 1) to remove PMA and calcium ionophore, resuspended in fresh culture medium and transferred to a fresh 24-well plate at $2x10^6$ cells/well.

Due to the low number of lymphocytes attainable from a single larval spleen, it was necessary to pool larval splenocytes to obtain sufficient cells for flow cytometric analysis and cytotoxicity assays. In order to prevent mixed lymphocyte reactions from occurring, cells from individual animals were cultured separately in flat-bottomed 96-well plates (Greiner) (at $1x10^6$/ml) and pooled only at the time of analysis/assay. All larval cells (both control and Tx) were cultured in medium supplemented with GFM (see section 2.2.3).

5.2.3 Generation of growth factor-rich medium

Growth factor-rich medium was generated as described in section 2.2.3

5.2.4 Flow cytometry

Flow cytometric analysis was carried out as described in section 2.2.4

5.2.5 Cytospins

Cytospins were prepared as described in section 3.2.5

5.2.6 Cell separation

Cell separation was carried out as described in section 3.2.6

5.2.7 Cytotoxicity assays

5.2.7.1 ^{51}Cr-release cytotoxicity assays

^{51}Cr-release assays were carried out as described in section 3.2.7.1

5.2.7.2 JAM assays

JAM assays were carried out as described in section 3.2.7.2

5.2.8 Apoptosis assays

5.2.8.1 Annexin-V-FITC

Annexin-V-FITC assays were carried out as described in section 3.2.8.1

5.2.8.2 ASP-staining

ASP-staining was carried out as described in section 3.2.8.2

5.2.9 Immunoprecipitation

5.2.9.1 Immunoprecipitation of biotin-labelled cells

(Fig. 5.2)

5-10x10^6 lymphocytes were washed twice in APBS (see appendix 1), resuspended in APBS containing 0.5mg/ml biotin (Vector laboratories) and rotated for 30 minutes at 4°C. Following centrifugation for 1 minute at 15,000rpm, the supernatant was discarded and the pellet resuspended in APBS containing 5mg/ml lysine (Sigma). The cell suspension was rotated at 4°C for 5 minutes and then washed three times in the APBS/lysine solution. The cell pellet was lysed on ice for 30 minutes in NP-40 protein lysis buffer (see appendix 2) containing 1%NP-40 and protease inhibitors and then centrifuged at 15,000rpm for 3 minutes to pellet cytoskeletal debris. *Xenopus* serum (1:200), anti-*Xenopus* Ig mAb's (10A9 and 11D5) and protein A sepharose CL-4B beads (Sigma) were added to the supernatant and rotated at 4°C for 2 hours to preclear (to remove unwanted antigens). The suspension was centrifuged at 15,000rpm for 1 minute, the pellet was discarded and protein A beads were added to the supernatant. The suspension was rotated at 4°C for 30 minutes for further preclearing. Following 1 minute centrifugation at 15,000rpm, the supernatant was transferred to a fresh tube and the appropriate antibody of interest was added together with protein A beads. The suspension was rotated at 4°C overnight. The suspension was then centrifuged at 15,000rpm for 30 seconds and washed twice in buffer A (see

appendix 2), twice in buffer B (see appendix 2) and once in buffer C (see appendix 2). The supernatant was discarded and the pellet resuspended in 1x denaturing SDS loading buffer (see appendix 2). Samples were boiled at 95°C for 5 minutes and spun briefly. Proteins were separated according to size by SDS-PAGE using Mini-Protean II gel apparatus (BioRad). Samples were run at 200v down a minigel of 10% acrylamide separating gel, 4% stacking gel (see appendix 2), together with pre-stained markers of 66kDa and 87kDa. The gel was then removed and washed in Towbin transfer buffer (see appendix 2) for 10-15 minutes to remove SDS. Transfer of proteins to nitrocellulose (Schleicher and Schuell) was carried out using the Trans-Blot R Electrophoretic Transfer cell (BioRad), which was assembled as described in section 2.2.8. Protein transfer to the nitrocellulose membrane was performed in Towbin transfer buffer at 100V, 250mA for 3-4 hours (limited to 250mA). The nitrocellulose was stained with Ponceau S stain (see appendix 2) to confirm transfer, destained in 5% acetic acid and washed in TBS (see appendix 2) for 20 minutes. Blocking solution (see appendix 2) was applied to the nitrocellulose for 2 hours at room temperature prior to the addition of streptavidin conjugated to HRP (Vector laboratories) diluted to 1:15,000 with blocking solution. The nitrocellulose was incubated for 30 minutes at room temperature, washed three times in TBS, incubated with chemiluminescent solutions (see appendix 2) for 1 minute in the dark and exposed to X-ray film (Fujifilm).

5.2.9.2 Immunoprecipitation of ^{35}S-labelled cells

(Fig. 5.3)

Cells were washed twice in APBS (see appendix 1), once in RPMI washing medium (see appendix 2) and incubated for 30 minutes in RPMI labelling medium (methionine and cysteine-free) (see appendix 2) at 3 x 10^7 cells/ml. Following centrifugation, cells were resuspended in RPMI labelling medium with 10mCi/ml ^{35}S-methionine and cysteine and incubated for 2 hours at 27°C 5%CO_2. After 2 hours, more medium was added and the cells placed back into the incubator overnight. Cells were washed twice in APBS/1%BSA and incubated for 1 hour on ice with the appropriate primary antibody. Following a further wash in APBS/BSA, cells were resuspended in 1% NP-

40 lysis buffer with added proteinase inhibitors (see appendix 2) and incubated on ice for 30 minutes with occasional vortexing. The lysate was centrifuged at 15,000rpm for 3 minutes and the supernatant transferred to a fresh tube. Protein A or G beads (Sigma) were added as appropriate and the tubes rotated at 4°C for 1 hour. The sample was then briefly centrifuged. The tube now contained a pellet of beads containing surface proteins linked to the appropriate antibody, and supernatant containing cytoplasmic proteins which had not as yet come into contact with antibody.

The supernatant containing unlinked cytoplasmic proteins (*Fig. 5.3*) was precleared with protein A or G beads (Sigma) at 4°C for 1 hour, centrifuged, and the supernatant incubated with the appropriate antibody and beads overnight at 4°C. The samples were centrifuged and the pellets washed twice in Net-NON (see appendix 2) and twice in Net-N (see appendix 2).

The pellet containing antibody-linked surface proteins (*Fig. 5.3*) was also washed twice in Net-NON and twice in Net-N.

Both surface and cytoplasmic precipitated proteins were resuspended in sample loading buffer containing the reducing agent β-mercaptoethanol (see appendix 2) and boiled at 95°C for 5 minutes. Proteins were separated according to size by SDS-PAGE using Mini-Protean II gel apparatus (BioRad). Samples were run at 200v down a minigel of 10% acrylamide separating gel, 4% stacking gel (see appendix 2), alongside ^{14}C and pre-stained markers. The gels were then soaked in Autofluor for 30 minutes, placed onto wet 3MM paper (Whatman), covered in cling film and dried at 80°C for 2 hours. X-ray film (Kodak) was placed onto the gels and exposed for the required length of time.

5.3 Results

5.3.1 Phenotypic and morphological changes associated with culture with PMA and Ca^{2+} ionophore

5.3.1.1 Adult *Xenopus*

The effects of various PMA/Ca^{2+} ionophore concentrations on cell surface antigen expression on thymocytes and control and Tx splenocytes were investigated *(Table 5.1)*. In each case, cells were cultured for 24 hours with PMA and calcium ionophore, washed in medium and cultured for a further 24 hours.

a) Preliminary trials with various concentrations of PMA/Ca^{2+} ionophore *(Table. 5.1)*

10ng/ml PMA (control spleen only)

48 hour culture of control splenocytes with 10ng/ml PMA resulted in a dramatic increase in NK cell antigen expression compared with medium-cultured cells; levels of α-NK mAb staining increased from ≈6% to 38% (1F8), 40% (4D4) and 44% (1G5). CD5 expression also increased from 65% to 72%, IgM levels were reduced slightly from 30% to 22%.

10ng/ml PMA + 100ng/ml Ca^{2+} ionophore (control spleen and thymus)

Further supplementation with 100ng/ml Ca^{2+} ionophore also caused a dramatic rise in 1F8, 4D4 and 1G5 staining in both spleen and thymus (1F8 levels rise to 33% and 50% respectively). CD5 expression on cultured splenocytes increased significantly to 91%, but remained relatively similar (69%) on thymocytes. IgM expression also remained unchanged in the thymus following 48 hour culture (4%), whereas levels decreased to a mere 5% in the spleen. AM22 staining (putative CD8α) increased from 23% to 34% in the spleen, but decreased from 62% to 35% in the thymus.

1ng/ml PMA + 100ng/ml Ca^{2+} ionophore (thymus only)

Culture of thymocytes in 1ng/ml PMA and 100ng/ml Ca^{2+} ionophore resulted in a dramatic increase in 1F8 staining (3% to 43%), but only a slight change in 4D4 and 1G5 staining (10% and 7% respectively). AM22 staining and CD5 expression decreased to 27% and 61% respectively, with IgM expression remaining relatively unchanged (5%).

1ng/ml PMA + 20ng/ml Ca^{2+} ionophore (thymus only)

This lower dose of calcium ionophore failed to achieve a dramatic increase in thymocyte 1F8 antigen expression – ≈14%, 9% and 6% stained positive for 1F8, 4D4 and 1G5 respectively. Effects on CD5, CD8 and IgM expression were comparable to cells stimulated with 1ng/mlPMA + 100ng/ml Ca^{2+} ionophore.

b) Use of 10ng/ml PMA + 20ng/ml Ca^{2+} ionophore *(Table. 5.2)*

10ng/ml PMA together with 20ng/ml Ca^{2+} ionophore was routinely used for the future investigations on the effects of culture in PMA/Ca^{2+} ionophore-supplemented media, as this combination induced consistent and reproducible expression of NK antigen. As both *Table 5.2* and *Fig. 5.4* demonstrate, 48 hour culture in medium supplemented with 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore resulted in a significant increase in NK antigen expression in control spleen and thymus, 1F8 staining levels increasing to 49% and 31% respectively. CD5 expression increased to 89% in the control spleen, but decreased slightly in the thymus (67%). Similarly, AM22 staining (putative CD8α) increased slightly in control spleen to 31%, but decreased significantly to 22% in the thymus. Surface IgM expression was reduced to 10% in control spleen and remained negligible in the thymus. It was clear that 48 hour culture was necessary to effect such changes, as 24 hour culture with 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore failed to cause an increase in 1F8 antigen expression in both control spleen and thymus.

In contrast to control splenocytes, 1F8, 4D4 and 1G5 staining on Tx splenocytes cultured for 48 hours with 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore, actually decreased slightly to 12%, 10% and 11% respectively. Surface IgM expression was

affected however, and decreased to just 12%. AM22 (putative CD8α) staining also decreased to 4%.

Fig. 5.5 demonstrates the increase in forward and side scatter of adult *Xenopus* splenocytes (both control and Tx) and thymocytes cultured for 48 hours in medium supplemented with PMA (10ng/ml) and Ca^{2+} ionophore (20ng/ml). This phenomenon represents the transformation of lymphocytes into lymphoblasts. Although there is significant apoptosis in the PMA/Ca^{2+} ionophore-treated cultures (low FS, high SS), the extent of this cell death (approximately 40-50% following 48 hour culture) was less than when higher doses of Ca^{2+} ionophore were used.

Dual colour flow cytometry was used to further explore the nature of cells displaying an increase in 1F8 antigen expression.

As *Fig. 5.6* demonstrates, following 48 hour culture with PMA and calcium ionophore, two populations of $2B1^{+ve}$ splenocytes and thymocytes were discernable, $2B1^{bright}$ and $2B1^{dull}$, the former showed significant co-staining with the α-NK mAb 1F8 (39% co-stain in the spleen, 20% in the thymus). Previous studies have shown that B-cells can express CD5 as a result of PMA/Ca^{2+} ionophore-induced stimulation (Jurgens *et al.*, 1995; Gravenor, 1996) and it is therefore most likely that the $CD5^{dull}$ population represents the B-cell subset, and the $CD5^{bright}$ population represents cells of the T-cell lineage. Approximately three-quarters of $AM22^{+ve}$ splenocytes and one-quarter of $AM22^{+ve}$ thymocytes became $1F8^{+ve}$ following PMA/Ca^{2+} ionophore-induced stimulation. AM20 (MHC-II) staining also revealed two subpopulations, i.e. MHC-II^{bright} cells (B-cells) and MHC-II^{dull} cells (T-cells). The MHC-II^{bright} B-cells remained $1F8^{-ve}$, whereas 42% of cells were MHC-$II^{dull}1F8^{+ve}$. Similarly, in the thymus, the majority of $1F8^{+ve}$ cells were also MHC-II^{dull}.

These experiments indicate that T-cells ($CD5^{bright}$, MHC-II^{dull}) can be driven to express the NK antigen identified by 1F8 following 48 hour culture with PMA/Ca^{2+} ionophore-supplemented media. Such activated T-cells are referred to as "NKT" cells in view of experiments carried out elsewhere (Rau *et al.*, 2002).

5.3.1.2 Larval *Xenopus*

PMA (10ng/ml) and calcium ionophore (20ng/ml) stimulation of larval splenocytes and thymocytes elicited increases in FS and SS similar to those observed in adults (*Fig. 5.7*). Following 48 hour culture with PMA and Ca^{2+} ionophore, some $2B1^{bright}$ larval T-cells became $1F8^{+ve}$, with 10% and 16% of splenocytes and thymocytes co-staining respectively. $AM20(MHC\text{-}II)^{bright}$ cells remained $1F8^{-ve}$, whereas 11% of splenocytes expressed both the 1F8 antigen and $AM20(MHC\text{-}II)^{dull}$. In contrast to adult *Xenopus*, IgM expression on larval splenocytes was not lowered by PMA/Ca^{2+} ionophore-induced stimulation and remained at 60%. The lack of 1F8 and D8 co-staining on splenocytes confirms the absence of the 1F8 antigen on B-cells.

5.3.2 Cytotoxic potential of PMA/Ca^{2+} ionophore-stimulated cells

After 48 hours *in vitro*, both non-stimulated and PMA/Ca^{2+} ionophore-stimulated Tx splenocytes displayed high levels of cytotoxicity (monitored by 6 hour JAM assays) towards B_3B_7 tumour targets (*Fig. 5.8*). Although unstimulated splenocytes from control frogs were moderately cytotoxic towards tumour cells, stimulated splenocytes were either unable to kill (*Fig. 5.8*), or their killing capacity was impaired (data not shown).

