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Development of antibody technology to identify natural killer cell surface antigens in Xenopus Laevis

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Development of Antibody Technology to Identify Natural Killer Cell Surface Antigens in *Xenopus Laevis*

Ralph Minter

Natural killer (NK)-like lymphocytes have recently been identified in thymectomised (Tx) *Xenopus* which are capable of spontaneous cytotoxicity towards the MHC-deficient, allogeneic thymus tumour cell line B_3B_7. This Thesis describes attempts to raise antibodies to *Xenopus* NK cell surface antigens by phage display and hybridoma technology.

The phage display technique was optimised for raising antibodies to novel, cellular antigens in a trial run using the *Xenopus* thymus tumour cell line B_3B_7. Having isolated a phage antibody which was shown by flow cytometry to bind B_3B_7 cells, the technique was then used to try and raise antibodies to *Xenopus* NK cells. Isolation of an NK-specific phage antibody was not achieved but phage antibody XL-6 was raised, which bound an antigen on *Xenopus* lymphocytes. Phage antibody XL-6, and soluble scFv derived from this, were able to identify a putative mature T cell population in the thymus and may be specific for an amphibian homologue of the mammalian leukocyte common antigen CD45.

Hybridoma technology was used to isolate three monoclonal antibodies, 1F8, 4D4 and 1G5, which were shown by flow cytometric analysis to identify a putative NK cell population in control and Tx *Xenopus*. Following immunomagnetic purification, 1F8-positive spleen cells from control and Tx animals were shown to kill the MHC-deficient tumour target B_3B_7, confirming that this antibody was specific for *Xenopus* NK cells. Western blotting experiments showed that 1F8, 4D4 and 1G5 identified a doublet of protein bands at 72 and 74 kilodaltons in *Xenopus* gut lymphoid lysates.

Initial attempts to isolate cDNA encoding a *Xenopus* NK cell surface antigen through immunoscreening a *Xenopus* gut cDNA expression library with antibody 1G5 were unsuccessful as was an attempt to clone a *Xenopus* homologue of the mammalian NK receptor NKR-P1 by PCR.

Development of Antibody Technology to Identify Natural Killer Cell Surface Antigens in *Xenopus Laevis*

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PhD Thesis

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Declaration

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

Signed: *R.L.Mint*

Date: 21-5-00

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Abbreviations

A_{260}	:	absorbance at 260nm
ADCC	:	antibody dependent cellular cytotoxicity
APBS	:	amphibian phosphate buffered saline
bp	:	base pairs
BSA	:	bovine serum albumin
CD	:	cluster of differentiation
cfu	:	colony forming unit
cpIII	:	coat protein III of bacteriophage M13
CTL	:	cytotoxic T lymphocyte
ddH_2O	:	double distilled water
dNTP	:	deoxynucleoside triphosphate
ELISA	:	enzyme-linked immunosorbent assay
FACS	:	fluorescence activated cell sorting
FCS	:	foetal calf serum
FITC	:	fluorescein isothiocyanate
HRP	:	horseradish peroxidase
IEL	:	intra-epithelial lymphocyte
IFN-γ	:	interferon-γ
Ig	:	immunoglobulin
IL	:	interleukin
IPTG	:	isopropyl-ß-D-thiogalactoside
ITAM	:	immunoreceptor tyrosine-based activatory motif
ITIM	:	immunoreceptor tyrosine-based inhibitory motif
kDa	:	kilodalton
KIR	:	killer cell inhibitory receptor
μg	:	microgram
μl	:	microlitre
mA	:	milli-amperes
mAb	:	monoclonal antibody
mg	:	milligram
MHC	:	major histocompatibility complex
ml	:	millilitre
ng	:	nanogram
NK	:	natural killer
NO	:	nitric oxide
OD_{550}	:	optical density (absorbance) at 550nm
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
PE	:	phycoerythrin
PEG	:	polyethylene glycol
pfu	:	plaque forming unit
psi	:	pounds per square inch
RBC	:	red blood cell

RFLP	:	restriction fragment length polymorphism
rpm	:	revolutions per minute
RT	:	room temperature
RT-PCR	:	reverse transcriptase polymerase chain reaction
scFv	:	single chain variable fragment
SDS-PAGE	:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOE-PCR	:	splicing by overlap extension polymerase chain reaction
TCR	:	T cell receptor
TNF-α	:	tumour necrosis factor-α
Tx	:	thymectomised
UV	:	ultra-violet
V	:	volts
X-gal	:	5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside

CHAPTER 1

General Introduction

This Chapter comprises a review of recent research into mammalian natural killer (NK) cells and examines evolutionary aspects of NK cells highlighted by the study of lower vertebrates. An analysis of the use of comparative models in biology is included which introduces the benefits of the amphibian *Xenopus laevis* as an immunological model. Finally, the technique of phage display is presented as a novel means of raising antibodies.