The use of annexin assays to probe for target cell apoptosis revealed that following culture for 6 hours with control splenocytes at an E:T ratio of 5:1, 9% intact (Gate A) B_3B_7 targets were annexin-V^{+ve} (*Fig. 5.9*). In contrast, following 6 hour culture with PMA/Ca^{2+} ionophore-stimulated splenocytes at the same E:T ratio, only 2% B_3B_7 cells were positive. Additional assays to monitor target cell apoptosis were carried out using the ASP assay. As *Table 5.3* shows, only 7% ASP^{+ve} B_3B_7 cells were found after co-culture with PMA/Ca^{2+} ionophore-stimulated cells at 5:1, compared to 19% when the effector population was cultured in medium alone.

In order to determine the cytotoxicity of $1F8^{+ve}$ T-lymphocytes ("NKT"-cells), control splenocytes were cultured for 48 hours in PMA/Ca^{2+}ion-supplemented media and immunomagnetically sorted using the 1F8 mAb. This 1F8-enriched population will contain NKT-cells together with a low level of true NK cells. This "NKT"-enriched population did not show the degree of cytotoxicity demonstrated by 1F8-enriched Tx populations (containing only NK cells) (in Chapter 3). JAM assays (*Fig. 5.8*) revealed the cytotoxicity of the "NKT"-enriched population to be a mere 12% at the highest E:T ratio. Similarly, annexin-V detection assays showed only 2% B_3B_7 target cells falling in gate A became apoptotic following incubation with 1F8-purified cells from stimulated control spleen in comparison to 32% following culture with 1F8-purified cells from Tx animals (*Fig. 3.9*). ASP-detection studies further confirmed this lack of significant cytotoxicity by the "NKT"-enriched population; only 10% B_3B_7 target cells became apoptotic in comparison to 30% following incubation with purified NK cells from Tx animals (*Table 3.1*).

5.3.3 Immunoprecipitation experiments on medium and PMA/Ca^{2+}ionophore-cultured cells

PMA and calcium ionophore stimulation was used to generate 1F8 antigen on the surface of T-cells from both thymus and spleen. PMA/Ca^{2+} ionophore-stimulated cells, together with various medium-cultured populations, including 1F8-sorted cells from Tx frogs, were surface-labelled with biotin and immunoprecipitated using the 1F8 mAb to further characterise the 1F8 antigen. Further immunoprecipitations were also carried out using ^{35}S-labelled cytoplasmic lysates of control splenocytes incubated for 48 hours with PMA (10ng/ml) and ionomycin (200ng/ml), which caused significant increases in 1F8 expression on the surface of T-cells.

5.3.3.1 Immunoprecipitations on biotin-labelled cells

As *Fig. 5.10* illustrates, three bands at approximately 60-65kDa were precipitated with the control IgG2b antibody (α-*Aspergillus niger* glucose oxidase, DAKO) and were therefore regarded as non-specific. Lysates from thymocytes cultured in

medium alone, produced no further precipitations, although the 1F8 mAb precipitated two faint bands of protein from control spleen lysates at approximately 45 and 55 kDa (the bands were faint and therefore not readily visible following scanning). Two bands of approximately 45 and 55kDa were observed following 1F8 immunoprecipitation with lysates of control splenocytes and thymocytes which had been cultured for 48 hours with PMA and calcium ionophore. 1F8 immunoprecipitation of lysates from Tx spleen also produced two protein bands, again at 45 and 55kDa. These bands were extremely strong in $1F8^{+ve}$ MACS-sorted cells.

5.3.3.2 Immunoprecipitations on ^{35}S-labelled cells

1F8 immunoprecipitations of ^{35}S-labelled cytoplasmic lysates of control splenocytes cultured for 48 hours in PMA (10ng/ml) and ionomycin (200ng/ml) *(Fig 5.11)* produced a unique protein band at approximately 55kDa which was not precipitated by the control antibody X71 (α-CTX). Precipitated surface proteins have not been included in this thesis as these experiments were carried out whilst visiting a collaborating laboratory in Rochester, New York. Unfortunately my stay was not long enough to enable me to obtain appropriate exposures of precipitated surface proteins.

5.4 Discussion

5.4.1 Phenotypic and morphological changes associated with culture with PMA and calcium ionophore

48 hour culture of splenocyte (both control and Tx) and thymocyte populations with PMA and Ca^{2+} ionophore results in significant increases in both size and granularity (*Fig. 5.5*) at all concentrations tested. Similar changes in morphology are also observed with stimulated control splenocytes and thymocytes of 7 week-old larvae, this phenomenon representing the transformation of lymphocytes into lymphoblasts. Dramatic changes in antigen expression are also observed, which vary with differing concentrations of PMA and calcium ionophore.

Preliminary trials using a variety of PMA and Ca^{2+} ionophore concentrations were initially performed using control splenocytes and thymocytes only. Control splenocyte cultures supplemented with 10ng/ml PMA show dramatic increases in NK antigen expression, heightened CD5 expression and a reduction in surface IgM. Further additions of 100ng/ml Ca^{2+} ionophore cause further increases in CD5 and CD8 expression and further reductions in surface IgM. Similar increases in NK antigen expression are observed when this PMA/Ca^{2+} ionophore concentration is added to thymocyte cultures, although CD5 and CD8 expression are seen to decrease. Alterations in concentrations of these mitogenic agents have more varying effects on thymocyte antigen expression in comparison to that of control splenocytes. Therefore thymocytes were used for the remainder of the preliminary trials. Low PMA concentrations (1ng/ml PMA + 100ng/ml Ca^{2+} ionophore) results in an increase in expression of the 1F8 antigen, but not 4D4 or 1G5 antigens, implying that the 1F8 antigen is more susceptible to changes in PMA concentrations than 4D4 and 1G5 antigens. Low concentrations of both mitogens (1ng/ml PMA + 20ng/ml Ca^{2+} ionophore) produces only slight increases in NK antigen expression.

As a result of these preliminary trials, it was decided that 10ng/ml PMA together with 20ng/ml Ca^{2+} ionophore would be routinely used for the future investigations on the effects of culture in mitogen-supplemented media, as this combination induces consistent and reproducible increases of NK antigen expression, and also causes less cell death than when higher doses of Ca^{2+} ionophore are used. Surface IgM expression is habitually lost, indicating activation of the B-cell population. It has been reported that PMA-activation of amphibian (Jurgens *et al.*, 1995), murine (Gravenor, 1996) and human (Zupo *et al.*, 1994) B-cells results in the induction of CD5 expression on the surface of the B-cells. This explains the overall increase of CD5 levels in control splenocyte populations following PMA/Ca^{2+} ionophore stimulation and also explains the lack of such an increase in the thymus, where B-cells are absent.

To effect the described changes in antigen expression following 24 hour exposure to PMA and Ca^{2+} ionophore, it is essential to allow a further culture period of 24 hours in medium alone. If this is not permitted, no increase in NK antigens are observed. This is perhaps not surprising given that PMA-induced DNA synthesis does not occur until after 24 hours (Ruben *et al.*, 2000).

In contrast to control splenocytes, PMA (10ng/ml) and calcium ionophore (20ng/ml) stimulation of Tx splenocytes has no affect on NK antigen expression, suggesting that the increases in NK antigen expression seen on cells from control frogs are occurring on T-cells. This is substantiated by dual colour flow cytometric analysis of PMA/Ca^{2+} ionophore-stimulated control splenocytes and thymocytes (see below). As in control splenocyte populations, decreases in surface IgM are apparent, again indicating activation of the B-cell population. However, as CD5 expression on activated B-cells is a T-cell-dependent phenomenon (Gravenor, 1996), heightened CD5 expression (as seen in stimulated control populations) is not observed in these T-cell-deficient models.

Dual colour flow cytometric analysis (*Fig. 5.6*) highlighted two populations of $CD5^{+ve}$ cells, $CD5^{bright}$ and $CD5^{dull}$. As mentioned previously, in the presence of T-cells (Gravenor, 1996), PMA-induced B-cells have the capacity to express $CD5^{dull}$ (Jurgens

et al., 1995; Gravenor, 1996) and it is therefore likely that the $CD5^{dull}$ population seen here represents the B-cell subset. These cells remain $1F8^{-ve}$. Induction of the 1F8 antigen occurs on the $CD5^{bright}$ population, suggesting that the NK antigen is being expressed by PMA-activated T-cells. Antibodies directed against MHC class-II (AM20) also discern two populations of lymphocytes, MHC-II^{bright} and MHC-II^{dull}, which correspond to B-cells and T-cells respectively (Gravenor, 1996). Co-staining with the 1F8 mAb confirms that NK antigen induction is specific to the T-cell (MHC-II^{dull}) population.

This notion is further supported by the observation that a proportion of the $CD8^{+ve}$ T-cell subset (identified by AM22 mAb) become $1F8^{+ve}$ upon stimulation with PMA and Ca^{2+} ionophore. This agrees with recent studies which show that immunomagnetically-purified *Xenopus* $CD8^{+ve}$ T-cells express the 1F8 antigen following PMA and ionomycin stimulation (Rau *et al.*, 2002). This subset expressing both T-cell and NK cell antigens is not simply NK cells which have undergone PMA/Ca^{2+} ionophore-induced increases in expression of both 1F8 and T-cell antigen, but instead are viable T-cells which have been induced to display NK antigens on their surface. These cells have been found to express TCRVβ6 mRNA and are denoted "NKT"-cells (Rau *et al.*, 2002). The emergence of 1F8 on larval splenic and thymic $CD5^{bright}$ cells following PMA and Ca^{2+} ionophore stimulation, suggests that "NKT"-cells can be identified prior to metamorphosis. A similar subset of cells has been recorded in catfish, where IL-2-mediated activation of T-cells results in the expression of the NK-like antigen NCC (Harris *et al.*, 1991).

5.4.2 Cytotoxic potential of PMA and Ca^{2+} ionophore-stimulated cells

All cytotoxicity assays employed concur that 48 hour culture of control splenocytes in media supplemented with PMA (10ng/ml) and Ca^{2+} ionophore (20ng/ml), impairs their cytotoxicity towards MHC-I deficient tumour target cells. 1F8-enriched stimulated control splenocytes containing "NKT"-cells together with a low level of NK cells display poor cytotoxicity. Such observations also relate to experiments by

Rau et al (2002), who reported that alloantigen-reactive *Xenopus* $CD8^{+ve}$ T-cells were left unable to kill in an MHC-specific fashion following further culture in PMA/ionomycin-supplemented media. Similarly, Ayroldi et al (1993) demonstrated PMA-mediated inhibition of the lytic potential of murine mature NK cells against YAC-1 NK-sensitive targets.

Tx splenocytes cultured in PMA/Ca^{2+} ionophore-supplemented media retain their ability to lyse tumour target cells, which implies that cytotoxic suppression of lymphoid populations cultured in PMA/Ca^{2+} ionophore is <u>not</u> due to a non-specific mechanism (such as PMA/Ca^{2+} ionophore-mediated damage to cell surface receptors). It may well transpire that cytotoxic inhibition is due to the formation of a suppressor T-lymphocyte subset which has the ability to inhibit the cytolytic potential of other cells (e.g. NK cells) within the effector population.

5.4.3 PMA, Ca^{2+} ionophore and ionomycin as a tool to generate 1F8 antigen

PMA and calcium ionophore were used here as a tool to generate high expression of the 1F8 antigen, which could then be characterised further by immunoprecipitations using biotin to label surface proteins and ^{35}S to metabolically label both surface and cytoplasmic proteins. Immunoprecipitations were also carried out using Tx splenocyte lysates to ensure that the 1F8 antigen generated on the surface of T-cells by PMA/Ca^{2+} ionophore stimulation is the same antigen as that naturally expressed by NK cells of Tx animals.

Biotin surface labelling shows that the α-NK mAb 1F8 precipitates two surface proteins from Tx 1F8-enriched splenocyte lysates and from PMA/Ca^{2+}-ionophore-stimulated control splenocyte and thymocyte lysates, at approximately 45 and 55kDa. The very faint bands apparent following 1F8 immunoprecipitation of lysates from 1F8-depleted Tx splenocytes is most likely due to the fact that approximately 5% $1F8^{+ve}$ cells remained in the 1F8-depleted population as demonstrated by flow cytometry (data not shown).

Immunoprecipitations using ^{35}S-labelled cytoplasmic lysates of stimulated control splenocytes herald an endogenously produced protein, also of 55kDa, precipitating specifically with the 1F8 mAb. (Very recent experiments indicate that 1F8-precipitation of ^{35}S-labelled surface lysates of stimulated control splenocytes also identifies a protein band of 55kDa, Dr. J Robert, Pers. Comm., Horton *et al.*, 2002, in preparation). It is not clear why immunoprecipitation following surface biotinylation gives both a 55 and 45kDa band, whereas immunoprecipitations following ^{35}S metabolic labelling yields only a 55kDa band. It is this larger protein which is common to both types of immunoprecipitation (biotin- and ^{35}S-labelling), suggesting this band to be the true NK antigen. Reducing agent is added to all SDS loading buffers signifying that this protein is a single chain and not dimeric. It is possible that the smaller 45kDa protein is co-precipitating with the 55kDa protein as a direct result of biotinylation. Alternatively, the smaller band may be the result of protein degradation. As all investigations are carried out using outbred *Xenopus*, together with the fact that results are 100% reproducible, it is unlikely for allelic polymorphism to be the cause of the two bands obtained following biotinylation. Preliminary deglycosylations experiments were carried out on biotin-labelled 1F8-immunoprecipitated proteins, but these failed to alter the size or number of protein bands found (data not shown), suggesting that the 10kDa difference in band size is not due to glycosylations of the 45kDa protein. It has also been suggested (J. Robert, Pers. Comm., University of Rochester, NY) that the 45kDa protein precipitated by 1F8 may not be cysteine/methionine-rich and therefore fails to label with ^{35}S.

These investigations have revealed a discrepancy between immunoprecipitation and Western blot data, where the 1F8 mAb identified proteins of 66-85kDa (Horton *et al.*, 2000). This difference may be due, in some way, to the fact that for Western blotting, the cell lysate is boiled prior to incubation with the 1F8 mAb, whereas in immunoprecipitation, 1F8 detects its antigen in its native state. In general, immunoprecipitations have proved to be more consistent, with the two different techniques described (both surface and metabolic labelling) concurring the identification of a 55kDa protein. Clearly, further studies are required to identify the molecular nature of the 1F8 antigen.

Table 5.1: Preliminary trials: Percentage of mAb-defined lymphocytes in adult *Xenopus* lymphoid tissues following culture in varying PMA/Ca^{2+} ionophore concentrations as determined by flow cytometry

CONTROL SPLEEN		mAb					
PMA/Ca^{2+}ion (ng/ml)	**Culture period**	**1F8 (anti-NK)**	**4D4 (anti-NK)**	**1G5 (anti-NK)**	**2B1 (anti-CD5)**	**AM22 (anti-CD8α?)**	**D8 (anti-IgM)**
-/-	**48 hr**	6 ± 1.2	6 ± 1.5	5 ± 0.33	65 ± 2.5	23 ± 2.1	30 ± 1.5
10/-	**48 hr**	38 + 2.0	40 + 1.5	44 + 1.2	72 + 3.8		22 + 3.8
10/100	**48 hr**	33 + 1.3	29 + 2.4	38 + 1.5	91 + 2.0	34 + 1.9	5 + 0.29

THYMUS		mAb					
PMA/Ca^{2+}ion (ng/ml)	**Culture period**	**1F8 (anti-NK)**	**4D4 (anti-NK)**	**1G5 (anti-NK)**	**2B1 (anti-CD5)**	**AM22 (anti-CD8α?)**	**D8 (anti-IgM)**
-/-	**48 hr**	3 + 0.33	3 + 0.67	2 + 0.41	76 + 2.8	62 + 2.5	2 + 0.33
10/100	**48 hr**	50 + 3.1	46 + 1.6	38 + 2.8	69 + 2.1	35 + 1.2	4 + 0.33
1/100	**48 hr**	43 + 1.5	10 + 1.4	7 + 1.2	61 + 2.8	27 + 2.5	5 + 0.33
1/20	**48 hr**	14 + 2.3	9 + 0.58	6 + 0.33	64 + 2.8	26 + 0.67	4 + 0.33

Values represent the mean percentage ± standard error where number of adult *Xenopus* analysed ≥3. "?" = "putative".

Table 5.2: Percentage of mAb-defined lymphocytes in adult *Xenopus* lymphoid tissues following culture in 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore as determined by flow cytometry

CONTROL SPLEEN		mAb					
PMA/Ca^{2+}ion (ng/ml)	Culture period	1F8 (anti-NK)	4D4 (anti-NK)	1G5 (anti-NK)	2B1 (anti-CD5)	AM22 (anti-CD8α?)	D8 (anti-IgM)
-/-	48 hr	6 ± 1.2	6 ± 1.5	5 ± 0.33	65 ± 2.5	23 ± 2.1	30 ± 1.5
10/20	24 hr	7 + 1.2					
	48 hr	49 + 2.4	45 + 4.5	41 + 3.5	89 + 1.6	31 + 1.5	10 + 1.7

THYMUS		mAb					
PMA/Ca^{2+}ion (ng/ml)	Culture period	1F8 (anti-NK)	4D4 (anti-NK)	1G5 (anti-NK)	2B1 (anti-CD5)	AM22 (anti-CD8α?)	D8 (anti-IgM)
-/-	48 hr	3 + 0.33	3 + 0.67	2 + 0.41	76 + 2.8	62 + 2.5	2 + 0.33
10/20	24 hr	5 + 1.2					
	48 hr	31 + 2.8	28 + 2.8	24 + 2.8	67 + 3.1	22 + 1.2	3 + 0.33

Tx SPLEEN		mAb					
PMA/Ca^{2+}ion (ng/ml)	Culture period	1F8 (anti-NK)	4D4 (anti-NK)	1G5 (anti-NK)	2B1 (anti-CD5)	AM22 (anti-CD8α?)	D8 (anti-IgM)
-/-	48 hr	20 ± 2.4	18 ± 2.0	15 ± 2.4	16 ± 3.8	10 ± 3.5	52 ± 2.1
10/20	48 hr	12 + 0.58	10 + 0.58	11 + 0.33	14 + 0.33	4 + 2.1	12 + 0.88

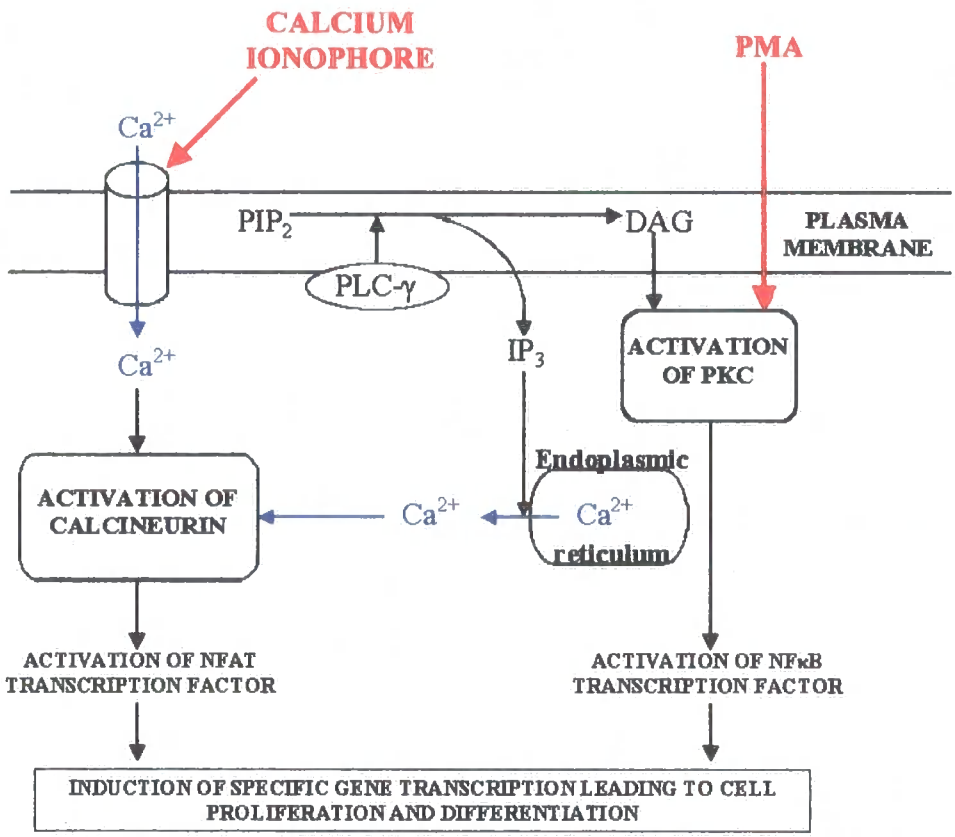
Values represent the mean percentage ± standard error where number of adult *Xenopus* analysed ≥3. "?" = "putative".

Table 5.3: Percentage of B_3B_7 target cells staining positive for ASP following 6 hour culture in medium alone, or after co-culture with *Xenopus* lymphoid populations cultured in medium alone, or in medium supplemented with 10ng/mlPMA and 20ng/ml Ca^{2+} ionophore

Target cells cultured with	**% ASP^{+ve} cells**
Medium	1 ± 0.33
Unseparated control splenocytes	19 ± 1.2
Unseparated control splenocytes + PMA/Ca^{2+}ion	7 ± 1.5
NKT-enriched	10 ± 0.5

Mean % ± standard error where number of adult *Xenopus* analysed =3

Fig. 5.1: Molecular mechanisms of mitogens



(Adapted from Abbas *et al.*, 1991)

Fig. 5.2: Flow diagram summarizing biotin immunoprecipitation.

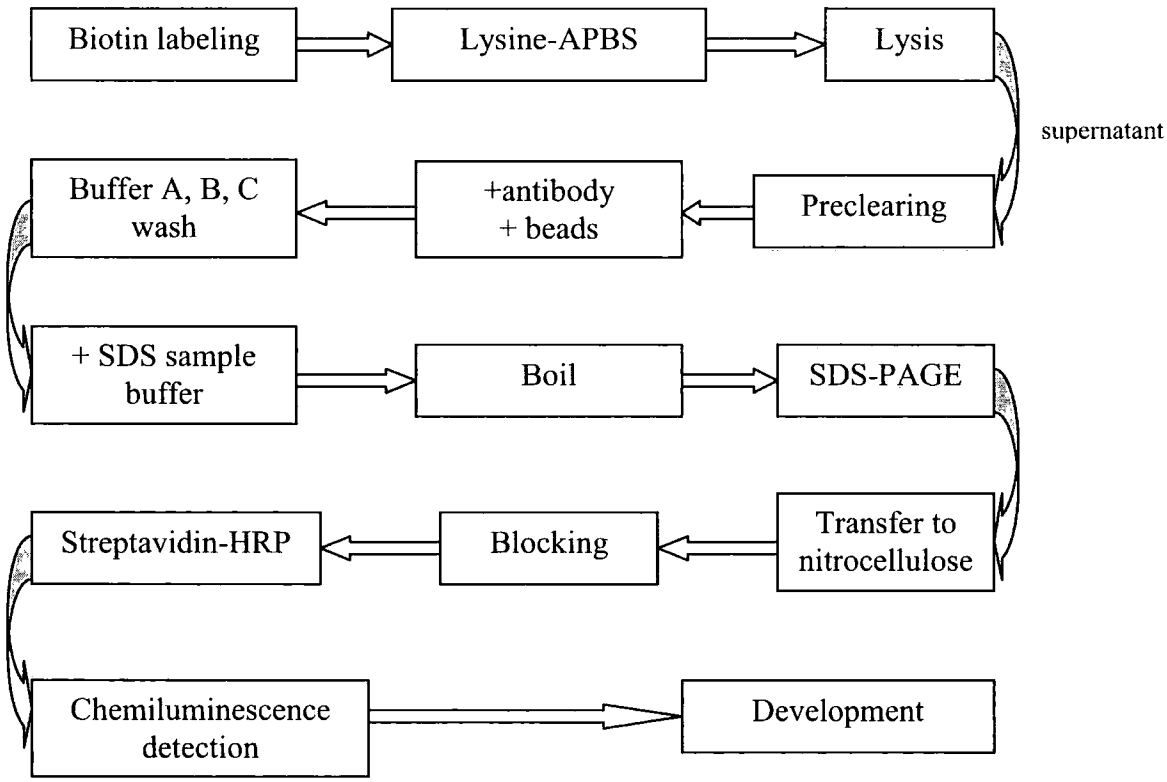


Fig. 5.3: Flow diagram summarizing ^{35}S sequential immunoprecipitation.

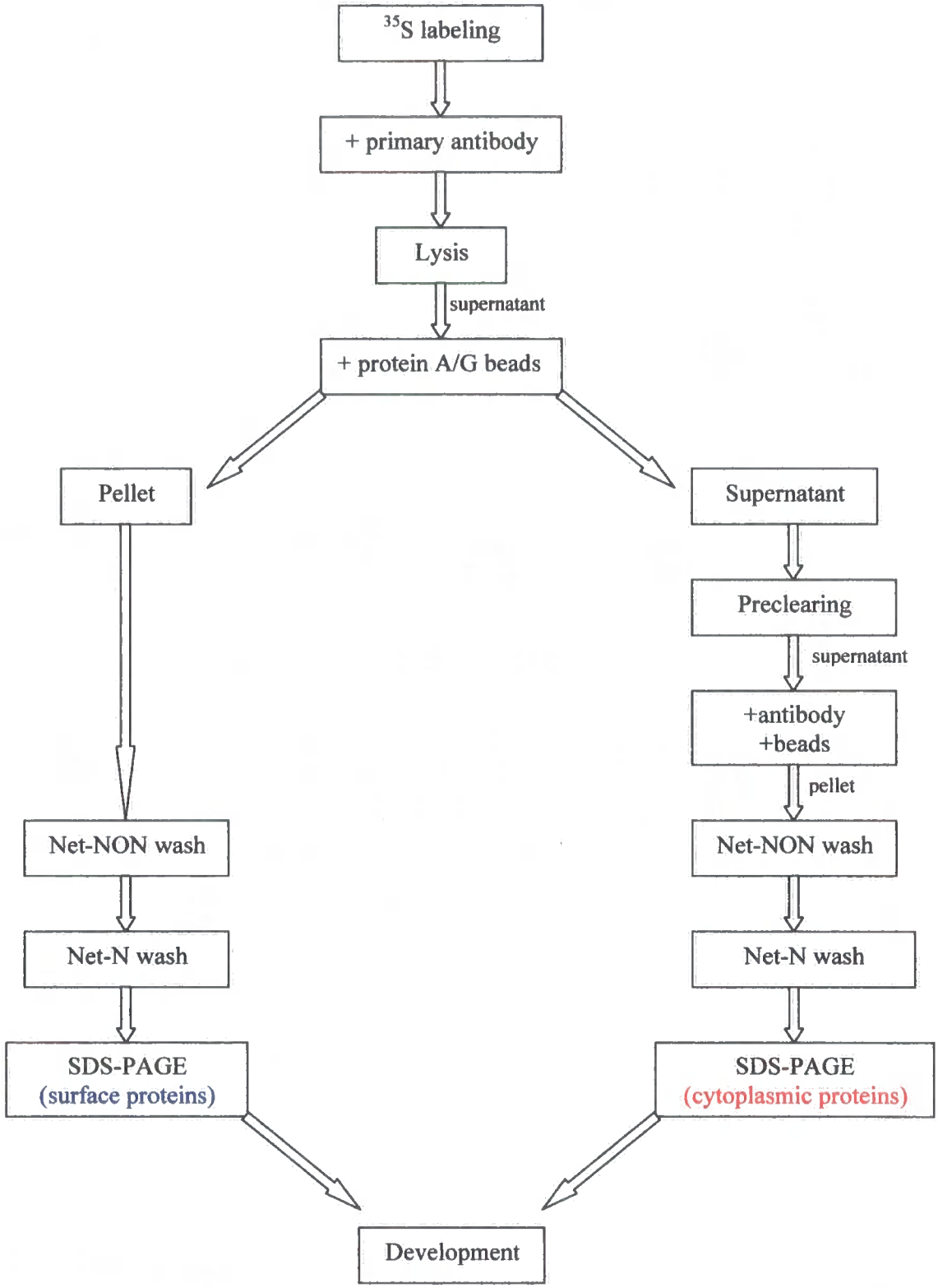
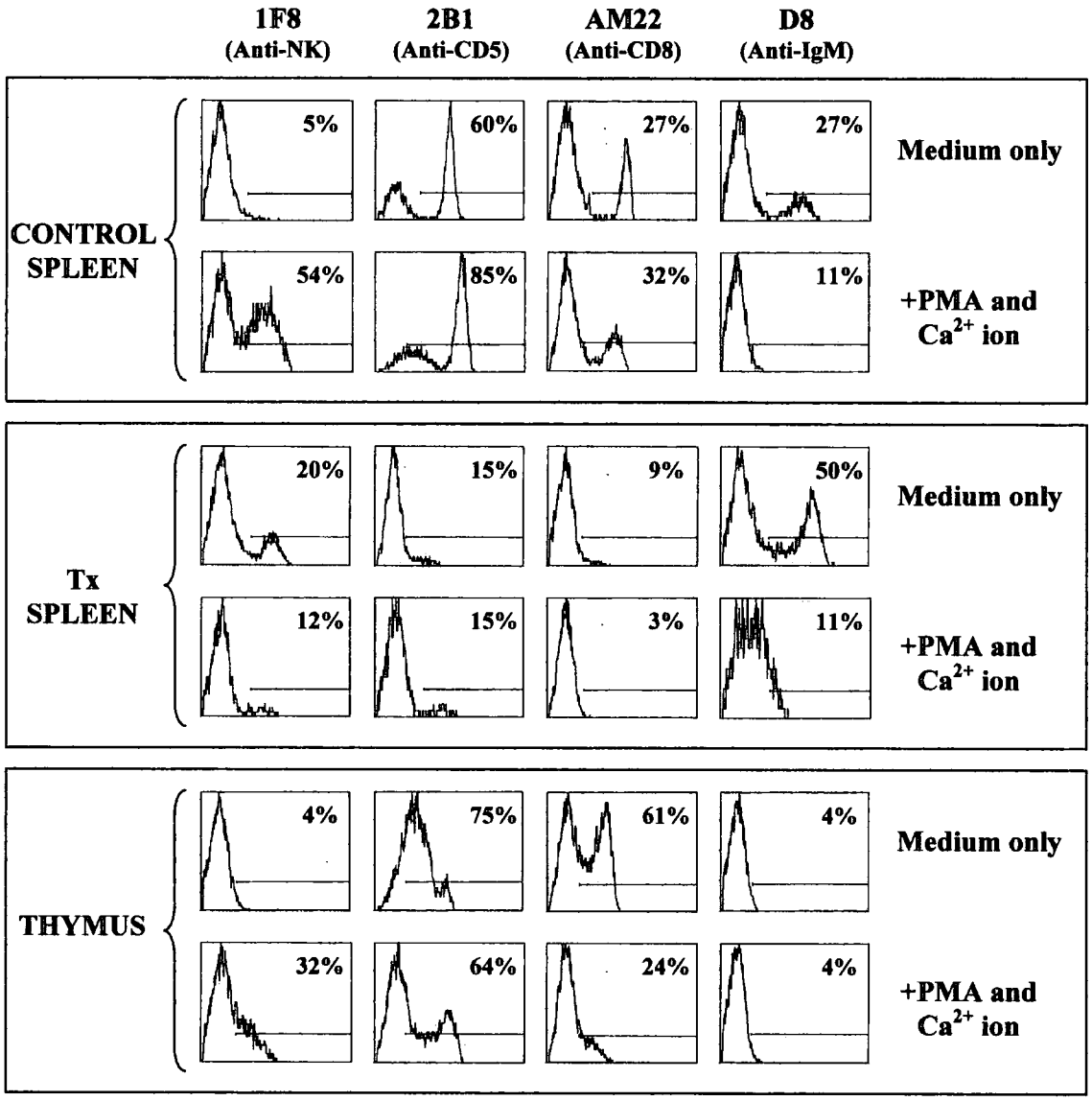