1.1 Natural Killer Cells

1.1.1 The Importance of Natural Killer Cells

NK cells are now widely accepted as a population of lymphocytes, distinct from T and B cells, which play a crucial role in innate resistance to viral infection and tumourigenesis. Their importance as a first line of defence lies in their ability to effect rapid cytotoxic responses without prior sensitisation. Recent research into NK cells has not only emphasised their effector role in innate immunity but has also highlighted additional regulatory functions in the acquired immune response. The unique niche occupied by NK cells in cellular immunity has prompted much investigation into the precise events which trigger NK activity. This increased understanding of NK activity is now beginning to be used for therapeutic benefit.

1.1.2 Discovery and Early Findings

Early work in several invertebrate species, most notably in the earthworm *Lumbricus*, identified populations of large, circulating cells with numerous dark-staining granules (Cooper, 1969). Initially these cells were shown to elicit cytotoxic responses in tissue graft rejection (Cooper, 1969) but were later found to be capable of spontaneous killing (Suzuki and Cooper, 1995).

One of the first demonstrations of spontaneous cytotoxicity in humans occurred when leukaemic blasts removed from a patient were shown in a chromium release assay to be lysed by lymphocytes from family members (Rosenberg *et al.*, 1972). In a control

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assay, peripheral blood cells removed from the non-leukaemic identical twin of the patient were not lysed thus eliminating the possibility of lysis due to histocompatibility differences. Further investigation showed that the spontaneously cytotoxic lymphocytes did not display the surface antigens characteristic of T cells or B cells (Ozer *et al.*, 1979). This new type of lymphocyte activity became known as 'natural killer' or 'NK' activity and the lymphocytes responsible were termed NK cells.

Early work on human NK cells showed that morphologically the majority are large granular lymphocytes with a high cytoplasm to nucleus ratio and numerous azurophilic granules (Timonen *et al.*, 1981). In comparison to mature T and B cells, they are relatively short lived and may survive only a few days in circulation (Trinchieri, 1989).

Studies on the distribution of human NK cells have shown that they typically comprise 10-15% of the lymphocyte population of peripheral blood and are also found in the red pulp of the spleen (Timonen and Saksela, 1980; Timonen *et al.*, 1981). NK cells have been identified in the liver and mucosal tissues such as the gastrointestinal tract (Timonen and Saksela, 1980; Timonen *et al.*, 1981). In the gastrointestinal tract, they are found scattered throughout the lamina propria at the base of the crypts and in the Peyer's patches of the ileum (Hogan *et al.*, 1985; Kummer *et al.*, 1995).

1.1.3 NK Cell Effector Functions

NK cells have been shown to exhibit innate cytotoxicity towards virally-infected cells and tumour cells as well as bacteria and other pathogens. Unlike CD8+ cytotoxic T lymphocytes (CTL's), NK cells do not require cognate interaction with antigenic peptides in association with the class I major histocompatibility complex (MHC) in order to be activated (Trinchieri, 1989). In fact the binding of MHC class I by NK cells has been shown to transmit inhibitory signals to NK cells as shown by experiments where class I-deficient tumour cells, susceptible to NK lysis, are protected by the expression of transfected class I genes (Quillet *et al.*, 1988). This finding led to the formulation of the 'missing self' theory which predicts a surveillance role for NK cells in search of cells which fail to express self class I MHC (Ljunggren and Karre, 1990). The elimination of such targets by NK cells would complement the activity of CTL's which are unable to destroy class I-deficient cells.

In addition to the non-MHC restricted cytotoxicity described above, NK cells are important mediators of antibody-dependent cellular cytotoxicity (ADCC). In ADCC, NK cells recognise the Fc portion of antibody molecules bound to the surface of target

cells and consequently lyse the antibody-coated cells. In both cases, the mechanism of lysis appears to be similar and involves the release of preformed cytolytic granules in close proximity to the plasma membrane of the cell being lysed (Trinchieri, 1989). Important among the components released are perforins which insert into the membrane as monomers and then polymerise to form pores which allow osmotic lysis of the target cell (Joag et al., 1989). The pores also allow NK-released granzymes to enter the target cell and trigger apoptosis (Robertson and Ritz, 1990). In addition, the surface expression of Fas ligand (FasL) and soluble release of TNF-related apoptosis-inducing ligand (TRAIL) by NK cells trigger apoptosis upon binding to their respective ligands Fas and DR4 or DR5 on target cells (Oshimi et al., 1996; Wiley et al., 1995; MacFarlane et al., 1997). Release of soluble tumour necrosis factor α (TNF-α) is also a major factor in NK-mediated cell lysis (Joag et al., 1989).