Fig. 5.4: Single colour flow cytometric data to demonstrate the percentage of mAb-defined lymphocytes in adult *Xenopus* lymphoid tissues following 48 hour culture in medium with or without supplements of 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore



Typical data is shown representing ≥ 3 data sets; markers were set to exclude 98% cells stained with control reagents

Fig. 5.5: Flow cytometric traces to demonstrate the change in FS and SS of adult *Xenopus* thymocytes and splenocytes following 48 hour culture in PMA and Ca^{2+} ionophore

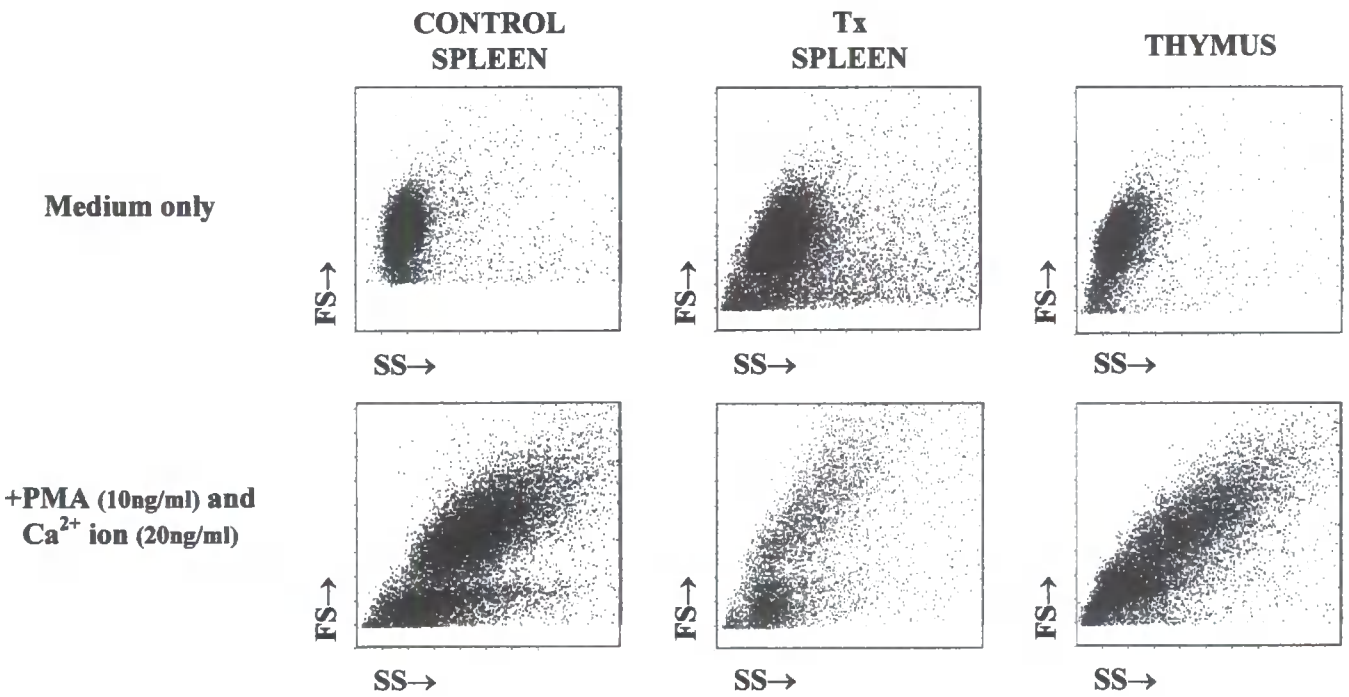
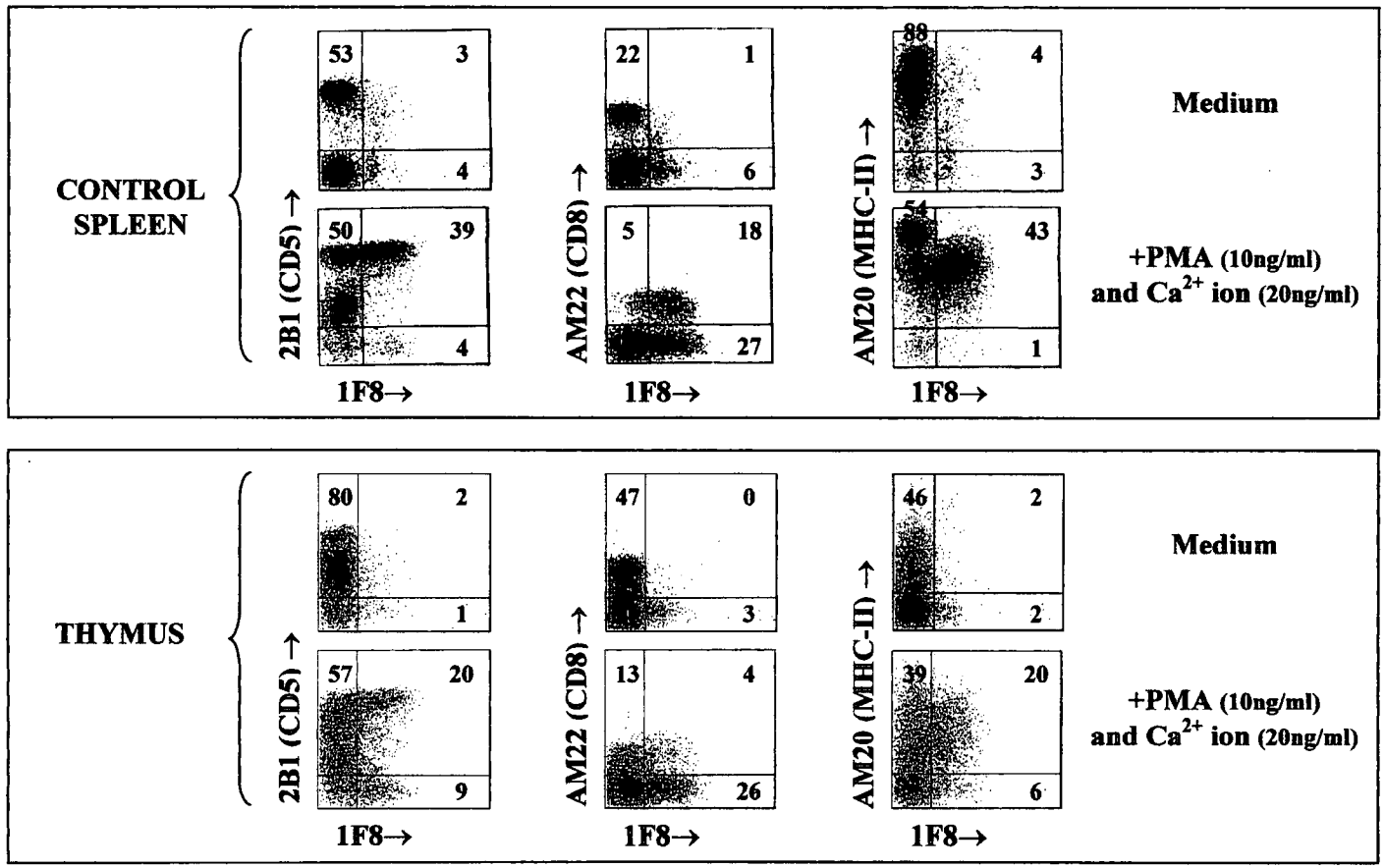
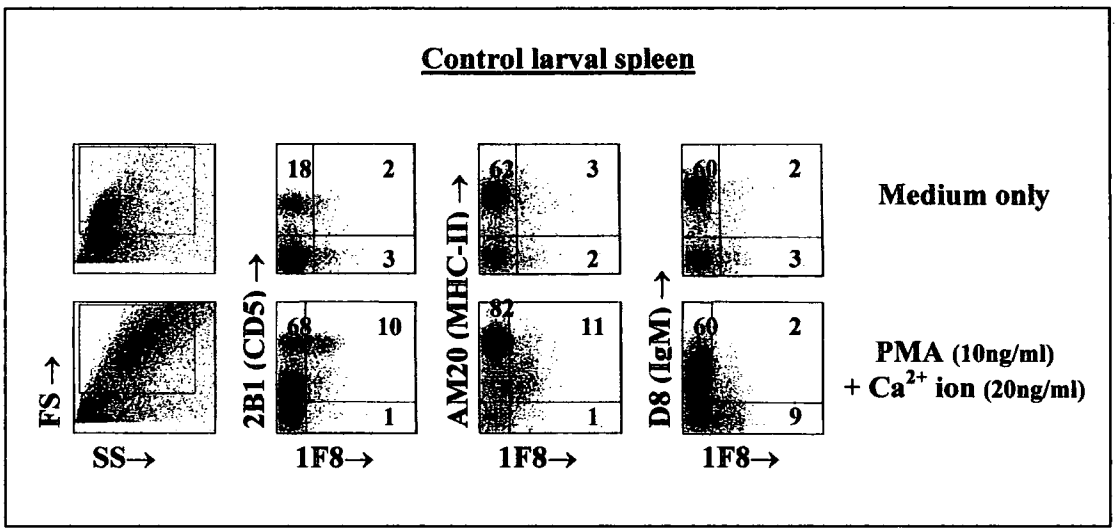


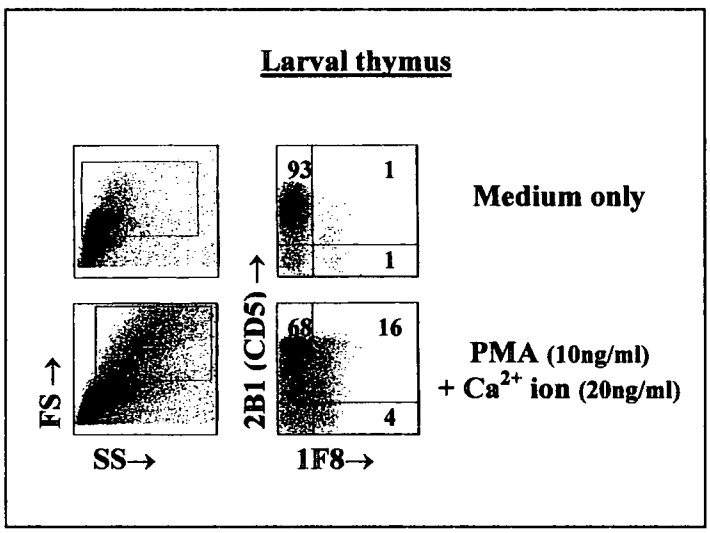
Fig. 5.6: Dual colour flow cytometric analysis to demonstrate the proportion of cell surface antigens on lymphocytes in adult *Xenopus* thymus and control and Tx spleen following 48 hour culture in medium with or without supplements of 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore



Typical data is shown representing ≥3 data sets; quadrants were set to exclude 98% cells stained with control reagents

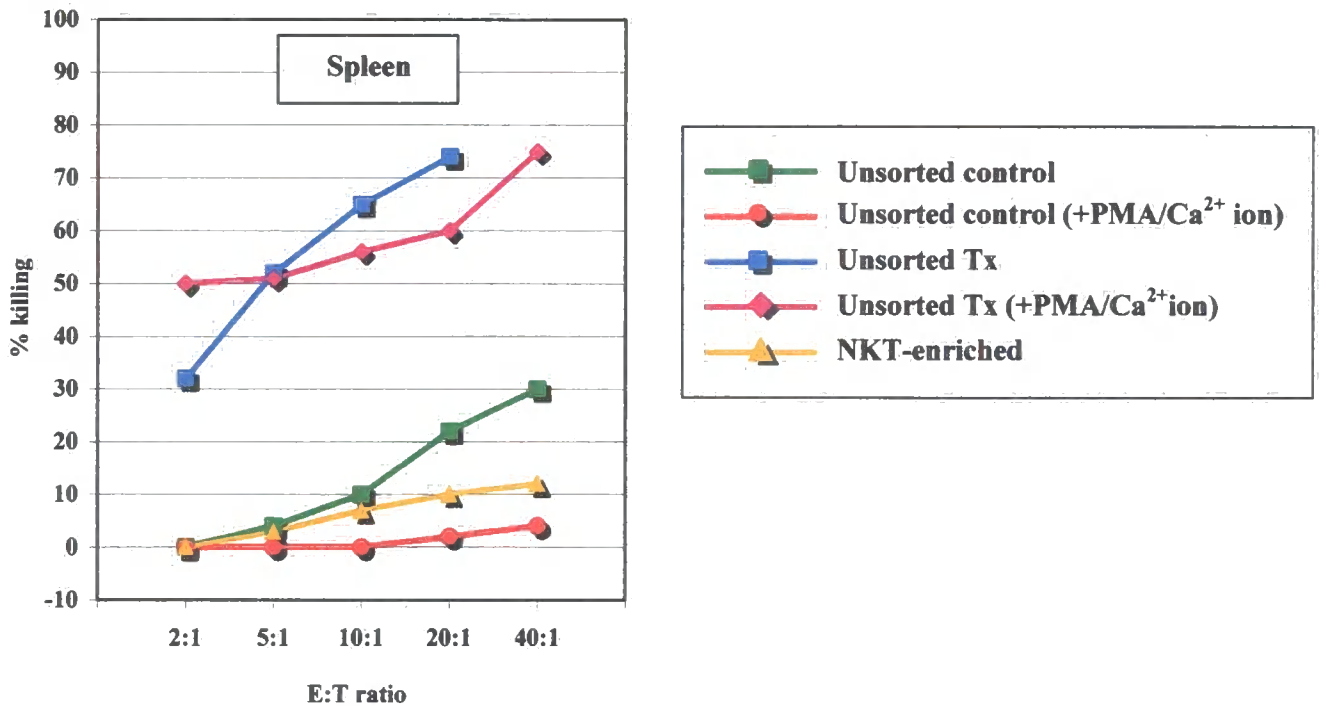
Fig. 5.7: Dual colour flow cytometric analysis to demonstrate the change in FS and SS, and the proportion of cell surface antigens on lymphocytes in 7 week-old larval *Xenopus* thymus and control spleen following 48 hour culture in medium with or without supplements of 10ng/ml PMA and 20ng/ml Ca^{2+} ionophore





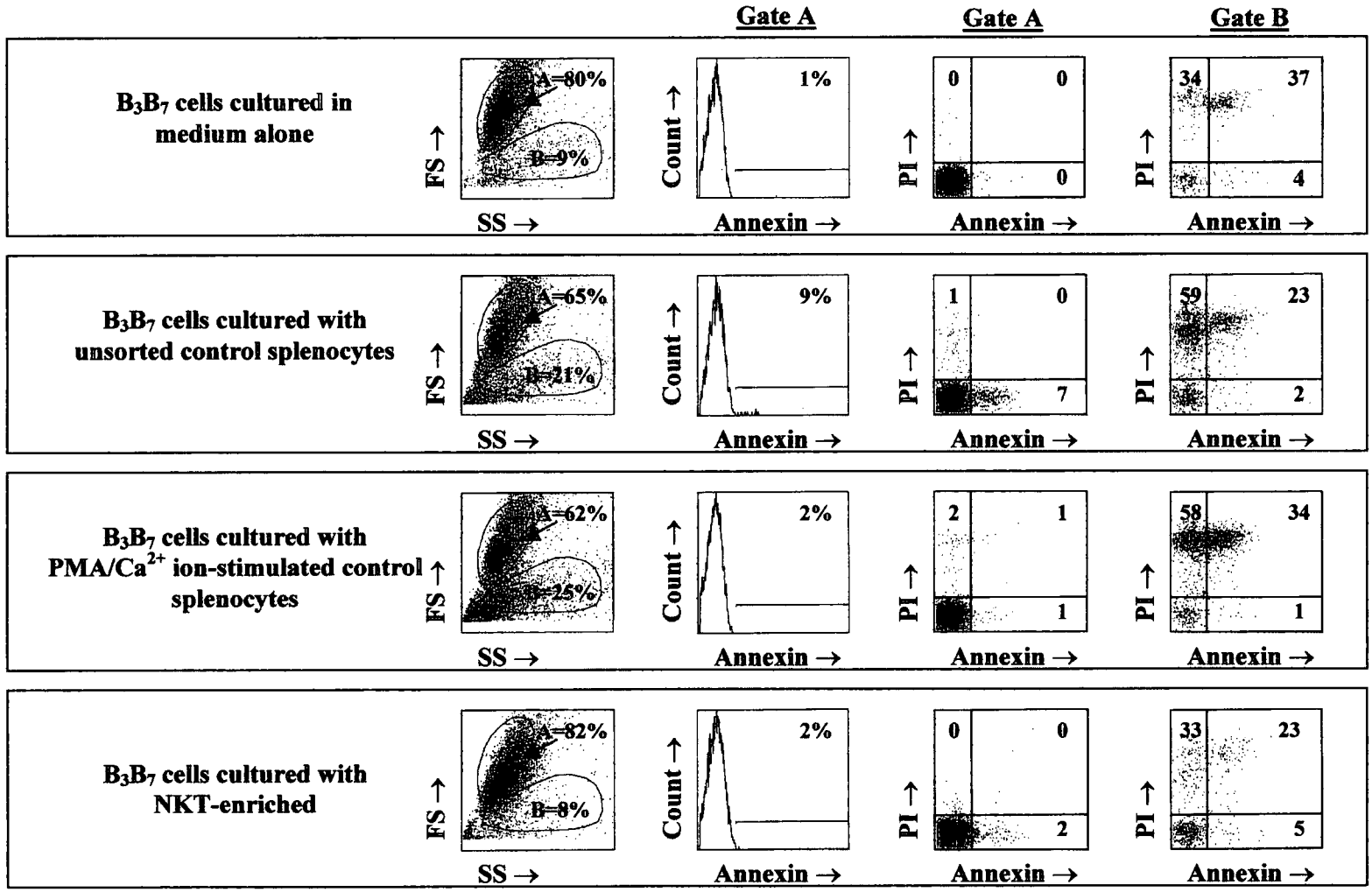
Typical data is shown representing ≥3 data sets; quadrants were set to exclude 98% cells stained with control reagents

Fig. 5.8: % DNA loss from B_3B_7 target cells following 6 hours co-culture with *Xenopus* lymphoid populations cultured in medium alone (+/- GFM), or in medium supplemented with 10ng/mlPMA and 20ng/ml Ca^{2+} ionophore



Values shown represent mean % killing. Control cells were cultured without GFM whereas Tx populations were cultured in GFM-supplemented media.

Fig. 5.9: Flow cytometric analysis of annexin-V/PI binding of B_3B_7 cells cultured for 6 hours either alone or after culture with *Xenopus* lymphoid populations pre-cultured in medium alone, or in medium supplemented with 10ng/mlPMA and 20ng/ml Ca^{2+} ionophore

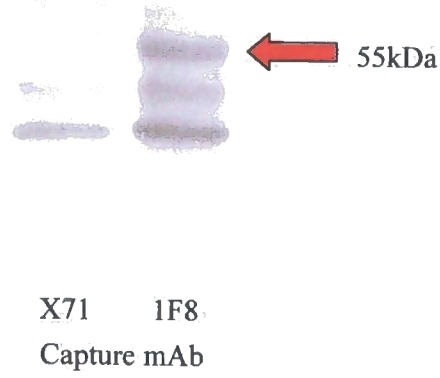


Typical data is shown representing 3 data sets; markers and quadrants were set to exclude 98% cells stained with control reagents. Gate A represents PI^{-ve} intact cells; gate B represents cells with lower FS/higher SS than intact population, many are PI^{+ve}. All effectors and targets were cultured at an E:T ratio of 5:1.

Fig. 5.10: Immunoprecipitation of surface biotinylated splenocytes and thymocytes cultured for 48 hours in medium alone or in medium supplemented with 10ng/mlPMA and 20ng/ml Ca^{2+} ionophore

The lysate from $5x10^6$ cells was used for each immunoprecipitation

Fig. 5.11: Immunoprecipitation of ^{35}S-labelled splenocytes cultured for 48 hours in medium supplemented with 10ng/mlPMA and 200ng/ml ionomycin



The cytoplasmic lysate from $5x10^6$ cells was used for each immunoprecipitation

CHAPTER 6

Expression and ontogeny of *Xenopus* β2-microglobulin

6.1 Introduction

Mammalian β2m is a 12kDa protein which non-covalently associates with the heavy chain of classical MHC class-Ia molecules (Salter-Cid *et al.*, 1998). β2m can also be found free in serum (Ono *et al.*, 1993), or as a subunit of non-classical MHC class-Ib proteins, such as CD1 (Porcelli *et al.*, 1998). It is essential for MHC-I surface expression and for the correct folding and loading of displayed peptides (Hansen *et al.*, 1988; Vitiello *et al.*, 1990). β2m is also thought to contribute to associations between murine MHC-I (H2-D) and the NK inhibitory receptor Ly49A. It has been proposed that such interactions are dependent on the presence of the β2m residue cluster [Lys-Thr-Thr-Gln], or that the 12kDa molecule plays a vital role in determining the conformation of the distal regions of MHC-I (Michaelsson *et al.*, 2001). β2m has also been implicated in the regulation of CD69 expression on T-cells (Paczek *et al.*, 2001). In mammals, β2m is coded for by one gene locus (Shand and Dixon, 2001) and although invariant in humans, the molecule does show some polymorphism in mice (Shum *et al.*, 1996; Owen, 1998). Structural similarities to Ig-like domains of MHC-I and MHC-II imply the existence of a common ancestor, most likely encoded in the MHC (Shum *et al.*, 1996). However, the gene locus of mammalian β2m is encoded outside the MHC, on chromosome 2 in mice and chromosome 15 in humans (Hay and Westwood, 1998), and it is therefore feasible that during evolution, the β2m gene became translocated to a location outside the MHC (Shum *et al.*, 1996).

Investigations into lower vertebrate β2m show the molecule is relatively conserved throughout evolution (Shand and Dixon, 2001), and may prove integral in determining the origin of MHC (Criscitiello *et al.*, 1998). As in mammals, chicken β2m is encoded by a single gene and is shown to be approximately 14.5kDa by SDS-PAGE (Skjodt *et al.*, 1986). Teleost β2m lacks amino acids 85 and 86 in exon 3 (Dixon *et al.*, 1993; Ono *et al.*, 1993; Shum *et al.*, 1996) due to a two codon deletion and is therefore two amino acids shorter than mammalian β2m (Ono *et al.*, 1993). Most teleost species (such as Zebrafish, Ono *et al.*, 1993) and channel catfish have one β2m gene locus and therefore most likely encode invariant β2m molecules

(Criscitiello *et al.*, 1998). Common carp β2m however, which has high similarity to rabbit β2m, has two gene loci (Dixon *et al.*, 1993), a feature most probably due to the tetraploid genome. Another tetraploid species, the Siberian sturgeon (belonging to the Chondrostei) also has two β2m gene loci (Lundqvist *et al.*, 1999) and shows high sequence homologies to warm-blooded vertebrates (Criscitiello *et al.*, 1998). Sturgeon β2m is similar in length to human β2m (due to the lack of the 2 codon deletions as seen in teleost fish, Lundqvist *et al.*, 1999). The rainbow trout also has a tetraploid genome, but has polymorphic β2m molecules encoded by multiple genes (Shum *et al.*, 1996). In fact, ten different β2m sequences have been cloned from an individual rainbow trout. The tetraploidy of the species does not account for such high β2m sequence diversity and it has therefore been proposed that the β2m gene may have remained in the MHC, subsequently undergoing duplications, giving rise to allele diversity (Shum *et al.*, 1996).

In our laboratory, a *Xenopus* β2m clone has recently been identified in a screen of a *Xenopus* spleen cDNA library (GenBank accession no. AF217962, Stewart *et al.*, 2002, in preparation). Random independent clones were sequenced and the seventh clone produced a significant match to β2m following a BLAST search of the GenBank database. The complete sequence of the insert was determined in both directions and the 5' end of the cDNA was extended by 5'-RACE to obtain the complete coding sequence, which was approximately 1.2kb in length.

The *Xenopus* β2m sequence was found to be an "intermediate" between fish and birds/mammals, displaying most homology with trout β2m. It possesses a pair of cysteines, characteristic of the β2m Ig domain structure, but was also found to have an extra cysteine residue, unique to *Xenopus* which the molecule presumably uses to covalently bind to MHC class-I heavy chains (unlike other β2m molecules, which do not possess an extra cysteine and bind non-covalently to MHC-I). This feature of *Xenopus* β2m was predicted by Dr. L. Du Pasquier, Pers. Comm., in view of the observation that MHC-I co-precipitates with β2m, the latter subsequently distinguishable from MHC-I as a 13kDa protein following denaturing SDS-PAGE (Flajnik *et al.*, 1984).

The aim of this chapter is to investigate the expression of β2m in both larval and adult cells and tissues. Four *Xenopus* cell lines are investigated for β2m expression at both the protein *(Fig. 6.3)* and mRNA *(Fig. 6.4)* level, some MHC-I^{+ve} such as the A6 kidney cell line (Rafferty, 1969), and the ff2 tumour cell line (derived from a male *ff* strain frog), and some MHC-I^{-ve} such as the 15/0 thymic lymphoid tumour cell line (derived from an LG15 clonal animal) and the B_3B_7 thymic lymphoid tumour cell line (derived from an *ff* animal Robert *et al.*, 1994). Expression of β2m is assessed through Western blotting, using a polyclonal antibody raised in mice and by RT-PCR using two sets of primers.

6.2 Methods

Further details of reagents are given in the appendices.

6.2.1 Extraction of lymphocytes

Lymphocytes were extracted and prepared as described in section 2.2.1. Larval tail sections were removed from the animal and mechanically homogenized.

6.2.2 Culture of cells

Lymphocytes from adults and larvae were cultured as described in section 2.2.2.

6.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA or RNA fragments according to size. 0.5g (1%) agarose (Gibco) was dissolved in 50ml TAE buffer (made up using RNase-free water if RNA was to be examined, see appendix 3) by microwaving for 1 minute. After a brief cooling period, 2.5µl ethidium bromide (10mg/ml) (Sigma) was added and the molten agarose was poured into a gel casting tray with combs (BioRad). The gel was left to set at room temperature for approximately 30 minutes, placed into the electrophoresis tank (BioRad) and immersed in TAE buffer containing 0.5µg/ml ethidium bromide. 1/6 volume of DNA/RNA sample loading buffer (see appendix 3) was added to the DNA or RNA samples, which were subsequently loaded into the wells. Gels were run at 90V for 20-30 minutes and DNA or RNA bands visualised using a Gel Doc 2000 transilluminator (BioRad).

6.2.4 Total RNA isolation

All apparatus in contact with RNA was previously soaked overnight in 0.1% DEPC (Sigma) and autoclaved for 30 minutes prior to use. RNase-free tips (Molecular Bio-Products) were used to pipette all solutions containing RNA.