1.1.3.1 NK Responses to Viral Infection

The importance of NK cells in the early regulation of viral infections has been well demonstrated with murine cytomegalovirus (MCMV) and herpes simplex virus (HSV) infection of mice. Suckling mice with low levels of NK activity are more susceptible to infection with these viruses and yet resistance can be induced by adoptive transfer of purified NK cells (Bukowski et al., 1985). Alternatively, resistant strains of mice treated with reagents that inhibit NK activity become more susceptible to viral infection (Welsh,1986). NK cells have been shown to inhibit MCMV infections in mice with severe combined immunodeficiency (SCID), which lack functional T and B cells, although these mice do not completely eradicate the virus. This emphasises the role of the NK cell in controlling the levels of replicating virus in the first few days of infection before the antigen-specific T cell response eradicates the virus at a later stage (Biron et al., 1983).

The important anti-viral effects of NK cells are further highlighted by the rare case of a patient completely deficient in NK activity who suffered a series of severe viral infections, including varicella zoster virus, human cytomegalovirus and HSV (Biron et al., 1989).

Human NK cells are capable of recognising and lysing virally-infected cells directly as demonstrated by in vitro killing assays (Santoli et al., 1978). The recognition step is not fully understood but could be due to downregulation of class I MHC expression by the virus (Brutkiewicz and Welsh, 1995), or the presence of viral peptides in the binding groove of class I interfering with the transmission of a negative signal to the

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NK cell (Chadwick *et al.*, 1992). There has also been some evidence to suggest that purified glycoproteins from several viruses can augment the cytolytic activity of NK cells *in vitro* and *in vivo* (Harfast *et al.*, 1980; Casali *et al.*, 1980; Arora and Houde, 1988).

The ability of activated NK cells to secrete interferon-γ (IFN-γ) is also strongly linked to anti-viral activity. IFN-γ has been shown to induce the production of the free radical nitric oxide (NO) from macrophages which in turn has a direct inhibitory effect on viral replication (Nathan, 1992; Karupiah *et al.*, 1993).

1.1.3.2 NK Responses to Bacteria and Other Parasites

Human NK cells have been shown to directly kill both gram positive and gram negative bacteria *in vitro* (Garcia-Penarrubia *et al.*, 1989). They can also lyse cells infected with *Salmonella typhimurium*, *Mycobacterium avium* or *Shigella flexneri in vitro* (Klimpel *et al.*, 1986; Katz *et al.*, 1990; Griggs and Smith, 1994). However, the evidence *in vivo* seems to suggest that the production of cytokines, particularly IFN-γ, is the most important contribution of NK cells to bacterial clearance. The prevention of NK cell IFN-γ production in SCID mice decreases the resistance to the pathogen *Listeria monocytogenes* (Tripp *et al.*, 1994). The IFN-γ mediated regulation of bacterial infection appears to be linked with production of the free-radical NO by macrophages and other cells (Glesch and Kaufmann, 1991; Beckerman *et al.*, 1993).

Direct killing of the fungus *Cryptococcus neoformans* and the protozoa *Toxoplasma gondii* and *Trypanosoma cruzi* by NK cells has also been shown *in vitro* (Hidore and Murphy, 1989; Hauser and Tsai, 1986; Hatcher and Kuhn, 1982). However, as with bacterial infections, it appears that *in vivo* the production of IFN-γ by NK cells is far more important for innate resistance to parasites than direct cytotoxic activity (Scott and Trinchieri, 1995).

1.1.3.3 NK Responses to Tumours

There is now considerable evidence that NK cells play a crucial role in the elimination of tumour metastases in the blood (Whiteside and Herberman, 1995; Vujanovic *et al.*, 1996). Early experiments showed that NK cells rapidly destroy tumour cells in the bloodstream of animal models (Riccardi *et al.*, 1980; Barlozzari *et al*, 1983) and these finding are borne out in human cancer patients where a link has been established between low levels of NK activity and high levels of metastasis (Whiteside and

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Herberman, 1994). This seems to apply to both haematological cancers, such as leukaemia, and solid tissue cancers such as breast cancer (Pross and Lotzova, 1993; Strayer *et al.*, 1986).