The cells to be used for total RNA extraction were washed in APBS (see appendix 1) and aliquotted into RNase-free 1.5ml centrifuge tubes (BDH) at a concentration of 5-10x10^6/tube. Cells were centrifuged at 15,000rpm, the supernatant was removed and the cell pellet resuspended in 1ml TRI reagent (Sigma). Cells were lysed by repeated pipetting and allowed to stand for 5 minutes at room temperature. 0.2ml chloroform was added per ml of TRI reagent used and the sample shaken vigorously for 15 seconds before being allowed to stand for 10 minutes at room temperature. The tube was centrifuged at 12,000g for 15 minutes and 4°C to separate the mixture into 3 phases, a red phase at the bottom of the tube containing protein, an interphase containing DNA, and an upper aqueous phase containing RNA which was transferred to a fresh RNase-free 1.5ml centrifuge tube. Care was taken to avoid the interphase containing DNA. 0.5ml isopropanol per ml TRI reagent was added, the samples mixed gently and placed at -20°C overnight to precipitate the RNA. The sample was centrifuged at 12,000g for 10 minutes at 4°C, the supernatant removed and 1.5ml 75% ethanol added to the RNA pellet. The sample was vortexed and centrifuged at 7,500g for 7 minutes at 4°C to pellet the RNA. The ethanol was removed and the RNA pellet air-dried for 5 minutes prior to resuspension in nuclease-free water (Ambion). A small aliquot of resuspended RNA was diluted in nuclease-free water and the quantity estimated by measuring the absorbance at 260nm (A_{260}) using a Helios β spectrophotometer (Thermo Spectronic). Purity of RNA was assessed by diluting an aliquot of RNA sample in RNase-free 10mM Tris (pH 7.5) and measuring the absorbance at 260nm (A_{260}) and 280nm (A_{280}) using a Helios β spectrophotometer (Thermo Spectronic). The A_{260}/A_{280} ratio indicates the purity of RNA. RNA was only used for future experiments if this value was between 1.9-2.1. Finally, the quality of RNA was assessed by running 8-10μg RNA down a 1% agarose gel (see section 6.2.3) to ensure no degradation had occurred.

6.2.5 PCR

For each sample of DNA/cDNA, two PCR's were performed, each using the 5' primer XLB2M-5 (see appendix 3) and one of the two 3' primers, XLB2M-3[1] or XLB2M-3[2] (see appendix 3).

The following components were added to a 0.5ml thin-walled centrifuge tube (Greiner) :-

10x PCR buffer (+ $MgCl_2$) (Boehringer Mannheim)	5µl
10mM dNTP's (Gibco)	1µl
10ng DNA template	4µl
XLB2M-5 primer (Invitrogen)	1µl
XLB2M-3[1]/3[2] primer (Invitrogen)	1µl
Taq DNA polymerase (Boehringer Mannheim)	0.5µl
Autoclaved, distilled water	37.5µl

The samples were overlaid with mineral oil and the following PCR reaction was performed in Perkin Elmer thermal cycler :-

94°C	2 minutes	1 cycle	(denaturation)
94°C	1 minute	35 cycles	(denaturation)
58°C	1 minute, 15 seconds		(annealing)
68°C	1 minute, 15 seconds		(elongation)
68°C	7 minutes	1 cycle	(elongation)

The resulting DNA was isolated from mineral oil by pipetting onto Parafilm®, transferred to a fresh 0.5ml centrifuge tube (Greiner) and stored at -20°C.

6.2.6 RT-PCR

The following components were added to an RNase-free 0.5ml thin-walled centrifuge tube (BDH) :-

Oligo dT_{12-18} (Gibco)	1µl
1µg total RNA	10µl
10mM dNTP mix (Gibco)	1µl

The tube was heated to 65°C for 5 minutes, chilled on ice and spun briefly to collect the contents of the tube. The following was then added :-

5x first strand buffer (Gibco)	4µl
0.1M DTT (Gibco)	2µl
RNase OUT (Gibco)	1µl

The contents of the tube were mixed and incubated at 42°C for 2 minutes. 1µl of Superscript II reverse transcriptase (Gibco) was added and the contents mixed by gentle pipetting. Control samples lacking reverse transcriptase enzyme were also included. All samples were overlaid with oil and tubes incubated at 42°C for 50 minutes and then 70°C for 15 minutes to inactivate the reactions. The resulting cDNA was isolated from mineral oil by pipetting onto Parafilm®, and 10% taken for PCR using XLB2M-5' and -3' primers (see section 6.2.5).

6.2.7 Western blots

Western blotting was carried out as described in section 2.2.8 with the following amendments. Following lysis, the amount of protein in each sample was assessed using the BioRad protein assay system. A calibration curve was constructed using known concentrations of BSA (Sigma) and the absorbance at 595nm (A_{595}) was read for each protein sample using a Helios β spectrophotometer (Thermo Spectronic). The same amount of protein was loaded down each lane of a minigel of 12% acrylamide separating gel, 4% stacking gel (see appendix 2). The primary antibody used was a mouse α-*Xenopus* β2m polyclonal antibody (raised against recombinant *Xenopus* β2m, Watson, MD unpublished), diluted to 1:500 in blocking buffer (see appendix 2).

6.3 Results

6.3.1 Preliminary observations

For each sample of total RNA, two RT-PCR's were performed, each employing the 5' primer XLB2M-5 and one of the two 3' primers, XLB2M-3^1 or XLB2M-3^2. As *Fig 6.1* demonstrates, PCR amplification from both peripheral blood cDNA and chromosomal DNA using XLB2M-3^1 produces a PCR product of ≈400bp, whereas when samples are amplified using the XLB2M-3^2 primer, a difference in the size of PCR product is observed depending on whether cDNA or DNA is used as the template. cDNA produces a product of ≈500bp whereas DNA produces a product of ≈1.3kb. This suggests the presence of an intron of ≈800bp between the annealing positions of XLB2M-3^1 and XLB2M-3^2. Use of the latter primer therefore provides a method of ensuring that the RT-PCR products obtained are derived from RNA templates and not from DNA contamination following RNA isolation.

Both RNA and protein samples were quantified and the same amount of each sample was used in RT-PCR and Western blots respectively.

6.3.2 Expression of β2m in adult cell populations

Both RT-PCR *(Fig. 6.2)* and Western blotting *(Fig. 6.3)* techniques respectively confirm the presence of β2m mRNA and β2m protein (13kDa) in adult peripheral blood, liver, spleen and thymus. There appears to be less β2m protein present in splenocyte lysates in comparison to those from liver and thymus, the latter proving to be relatively β2m-rich *(Fig. 6.3)*. Peripheral red blood cell lysates contain lower amounts of the 13kDa protein, but also contain a slightly larger 14kDa protein also staining positive with the α-β2m antibody.

6.3.3 Expression of β2m in *Xenopus* cell lines

Four *Xenopus* cell lines with varying expression of MHC proteins (see Introduction) were investigated for β2m expression at both the protein *(Fig. 6.3)* and mRNA *(Fig. 6.4)* level, the A6 kidney cell line (Rafferty, 1969), the 15/0 tumour cell line, the ff2 tumour cell line and the B_3B_7 tumour cell line (Robert *et al.*, 1994). RT-PCR demonstrates the presence of β2m mRNA in each of the cell lines investigated, even in the B_3B_7 and 15/0 cell lines which lack expression of MHC class-Ia proteins. Similarly, Western blotting reveals a protein of 14kDa identified by the α-β2m antibody in lysates of A6, ff2 and B_3B_7 cells lines (15/0 cells were not tested). ff2 and A6 cell lines appear to have comparable expression of the β2m protein, whereas expression in B_3B_7 cells is significantly lower.

6.3.4 Expression of β2m in larval tissues

RT-PCR establishes the presence of β2m mRNA in liver and tail of stage 57 *(Fig. 6.5)*, 56 *(Fig. 6.6)* and 54 *(Fig. 6.7)* larvae. Transcripts were also found in thymus of stage 57 and 54 larvae (stage 56 larval thymus was not tested). Larval tissue was not used in Western blotting due to limited numbers of tadpoles.

6.5 Discussion

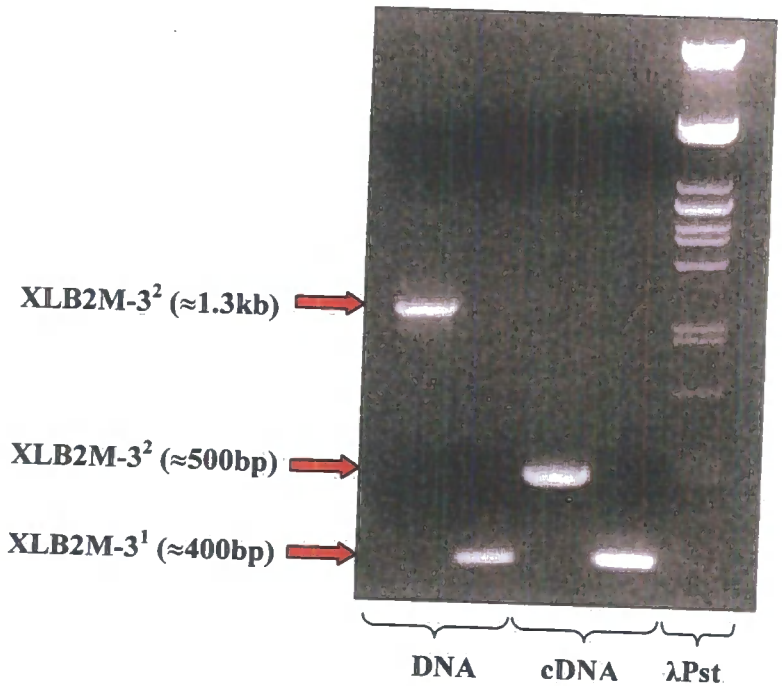
All adult cell populations tested (spleen, liver, thymus and red blood cells) possess β2m mRNA transcripts as demonstrated by RT-PCR. Western blotting techniques employing a recently generated α-β2m polyclonal antibody also establish the expression of the 13kDa β2m protein in spleen, liver and thymus. Given the MHC-I^{+ve} phenotype of these cells, the presence of the β2m protein was expected as this molecule is essential for MHC-I surface expression (reviewed in Criscitiello *et al.*, 1998). As all protein lysates were standardised using the BioRad protein assay system, the relative amounts of β2m protein in each sample can be compared. Thymocytes are a rich source of β2m in comparison to cells of liver and spleen. Peripheral red blood cells also express the 13kDa β2m protein, present in spleen, liver and thymus, although there appears to be considerably less of this protein in comparison. In the common carp, peripheral erythrocytes fail to express β2m, and red blood cells from the spleen are at best $\beta2m^{dull}$ (Rodrigues *et al.*, 1998b). *Xenopus* red blood cells also express a 14kDa protein which cross-reacts with the β2m antibody. It may transpire that the larger band represents a glycosylated form of the 13kDa band, although deglycosylation experiments would be necessary to confirm this.

Four *Xenopus* cell lines were investigated for β2m expression at both the mRNA and protein levels, the A6 kidney cell line (Rafferty, 1969), the 15/0 thymic lymphoid tumour cell line, the ff2 thymic lymphoid tumour cell line and the B_3B_7 thymic tumour cell line (Robert *et al.*, 1994). The A6 cell line is MHC-I^{low} (determined by Northern blotting and flow cytometry, Dr. J. Robert, University of Rochester, NY, Pers. Comm.) and it therefore follows that they also express *Xenopus* β2m mRNA and the 14kDa β2m protein. Similarly, the ff2 tumour cell line has been proved to express both MHC-I transcripts (through Northern blotting, Robert *et al.*, 1994) and surface MHC-I proteins (determined by flow cytometry and Western blotting, Robert *et al.*, 1994) and are therefore also not surprisingly found to express *Xenopus* β2m mRNA and protein (14kDa). Both 15/0 and B_3B_7 cell lines do not express MHC-Ia transcripts or MHC-Ia protein (Robert *et al.*, 1994), although RT-PCR has established the presence of β2m mRNA in both cell lines. B_3B_7 cell lysates were also assessed

for β2m protein through Western blotting and are found to contain the 14kDa protein as seen in A6, ff2 and peripheral red blood lysates. As mentioned previously, the slower migrating 14kDa β2m protein expressed by these cell lines may be a glycosylated form of the 13kDa β2m protein expressed by lymphoid cell populations. B_3B_7 cells however express considerably less β2m protein in comparison to the MHC-I^{+ve} A6 and ff2 cells. Flow cytometry using the α-β2m antibody would establish whether the β2m present in MHC-I-deficient B_3B_7 cell lysates resides inside the cell or is expressed at the cell surface. Unfortunately, the polyclonal α-β2m antibody fails to detect β2m in flow cytometry and so the location of the β2m protein was not determined. It is also possible that the β2m in B_3B_7 and 15/0 cells is associating with MHC-Ib molecules, as these cell lines are known to express MHC-Ib transcripts (Robert *et al.*, 1994).

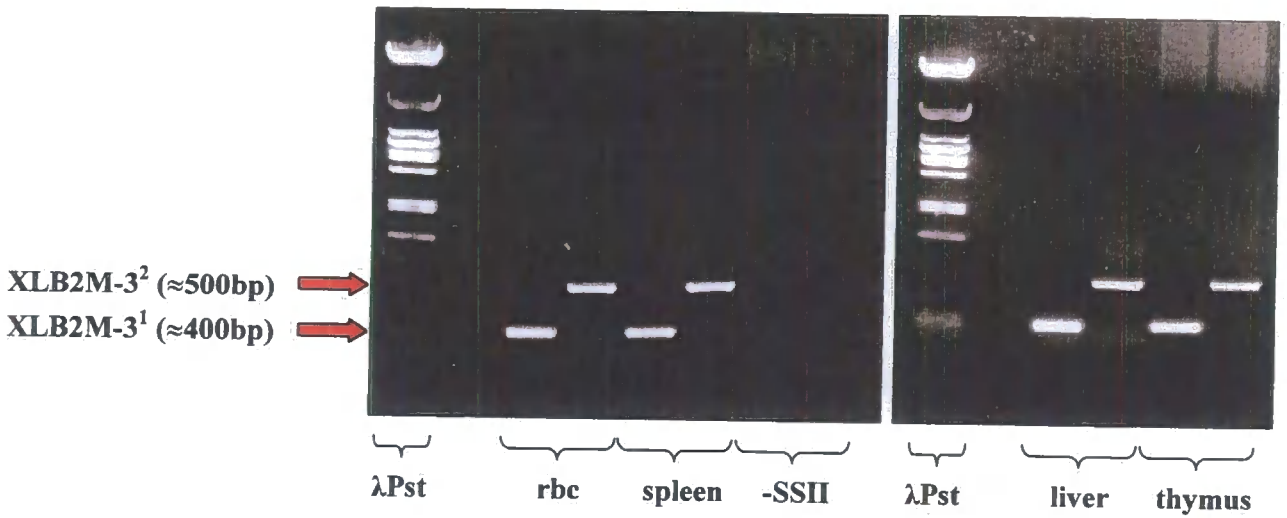
In *Xenopus* larvae, surface expression of MHC-I protein is first detectable at low levels in the spleen of stage 55-56 larvae (34 days of age) (Du Pasquier and Flajnik 1990; Rollins-Smith *et al.*, 1997). It has recently been shown by Salter-Cid et al (1998) that class-Ia transcripts are hardly detectable before metamorphosis and that class-Ib is undetectable in any larval stage. No class-Ia mRNA is detectable in thymus, spleen or skin in tadpoles, whereas low level class-Ia message is present in intestine and lung in stage 54-56 larvae (Salter-Cid *et al.*, 1998). RT-PCR reveals the presence of β2m transcripts in thymus, tail and liver in tadpoles as early as stage 54 (≈5 weeks), which suggests that *Xenopus* β2m transcription precedes MHC class-I. This is also true of mammalian β2m (Jaffe *et al.*, 1991), whereas in carp, it is class-I transcription which precedes that of β2m (Rodrigues *et al.*, 1998a). The presence of *Xenopus* β2m mRNA however, does not automatically prove the existence of β2m protein. Unfortunately, the expression of β2m protein could not be tested with Western blotting due to limited numbers of tadpoles available. Clearly further studies are required to establish when β2m proteins are first expressed during ontogeny, in order to gain a better understanding of the slow emergence of MHC class-I proteins in *Xenopus*.