The cytolytic ability of NK cells is clearly important in the elimination of tumour cells and studies *in vitro* have shown that NK cells are capable of inducing necrosis, through the release of perforin, and apoptosis in tumour cell targets (Vujanovic *et al.*, 1994; Young, 1989; Whiteside *et al.*, 1998). NK-derived cytokines may also play a part in slowing the growth of tumours and increasing their susceptibility to lysis by other effector cells (Whiteside and Herberman, 1992).

NK recognition of tumour cells is only poorly understood at the moment, although it has been shown that many tumour cell lines which make good targets for NK-mediated cytolysis have reduced expression of class I MHC on their cell surface (Harel-Bellan, 1986; Storkus, 1987).

The ability of NK cells to control tumour metastases has been investigated as a means of therapy for cancer patients. Two methods which have demonstrated anti-tumour effects in animal models are the activation of endogenous NK cells and adoptive transfer of activated NK cells (Whiteside *et al.*, 1998). Unfortunately, the small number of clinical trials carried out in humans have been performed on patients in the advanced stages of metastasis and have not shown a clear therapeutic effect (Whiteside *et al.*, 1998). However, a greater understanding of NK cell biology is likely to facilitate better design of NK-mediated therapies.

1.1.4 The Regulatory Role of NK Cells

NK cells were once thought to be merely effectors of innate cytotoxic responses and to interact only with the non-specific cells of the immune system. However, it has recently been suggested that NK cells have an additional, regulatory role in acquired immune responses. Although this regulatory role is poorly understood, there are several examples of NK cells being involved in the development of antigen-specific T and B cell responses.

NK cells were shown to be crucial for the differentiation of active CTL's in a mixed lymphocyte culture reaction (Kos and Engleman, 1995). Furthermore, it appeared that direct contact between NK cell and T cell was required for CTL development as NK-derived soluble factors could not elicit the same response. Separate experiments have

also shown NK cells to be required for the development of tumour-specific CTL's (Kurosawa *et al.*, 1995).

NK cells are also thought to participate in the humoral response to T cell independent (TI) antigens associated with bacterial and viral infection. NK cell production of IFN-γ was required for antibody secretion by B cells in response to TI antigens *in vitro* (Snapper *et al.*, 1996). Evidence has also been found *in vivo* for an NK cell involvement in B cell responses to TI antigens (Wilder *et al.*, 1996).

In addition, the production of IFN-γ by NK cells was shown to favour the development of T helper type 1 (Th1) cells in the mouse following injection of leishmanial antigens (Scharton and Scott, 1993; Romagnani, 1992). Depletion of NK cells in both human and mouse studies has been shown to interfere with the IFN-γ-mediated development of Th1 clones (Manetti *et al.*, 1993; Afonso *et al.*, 1994). However NK cells are not always required for the development of Th1 cells and it is thought that they may be more important in response to antigens which induce a strong IL-4 response in the absence of adjuvant (Trinchieri and Scott, 1995).

Although the regulatory role of NK cells has yet to be clearly defined, evidence does seem to suggest that NK cells play a crucial part in certain antigen-specific T and B cell responses.

1.1.5 Surface Receptors on NK Cells

Studies of surface molecules on NK cells have been complicated by the fact that many are also expressed on related cells, most notably T cells. For example the molecule CD56, which is present on more than 95% of human NK cells, is also present on a small population of T cells (Ritz *et al.*, 1988). Significantly the T cells which express CD56 are, like NK cells, capable of non-MHC restricted cytotoxicity which suggests a strong developmental relationship between NK cells and T cells (Lanier *et al.*, 1986a). As a result of this close relationship, NK cells have become defined as much by the absence of T cell receptor (TCR) and CD3 expression as by the presence of 'NK-specific' molecules such as CD56.

Despite the problems resulting from this close relationship with T cells, a number of NK cell surface molecules have been discovered in recent years which have enabled greater understanding of the ways in which NK responses are switched on and off.

1.1.5.1 Inhibitory NK cell Receptors

Inhibition of NK cell activity occurs as a direct result of interaction with self MHC class I molecules (Shimizu and DeMars, 1989; Storkus *et al.*, 1989). As mentioned previously this infers a role for NK cells in searching for and lysing cells which lack normal expression of class I MHC, such as some tumour cells and virally infected cells (Ljunggren and Karre, 1990). The inhibitory receptors on NK cells which bind to MHC class I molecules have been extensively studied in humans and rodents.

The predominant inhibitory receptors in humans, known collectively as killer cell inhibitory receptors (KIR's), are members of the immunoglobulin (Ig) superfamily. These can be divided into the KIR2D and KIR3D subfamilies, which have two and three extracellular Ig-like domains respectively (reviewed in Lanier, 1998a). KIR's are generally expressed as monomers but one isoform, known as KIR3DL-NKAT4, can appear as a disulphide-linked homodimer (Pende *et al.*, 1996; Dohring *et al.*, 1996a).

Different KIR's are specific for different class I MHC molecules, for example the KIR2D and KIR3D subfamilies bind HLA-C and HLA-B ligands respectively, whereas the KIR3DL-NKAT4 binds only HLA-A molecules (Moretta *et al.*, 1993; Litwin *et al.*, 1994; Pende *et al.*, 1996; Dohring *et al.*, 1996a). This ligand-binding diversity of KIR molecules is generated by alternative splicing of messenger RNA (mRNA) and allelic polymorphism of the 12 estimated KIR genes located on chromosome 19 (Dohring *et al.*, 1996b; Selvakumar *et al.*, 1997). Different KIR's are expressed on overlapping subsets of NK cells which produces a diverse repertoire of receptors for MHC class I (Moretta *et al.*, 1990). In addition, KIR molecules are also expressed on a subset of CTL's, again emphasising the similarities between NK cells and some T cells (Mingari *et al.*, 1997).

The mechanism of inhibition through KIR molecules is linked to the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM's) in the cytoplasmic domain. All inhibitory KIR molecules contain two ITIM's which, after phosphorylation, recruit SH2-containing tyrosine phosphatase-1 (SHP-1) or SHP-2 (Burshtyn *et al.*, 1996; Campbell *et al.*, 1996; Fry *et al.*, 1996). The exact pathway for inhibition has not yet been defined but it is apparent that SHP-1, and possibly SHP-2, are required (reviewed in Vivier and Daëron, 1997).

Surprisingly, the major inhibitory molecules on murine NK cells, known as the Ly-49 family, are structurally unrelated to the human KIR molecules. Instead, they are C-type

lectin-like receptors, which form disulphide-linked homodimers in the plasma membrane (Mason *et al.*, 1995; Stoneman *et al.*, 1995). Despite this fundamental difference in structure, there are many striking similarities between KIR and Ly-49 receptors. Ly-49 proteins are encoded by a family of nine genes (Brown *et al.*, 1997) in which diversity is achieved by allelic polymorphism and alternative splicing of mRNA (Smith *et al.*, 1994; Silver *et al.*, 1996). Different Ly-49 receptors have specificities for different MHC class I ligands, and are expressed on overlapping subsets of NK cells to produce a diverse repertoire (Brennan *et al.*, 1994). Ly-49 can also be expressed on a subset of T cells (Chan and Takei, 1989). Finally, and perhaps most significantly, Ly-49 molecules possess ITIM's in their cytoplasmic domain which recruit SHP-1 in order to inhibit NK cells (Nakamura *et al.*, 1997).

A third set of inhibitory receptors are the CD94/NKG2 heterodimers which are present on human, mouse and rat NK cells, as well as a subset of T cells in humans (reviewed in Lanier, 1998a). These heterodimers are, like the murine Ly-49 proteins, members of the C-type lectin superfamily (Lazetic *et al.*, 1996). The CD94 subunit appears to be invariant and acts primarily as a chaperone to transport NKG2 to the cell surface (Lazetic *et al.*, 1996), whereas NKG2 is encoded by a family of four genes, designated NKG2A, NKG2C, NKG2E and NKG2D/F (Houchins *et al.*, 1991). CD94/NKG2A is the best studied of these heterodimers and possesses two ITIM sequences in the cytoplasmic domain of the NKG2A subunit, consistent with its role as an inhibitory receptor (Houchins *et al.*, 1997). Recent studies have shown that CD94/NKG2A is specific for the non-classical class I molecule HLA-E (Braud *et al.*, 1998a). HLA-E is an unusual molecule in that it requires binding of a leader peptide of a classical class I molecule before it can be expressed on the cell surface (Braud *et al.*, 1998b). As a result it has been proposed that HLA-E can be used by NK cells to detect events that disrupt the synthesis or transport of classical class I molecules (Lanier, 1998b).