Fig. 6.1: β2m RT-PCR of peripheral red blood cell cDNA and DNA



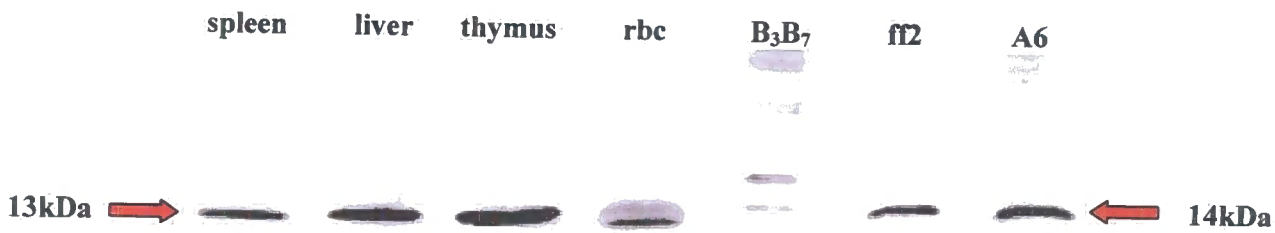
Pst-digested λDNA is shown as a marker.

Fig. 6.2: β2m RT-PCR of adult *Xenopus* cell populations



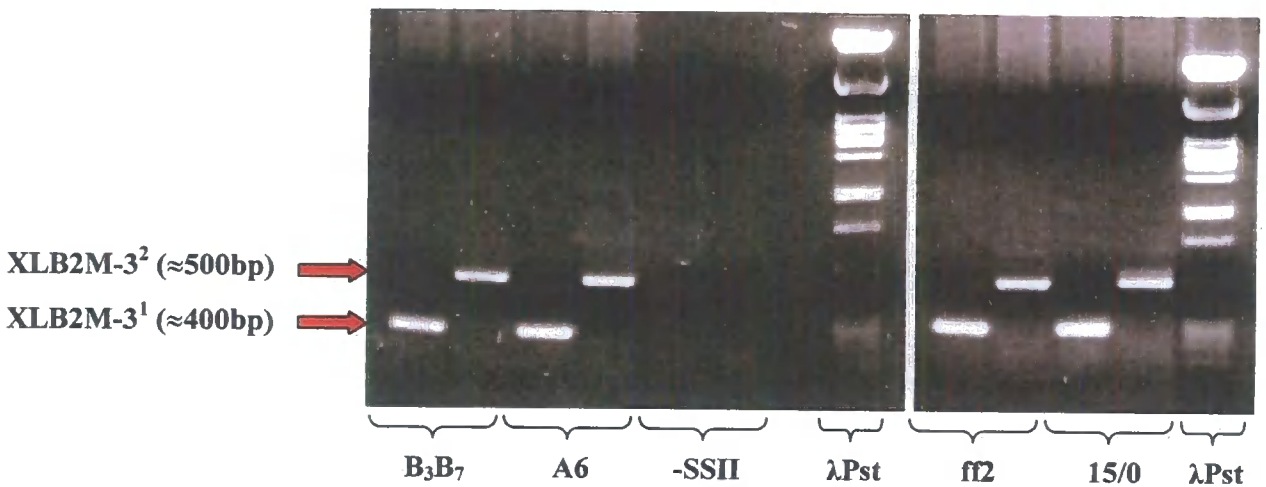
The same amount of RNA was used in each RT-PCR. Control RT-PCR's were also carried out which lack the addition of Superscript II reverse transcriptase (-SSII). Pst-digested λDNA is shown as a marker. Key: rbc = peripheral red blood cells.

Fig. 6.3: β2m Western blots of *Xenopus* adult lymphoid cell populations and tumour cell lines



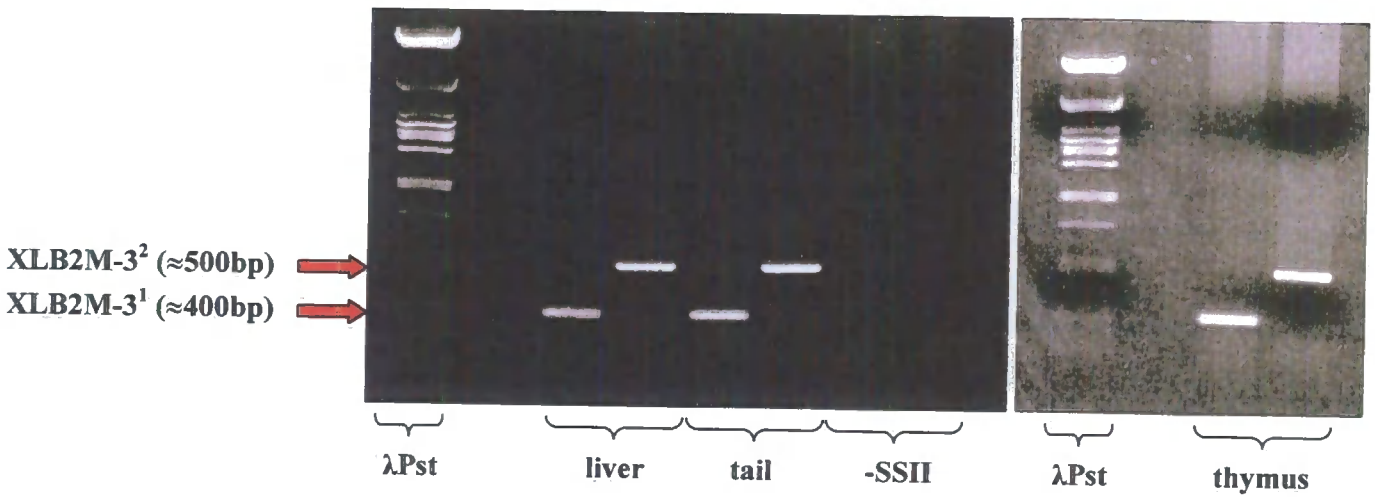
The same amount of protein was run down each lane. Key: rbc = peripheral red blood cells. B_3B_7, ff2, A6 = *Xenopus* cell lines

Fig. 6.4: β2m RT-PCR of *Xenopus* tumour cell lines



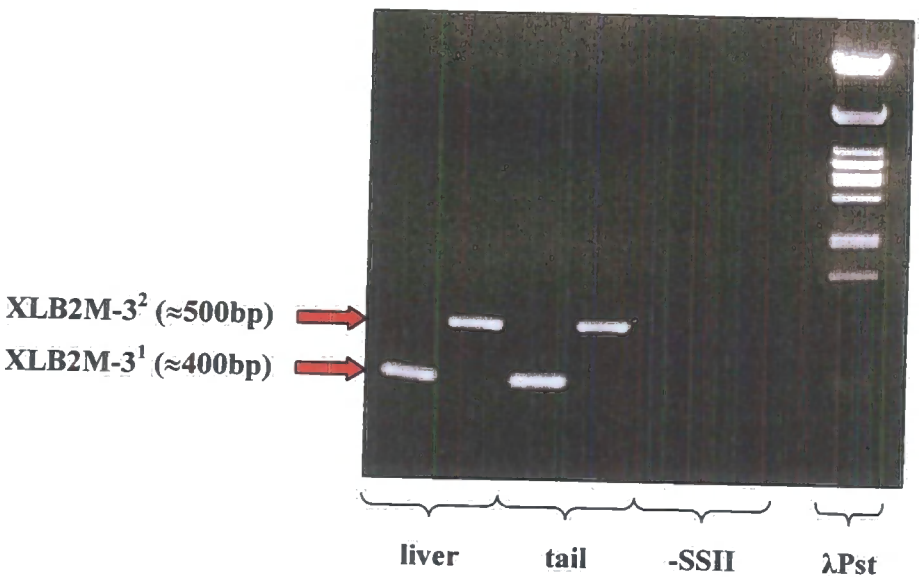
The same amount of RNA was used in each RT-PCR. Control RT-PCR's were also carried out which lack the addition of Superscript II reverse transcriptase (-SSII). Pst-digested λDNA is shown as a marker. B_3B_7, A6, ff2, 15/0 = *Xenopus* cell lines

Fig. 6.5: β2m RT-PCR of stage 57 larval *Xenopus* tissues



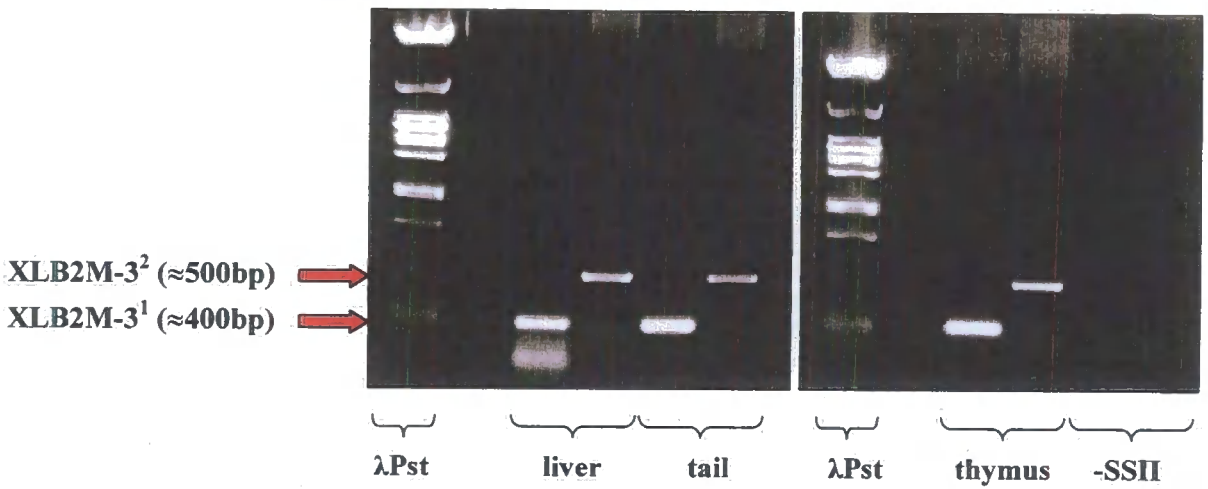
The same amount of RNA was used in each sample. Control RT-PCR's were also carried out which lack the addition of Superscript II reverse transcriptase (-SSII). Pst-digested λDNA is shown as a marker.

Fig. 6.6: β2m RT-PCR of stage 56 larval *Xenopus* tissues



The same amount of RNA was used in each sample. Control RT-PCR's were also carried out which lack the addition of Superscript II reverse transcriptase (-SSII). Pst-digested λDNA is shown as a marker.

Fig. 6.7: β2m RT-PCR of stage 54 larval *Xenopus* tissues



The same amount of RNA was used in each sample. Control RT-PCR's were also carried out which lack the addition of Superscript II reverse transcriptase (-SSII). Pst-digested λDNA is shown as a marker.

CHAPTER 7

Conclusions and future directions

The initial aim of the work described in this Thesis has been to probe the lymphoid organ distribution, phenotype and function of the lymphocyte subset identified by the recently generated α-*Xenopus* mAb's 1F8, 4D4 and 1G5, presumed to be α-NK mAb's. The ontogeny of this candidate NK cell subset has also been examined, and the induction of expression of the 1F8 antigen on T-cells following *in vitro* stimulation using PMA and calcium ionophore was used to explore the biochemical nature of this NK-associated antigen. The Thesis also examines the expression patterns of *Xenopus* β2m in cells from both adult and larval *Xenopus* and also in MHC class-I deficient tumour cells.

The three candidate α-NK mAb's 1F8, 4D4 and 1G5 were shown to identify large lymphocytes (12μm in diameter), which are present in spleen, liver and intestine, but absent in the thymus of control adult *Xenopus*. Removal of the thymus gland early in larval life (thymectomy) results in the eradication of T-cells from the animal causing subsequent increases in the proportion of candidate NK cells, thereby making the thymectomised (Tx) model integral in the study of this subset of lymphocytes.

The candidate NK cells identified by the 1F8 (the mAb with the most consistent staining pattern of NK cells) displayed phenotypic and functional characteristics generally attributed to NK cells. Firstly, flow cytometry revealed the lack of surface IgM on $1F8^{+ve}$ cells, indicating that they do not belong to the B-cell subset. Furthermore, RT-PCR (Rau *et al.*, 2002) has demonstrated the absence of TCRVβ transcripts in 1F8-enriched cells from Tx frogs, signifying no relation to the T-lymphocyte subset. It was interesting to note that in liver and intestine, cells expressing the 1F8 antigen also constitutively express low levels of the CD5 antigen. High levels of CD5 are expressed only on *Xenopus* T-cells, and this lower CD5 expression on NK cells (also seen on PMA-stimulated B-cells) is perhaps indicating the presence of an "activated" NK population. When splenic NK cells are purified using the 1F8 mAb, they also frequently become $CD5^{lo}$. These purified candidate splenic NK cells are also found to express low levels of CD8 (using the AM22 mAb, thought possibly to be directed against the α chain of CD8). They do not however, appear to express the β chain of this receptor, that the F17 mAb is believed to bind.

Overall, we may conclude that $1F8^{+ve}$ cells of *Xenopus* are neither B-cells nor T-cells. However, in order to establish if $1F8^{+ve}$ cells are indeed NK cells, it was necessary to assess the cytolytic activity of this candidate effector population. ^{51}Cr-release and DNA fragmentation assays have shown that cultured unsorted splenocytes from control and Tx animals display NK-like activity against MHC-I deficient allogeneic tumour targets, but not towards MHC-I^{+ve} lymphoblasts, confirming previous studies by Horton et al (1998b). Tx populations consistently require further additions of T-cell-derived growth factors in the culture medium to promote cytotoxicity, which is suggestive of LAK (lymphokine-activated killing) activity. Mammalian effector cells displaying LAK activity are often able to lyse a wide array of targets (Brooks *et al.*, 1983). It would be of interest to assess if differing periods of GFM-culture of Tx NK cells has a bearing on target promiscuity, or indeed on degree of cytotoxicity.