The question of why the predominant inhibitory NK receptors of rodents are C-type lectins while in humans they are Ig-like molecules has yet to be answered. One theory is that the CD94/NKG2 system, which is present in rodents and humans, evolved first. These receptors detect more general deficiencies in class I expression which might result from certain viral infections (Wiertz et al., 1997). As an improvement to this system, the Ly-49 receptors in mouse and the KIR molecules in humans may have evolved to provide greater specificity and sensitivity (Lanier *et al.*, 1998).

The recent progress in molecular characterisation of inhibitory receptors has facilitated the discovery of several novel Ig-like molecules which can inhibit NK cells. These

include the p49 molecule expressed on human NK cells and a subset of T cells (Cantoni *et al.*, 1998) and the murine gp49 which is expressed on NK cells and mast cells (Rojo *et al.*, 1997; Wang *et al.*, 1997). The human p49 protein is inhibited specifically by HLA-G1 molecules expressed on the human trophoblast during pregnancy (Ponte *et al.*, 1999). The relative importance of these new inhibitory receptors, compared to the existing KIR's, has yet to be demonstrated.

1.1.5.2 Activating Receptors on NK cells

In contrast to the current understanding of inhibitory receptors on NK cells, there are many gaps in the knowledge about receptors which activate NK cells. Despite an intensive search, no predominant receptor which is highly antigen specific, equivalent to the TCR of T cells or the surface Ig of B cells, has been found on NK cells. Instead the search has highlighted an array of activating receptors with an ability to bind fairly broad ranges of targets.

One of the best studied activating receptors on NK cells is the low affinity receptor for IgG, known as CD16 or $Fc\gamma RIII$. CD16 is expressed on most mouse and human NK cells as well as on activated monocytes and a subset of T cells (Lanier *et al.*, 1986b; Phillips *et al.*, 1991). Following ligation to the Fc portion of an IgG molecule, CD16 becomes associated with the γ subunit of the high affinity IgE receptor ($Fc\epsilon RI-\gamma$) or the ζ subunit of CD3 in human NK cells (Lanier *et al.*, 1991). Both $Fc\epsilon RI-\gamma$ and the ζ subunit possess immunoreceptor tyrosine-based activation motifs (ITAM's) in their cytoplasmic domains and these are consequently phosphorylated by src-family tyrosine kinases (Salcedo *et al.*, 1993). This begins a cascade of biochemical events which leads to secretion of cytokines and activation of ADCC mechanisms (Perussia et al., 1984; Anegon et al., 1988). CD16 is thought only to be involved in cytotoxic responses to antibody-coated targets (ADCC) and not in any other NK-mediated cytotoxicity.

The NKR-P1 family of proteins are members of the C-type lectin superfamily and have been primarily studied in rodents although a human homologue has also been found (Lanier *et al.*, 1994). They are expressed as disulphide-linked homodimers on the surface of most NK cells and a subset of T cells (Chambers et al., 1989; Giorda et al., 1990). Monoclonal antibodies (mAb's) to NKR-P1 are able to induce a rise in intracellular Ca^{++} levels in NK cells, indicative of activation, and trigger cytotoxic responses and cytokine production (Chambers *et al.*, 1989; Ryan *et al.*, 1991; Arase *et al.*, 1996). The triggering mechanism of NKR-P1 is probably related to a motif in the

cytoplasmic region, also found on CD4 and CD8, which can interact with the src-family kinase p56[lck] and thus initiate signal transduction (Turner *et al.*, 1990; Campbell & Giorda, 1997). In addition, NKR-P1 interacts with the molecule FcεRI-γ (Arase *et al.*, 1997) and this association may be equally important as FcεRI-γ has been shown to play a role in signal transduction from other receptors, such as CD16 (Lanier et al., 1991). The ligand for the NKR-P1 receptors is unknown although recombinant NKR-P1 has been shown to bind certain synthetic carbohydrates which may be expressed on tumour and lymphoblastoid cells (Bezouska et al., 1994).

The KIR, Ly-49 and CD94/NKG2 families of inhibitory receptors which bind class I MHC molecules all have variants which do not express ITIM's and have the ability to activate rather than inhibit NK cells. When bound by the appropriate class I ligand, the activating KIR receptor p50 has been shown to increase Ca^{++} levels in NK cells and induce cytotoxic responses (Moretta *et al.*, 1995). The ability to be activated upon binding class I MHC is in agreement with the observation that rat and mouse NK cells are capable of killing MHC-mismatched donor cells (Ohlen *et al.*, 1989; Rolstad *et al.*, 1997). The signal transduction mechanism of the activating forms of KIR, Ly-49 and CD94/NKG2 is probably due to the presence of a charged amino acid in the transmembrane domain (Houchins *et al.*, 1991; Biassoni *et al.*, 1996; Mason *et al.*,1996). This residue is thought to allow the binding of a small dimeric molecule known as DAP-12 which contains an ITAM in its cytoplasmic domain (Lanier *et al.*, 1998c). Once phosphorylated, DAP-12 is able to initiate a cascade of biochemical events which lead to NK cell activation (Lanier *et al.*, 1998c).

Many other membrane receptors have been linked to NK cell activation as mAb's against these molecules can stimulate NK-mediated lysis of target cells. These include the human CD2, CD44 and DNAM-1 molecules, mouse 2B4 and Ly6 and mouse and human CD69 which are all expressed on a variety of cells other than NK cells (reviewed in Long and Wagtmann, 1997). Also important in NK cytotoxicity are cell adhesion molecules which can allow conjugation to target cells prior to lysis. The most important of these are LFA1 (CD11a/CD18) and CD2 which bind the respective ligands ICAM-1 (CD54) and LFA-3 (CD58) (reviewed in Robertson and Ritz, 1990). Some of these molecules are probably only co-stimulatory and thus unable to initiate NK cell activation alone.

More recently, a number of novel activating receptors which show a high level of specificity for NK cells have been discovered. Two of these are the NKp44 and NKp46 molecules cloned from human NK cells, which are members of the

immunoglobulin superfamily (Pessino *et al.*, 1998; Cantoni *et al.*,1999). Both are specifically expressed on NK cells and when cross-linked by mAb's are capable of inducing lysis of tumour cell targets (Sivori *et al.*, 1997; Vitale *et al.*, 1998). NKp44 and NKp46 are similar in that they each contain a charged amino acid in their transmembrane domain which attract the signal transducing molecules DAP-12 and the ζ-subunit of CD3 respectively (Vitale *et al.*, 1997). Homologues of both these receptors have been found in the mouse (Pessino et al., 1998; Biassoni et al., 1999) which implies a fundamental role in NK cell activation.

Finally, a receptor which shows remarkable conservation through vertebrate evolution is the nonspecific cytotoxic cell receptor protein-1 (NCCRP-1), originally discovered on the surface of nonspecific cytotoxic cells (NCC) in teleost fish (Evans *et al.*, 1988). NCC are the teleost equivalent of mammalian natural killer cells and display spontaneous cytotoxicity towards certain tumour cell targets (reviewed in Evans and Cooper, 1990). Monoclonal antibodies to NCCRP-1 were found to block NCC recognition and subsequent lysis of tumour targets (Evans *et al.*, 1988). This trait was also apparent when the antibody was applied to human NK cells, although the high concentration of antibody required for an effect on human NK cells casts some doubt on these findings (Harris *et al.*, 1991). Teleost NCCRP-1 has now been sequenced (Jaso-Friedmann, *et al.*, 1997) and recent work suggests that it may be a type III membrane receptor capable of recognising antigen (Evans *et al.*, 1998).

1.1.5.3 A Balance between Activating and Inhibitory Receptors

It is clear that there are a large array of molecules expressed on NK cells which can trigger cytotoxicity in a fairly simple and nonspecific manner. Most of the ligands for activating receptors have yet to be identified but it is unlikely that they are highly specific (reviewed in Long and Wagtmann, 1997). In fact, some of these ligands are expressed on healthy cells which predicts a requirement for control mechanisms to prevent NK cells destroying self tissue. It would appear that this control is achieved through the more specific signals received via the various MHC class I-binding inhibitory receptors. These inhibitory receptors, through SHP-1, have been shown to block crucial steps in the transduction of activating signals and thus override potentially self-destructive cytotoxicity (Binstadt et al., 1996; Valiante et al., 1996). However, the inhibition is not absolute and it has been shown that inhibitory signalling through human KIR can be overcome using agonist mAb's to several activating receptors simultaneously (Lanier et al., 1997). This suggests that the responses of NK cells are held in check by a balance of positive and negative signalling events through

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numerous receptors, with certain environments able to tip the balance towards a cytotoxic response.

1.1.6 Evolutionary Studies of NK Cells

Although the vast majority of NK research has been performed in mammals, there is an increasing body of evidence to suggest that naturally cytotoxic cells are an important part of the immune systems of more primitive species. The importance of cytotoxic immune effectors may even stretch as far back through animal evolution as the invertebrates, as the earthworm displays some degree of spontaneous cytotoxicity towards tumour cell targets (Cossarizza *et al.*, 1996).