Immunomagnetic sorting enabled the isolation of the $1F8^{+ve}$ candidate NK cells, which proved to be extremely effective killers of MHC-I^{-ve} tumour targets following culture in GFM-supplemented media. Populations depleted of candidate NK cells (containing B-cells, macrophages, red blood cells etc) failed to kill the tumour cells. Previous studies on catfish effector cells investigated the time course of cytotoxicity (Hogan *et al.*, 1999). These studies show that although target cell cytoplasmic leakage caused by these effector cells (demonstrated by ^{51}Cr-release) increases over a co-culture period of 4 hours, DNA fragmentation as measured by JAM assays occurs within 1 hour of co-culture and does not increase further after this time (Hogan *et al.*, 1999). The assays reported in this Thesis involved fixed co-culture periods of 6 hours and it may be of interest to perform a time course study to assess the levels of ^{51}Cr release and/or DNA loss for the target cells over the 6 hour period.

The phenotypic and functional data reported here on *Xenopus* $1F8^{+ve}$ lend strong support to the contention that these cells represent NK cells.

The cytotoxic mechanism used by $1F8^{+ve}$ NK cells was further investigated through apoptosis-detection studies using antibodies directed against annexin-V and ASP (apoptosis-specific protein). This data established that *Xenopus* NK cells kill their tumour target cells by inducing apoptosis. Future investigations could establish

whether *Xenopus* NK cells do this by using perforins/granzymes or the Fas/FasL system. Preliminary evidence for the presence of the Fas/FasL system in *Xenopus* has already been presented. Anti-human Fas antibodies have been shown to specifically bind to adult *Xenopus* splenocyte suspensions (Mangurian *et al.*, 1998), an interaction which improves with the addition of the apoptosis-inducer phytohaemagglutinin (PHA). It therefore follows that *Xenopus* splenocytes most likely express surface molecules homologous to human Fas, which would demonstrate the evolutionary conservation of this apoptosis-induction mechanism. Should such homology extend throughout components of the apoptotic pathway, it is also conceivable that mammalian caspase modulators could be used to probe the apoptotic killing mediated by *Xenopus* NK cells.

The biochemical nature of the 1F8 antigen present on the surface of *Xenopus* NK cells has been examined through Western blotting and immunoprecipitation. Unfortunately, data from the two techniques are contradictory. Whilst Western blot data shows the 1F8 antigen present on the surface of Tx splenocytes to be 66-85kDa in size, 1F8-immunoprecipitation of biotinylated lysates identifies proteins of 45 and 55kDa. The latter technique has proved to be more consistent and as the mAb in this scenario is detecting its antigen in its native state (compared to Western blotting where the protein is boiled prior to antibody detection), the 45-55kDa value appears to be a more likely candidate for the 1F8 antigen. Clearly, further molecular studies are required to probe the nature of the 1F8 antigen. These studies are even more crucial now that it known from *in vivo* mAb injection experiments that the 1F8 antigen appears to play an important role in α-tumour immunity (Rau *et al.*, 2002). Furthermore, *in vitro* cross-linking with the 1F8 mAb should address the role of the 1F8 antigen in target cell recognition. To date, no affect on tumour cell killing was observed when the 1F8 mAb was added to NK cell/target cell mixtures, or when NK effectors were pre-treated with the 1F8 mAb (unpublished observations, this laboratory). It may also be of interest to study factors thought to regulate NK cytotoxicity such as TGF-β, which appears to have an inhibitory affect on murine NK activity (Bellone *et al.*, 1995; Hunter *et al.*, 1995). The effect of this molecule on *Xenopus* NK cells would be interesting to note.

Phenotypic studies on *Xenopus* larvae have revealed that cells expressing the 1F8 antigen (albeit at low levels) appear to emerge as late as ≈7 weeks of age (stage 56/58), which is ≈5 weeks after T- and B-cells become detectable. This late appearance of NK cells comes approximately 2 weeks after the ontogeny of MHC class-Ia expression (Rollins-Smith *et al.*, 1997; Salter-Cid *et al.*, 1998). 1F8 antigen expression on splenocytes increases by 3 months of age, although these NK cells are still few in number in comparison to adults. Given the absence of NK cells in young larvae, it follows that both ^{51}Cr-release and DNA fragmentation assays have failed to detect α-tumour cytotoxicity in GFM-cultured splenocyte populations (from both control and Tx). It may transpire that the very low percentage of 1F8^{lo} splenocytes detectable by flow cytometry in late larval life may be competent to kill, but cytotoxicity is masked when unseparated splenocytes are assayed. In this respect, 1F8-enrichement is essential, but unfortunately is also impractical due to the high number of larvae necessary to obtain sufficient effector numbers. Significant albeit low level of specific tumour killing is first detectable in splenocyte populations from 3-4 month old froglets. This low level of cytotoxicity may relate to the low intensity of 1F8 antigen expression. By 6 months of age, *Xenopus* splenocyte populations display cytotoxicity towards tumour target cells comparable to that of 1 year-old adults (Horton *et al.*, 1998b).

Investigations into the effects of PMA and calcium ionophore on *Xenopus* lymphocyte populations revealed that stimulation of control splenocyte and thymocytes with PMA (10ng/ml) and calcium ionophore (20ng/ml) results in significant increases in size and granularity together with marked changes in phenotype. Surface IgM expression is habitually lost, indicating activation of the B-cell subset. This subset also becomes CD5^{lo}, a phenomenon which is T-cell-dependent (Gravenor, 1996). The most useful observation however, was the dramatic increase in NK antigen expression on the surface of both splenocytes and thymocytes. It is now apparent that such increases are attributable to induction of 1F8 antigen expression on T-cells, this subset thereby being termed "NKT"-cells. It follows that such increases in NK antigen expression following stimulation are not seen in T-cell deficient Tx splenic populations. Similar emergence of 1F8 on larval splenic and thymic T-cells following PMA and calcium ionophore stimulation suggests that such

"NKT"-cells can be identified prior to metamorphosis. It appears that "NKT" cells are not only found following *in vitro* PMA/Ca^{2+} ionophore-induced stimulation. They also exist in low numbers constitutively in the spleen of normal *Xenopus* (Rau *et al.*, 2002).

1F8-enriched stimulated control splenocytes containing "NKT"-cells together with a low level of NK cells were shown to display poor cytotoxicity versus tumour cells. These observations relate to work by Rau et al (2002), who reported that alloantigen-reactive *Xenopus* $CD8^{+ve}/1F8^{+ve}$ T-cells were unable to kill in a MHC-restricted manner following culture in media supplemented with PMA and ionomycin. However, as PMA/calcium ionophore-stimulated Tx splenocytes retain their ability to kill tumour target cells, it is unlikely that a non-specific mechanism (such as cell surface receptor damage) is responsible for the suppression of cytotoxicity. It may well be that PMA and calcium ionophore stimulation generates a suppressor T-cell population (absent in Tx animals), which are capable of inhibiting the cytolytic functions of other cells in the effector population (e.g. NK cells).

The increases in 1F8 antigen following PMA/calcium ionophore stimulation provided the opportunity to study the 1F8 antigen through immunoprecipitation. 1F8 immunoprecipitation following both surface labelling with biotin and metabolic labelling with ^{35}S identified a common protein of 55kDa (also seen in Tx splenocyte lysates as described earlier). Immunoprecipitations with biotin-labelled lysates also produced a band at 45kDa. However, it is the larger protein which was consistent throughout the techniques employed, suggesting that this band is the true NK antigen.

The fortunate identification of a *Xenopus* β2m clone in a random screen of a *Xenopus* spleen cDNA library (paper in preparation) and the subsequent generation of β2m primers and polyclonal α-β2m antibody has enabled investigation into β2m expression in various adult and larval tissues at both the RNA and protein level. All adult cell populations tested (spleen, liver, thymus, peripheral red blood cells) possess β2m transcripts (demonstrated by RT-PCR) and express the 13kDa β2m protein (shown by Western blotting). This was not surprising given the MHC-I^{+ve} phenotype of these cells. Peripheral red blood cell lysates were also shown to contain a 14kDa protein,

also identified in lysates of various adult cell lines (including the MHC-Ia^{-ve} 15/0 and B_3B_7 tumour cell lines). This slightly larger β2m protein may be a glycosylated form of the 13kDa protein expressed in adult tissues. Larval tissues at various stages of development were also examined by RT-PCR for β2m expression. β2m transcripts were found in thymus, tail and liver as early as stage 54 (≈5 weeks of age). At this time point, MHC class-Ia transcripts are only present in restricted distribution e.g. intestine and gills. Transcripts are not present in thymus, spleen or skin (Salter-Cid *et al.*, 1998), suggesting that like mammalian β2m (Jaffe *et al.*, 1991), *Xenopus* β2m transcription precedes that of MHC class-I.

In conclusion, a recently generated mAb, 1F8 has been shown to identify an NK cell subset in *Xenopus*, which displays specific cytotoxicity against MHC-deficient tumour target cells. The ontogeny of these cells has also been investigated in MHC class-I deficient larvae. NK cells are found to emerge in 6-7 week-old larvae, some 2 weeks after MHC-Ia is first detected, although these cells do not appear to be functionally competent until 6 months of age. Generation of *in vitro* "NKT"-cells has been possible through PMA/calcium ionophore stimulation of T-cells, a finding which has also presented the opportunity to biochemically characterise (through immunoprecipitation) the 1F8 antigen, which appears to be 55kDa in size. Finally, the expression and ontogeny of β2m in *Xenopus* tissues and cell lines has been addressed and has demonstrated the presence of β2m in larval tissues of tadpoles as young as ≈5 weeks of age.

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Appendix 1: General cell culture, flow cytometric analysis and immunostaining

ABC solution: 50µl reagent A, 50µl reagent B, 3ml wash buffer

APBS (pH 7.4): 6ml 10x PBS, 78ml ddH_20, filtered through a 0.2µ filter

B_3B_7 medium: 400ml serum-free medium, 120ml ddH20, 40ml A6 cell supernatant, 10ml FCS, 1.3ml kanamycin, filtered through a 0.2µ filter

Blocking buffer: 1g BSA, 100ml APBS

CMF media (neutral pH): 15ml 10x HBSS, 15ml 10x HEPES, 120ml ddH_20, 0.15g BSA, filtered through a 0.2µ filter

CMF/EDTA/DTT: 28.8ml CMF, 1.2ml 0.02% EDTA, 300µl 10mM DTT, filtered through a 0.2µ filter

DAB substrate: 10µl of 30% H_20_2 (diluted to 1:10 in wash buffer), 3ml 0.5mg/ml DAB, filtered through a 0.2µ filter

Diluting buffer: 9ml APBS, 1ml Goat serum

FACS medium (pH 7.4): 6.6g NaCl, 1.5g Na_2HPO_4, 0.2g KH_2PO_4, 1g BSA, 1g Sodium azide, 1000ml ddH_20, filtered through a 0.2µ filter

HEPES (10x solution) (pH 7.2): 1.19g HEPES, 1.05g $NaHCO_3$, 50ml ddH_20

Serum-free medium: 500ml Iscoves medium, 5ml NEAA, 5ml penicillin/streptomycin, 0.5ml insulin, 0.5ml 2-Me, 1.5ml primatone

Wash buffer: 0.1g BSA, 100ml APBS

Wright-Giemsa stain: 1.53g/L Wrights stain, 2.5g/LGiemsa stain, 100ml/L glycerol

Appendix 2: Protein analysis

Blocking solutions:

Immunoprecipitations: 3g BSA, 100ml APBS, 0.2ml Tween 20

Western blotting: 5g Milk powder, 100ml TBS, 0.2ml Tween 20

Buffer A: 100µl 1M Tris (pH 7.5), 500µl 3M NaCl, 20µl NP-40, 40µl 500mM EDTA, 9.34ml ddH_20

Buffer B: 100µl 1M Tris (pH 7.5), 1.67ml 3M NaCl, 20µl NP-40, 40µl 500mM EDTA, 8.17ml ddH_20

Buffer C: 100µl 1M Tris (pH 7.5), 9.99ml ddH_20

Chemiluminescent solutions:

Solution 1: 50µl Luminol (250mM in DMSO), 22µl p-Coumaric acid (90mM
in DMSO), 500µl 1M Tris (pH 8.5), 4.5ml ddH_20

Solution 2: 3.2µl 30% H_2O_2, 500µl 1M Tris (pH 8.5), 4.5ml ddH_20

Net-N: 150mM NaCl, 5mM EDTA, 50mM Tris pH8, 0.5% NP-40, 0.05% sodium azide

Net-NON: Net-N, 1mg/ml Ovalbumine, 0.3-0.6M NaCl

NP-40 protein lysis buffer: 10µl NP-40, 50µl 1M Tris (pH 8), 50µl 3M NaCl, 1µl 1M $MgCl_2$, 896µl ddH_20

Ponceau S stain: 0.2g Ponceau S, 5ml Acetic acid, 95ml dH_20

Ponceau S destain: 5ml Acetic acid, 95ml dH_20

RPMI labelling medium: 18.75ml RPMI washing medium, 1.25ml dialysed FCS (5%), 5ml dialysed A6 supernatant (20%) (optional), filtered through a 0.2µ filter

RPMI washing medium: 200ml RPMI medium, 60ml ddH_20, 2.6ml L-Glutamine, 2.6ml Penicillin/streptomycin, 150µl kanamycin, filtered through a 0.2µ filter

Sample loading buffer (5x): 2.5ml 1M Tris (pH 6.8), 0.78g DTT/1%β-mercaptoethanol, 1.0g SDS, 0.05g Bromophenol blue, 5ml Glycerol, 2.5ml ddH_20

SDS/PAGE gels:

	10% separating	**4% stacking**
Sterile ddH_20	3.61ml	2.8ml
0.5M Tris (pH 6.8)	-	1.25ml
1.5M Tris (pH 8.8)	2.5ml	-
30% acrylamide/Bis (29.2% acrylamide, 0.2% bisacrylamide)	3.33ml	0.67ml
2% SDS	0.5ml	0.25ml
10% ammonium persulphate	0.05ml	0.025ml
TEMED	0.005ml	0.005ml
	10ml total	5ml total

SDS/PAGE running buffer (10x solution): 15g Tris base, 72g Glycine, 5g SDS, 500ml dH_20

Towbin transfer buffer: 3.03g Tris base, 14.4g Glycine, 200ml Methanol, 800ml dH_20

Tris-buffered saline (TBS) (10x solution, pH 7.5): 6.1g Tris base, 43.8g NaCl, 500ml dH_20

Appendix 3: RNA/DNA analysis

Primers: **XLB2M-5** : 5'- TGG TCA AGG TTT ACA CTG CG -3'

XLB2M-3[1]: 5'- GGG AGA CCA CAC ATT CCA CT -3'

XLB2M-3[2]: 5'- GCT CTT AAC TGC CGC CAT AC -3'

Sample loading buffer (6x): 0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 30% Glycerol, made up in ddH_20

TAE buffer (50x solution): 242g Tris base, 57.1ml Acetic acid, 100ml 0.5M EDTA (pH 8), 842.9ml dH_20