1.1.6.1 The Prevalence of Natural Cytotoxicity in Lower Vertebrates

Of the lower vertebrate animals, naturally cytotoxic cells have been identified in fish, amphibians, reptiles and birds (Evans and Cooper, 1990; Ghoneum et al., 1990; Sherif and Elridi, 1992; Göbel *et al.*, 1994). As mentioned previously, teleost fish have NCC, a population of lymphoid cells which are innately cytotoxic towards tumour cell targets (reviewed in Evans and Cooper, 1990). Strong evidence of the relatedness of these fish NCC to mammalian NK cells comes from the finding that an antibody to NCCRP-1 on fish NCC also binds specifically to human NK cells (Harris *et al.*, 1991).

Initial studies of amphibians highlighted the presence of naturally cytotoxic cells capable of lysing tumour cell targets (Ghoneum et al., 1990). Further study of the amphibian *Xenopus laevis* has shown that a non-T, non-B lymphoid cell population displays cytotoxicity towards an allogeneic, MHC-deficient thymus tumour target B_3B_7, which is strongly suggestive of NK activity in these animals (Horton *et al.*, 1996).

Cytotoxic activity towards the NK-sensitive human tumour cell line K562, was observed in peripheral blood mononuclear cells of the snake *Psammophis sibilans* (Sherif and Elridi, 1992). Similar activity was found in spleen and thymus populations, and further work is required to attribute this killing to a reptilian equivalent of NK cells.

In the chicken, candidate NK cells have been identified which, like mammalian NK cells, have been shown to lyse an NK-sensitive tumour cell line (Göbel *et al.*, 1994). These avian lymphoid cells are able to bind chicken IgG, suggesting the presence of Fc

receptors and an ability to kill by ADCC (Göbel *et al.*, 1994). They also lack expression of the TCR/CD3 complex on the cell surface, although they do express cytoplasmic CD3 and surface CD8, indicating that these cells have much in common with T cells (Göbel *et al.*, 1994).

1.1.6.2 Insights into the Evolution of Cytotoxic Lymphocytes

Despite being acknowledged as two distinct populations of lymphocytes, NK cells and CTL do bear many striking similarities. T cell surface receptors such as CD2 and CD8 can be expressed on NK cells and most of the NK-defining molecules, including CD16, CD56, KIR, Ly-49, CD94/NKG2 and NKRP-1, are expressed on subsets of CTL (reviewed in Lanier, 1998a). From a functional viewpoint, the subset of CTL which express these NK-defining molecules such as CD56 are, like NK cells, capable of non-MHC restricted cytotoxicity (Lanier *et al.*, 1986a). In addition, both NK cells and CTL can be stimulated by the cytokines interleukin (IL)-2 or IL-12 (Smith, 1988; Kobayashi *et al.*, 1988) and furthermore, extended culture of CTL with recombinant IL-2 increases the amount of non-MHC restricted cytotoxicity in these cells (Dianzani et al., 1989).

Many of the proteins involved in signal transduction in T cells, namely the CD3 ζ-chain, the tyrosine kinases p56^{lck} and ZAP-70, phospholipase C, MAP kinase, p21 ras and SHP-1, are also utilised by NK cells (reviewed in Lanier, 1998a). The perforin-dependent and Fas-mediated cytolytic effector mechanisms are also shared by both NK cells and CTL (Valiante and Parham, 1996).

These similarities between NK cells and CTL, as well as the prevalence of NK cells in some very primitive organisms, have given rise to the idea that NK cells are the evolutionary fore-runners of cytotoxic T cells (Janeway, 1989). It is proposed that a primitive immune system would tend to consist of a few multi-specific effector cells, such as NK cells. The consequent acquisition by NK cells of clonally distributed, antigen-specific receptors, at some point in animal evolution, could conceivably have turned them into cytotoxic T cells (Janeway, 1989).

1.2 Comparative Studies in Biology

The study of comparative animal models allows the identification of biological phenomena which are conserved throughout animal evolution. In the case of comparative immunology, where such phenomena are intrinsically linked to the

survival of the animal, the conservation of these features strongly underlines their immunological significance.

1.2.1 The *Xenopus laevis* Comparative Model

As an immunological model for the investigation of NK cells, the amphibian *Xenopus laevis* offers certain advantages over other animal models. Firstly, the *Xenopus* immune system is the best characterised of the lower vertebrates and shows a high level of similarity with the immune systems of mammals. All the important lymphoid organs are present, including thymus, spleen, liver, kidney and gut-associated lymphoid tissue (Plytycz and Bigaj, 1983; Katagiri and Tochinai, 1987). B and T cells, including T-helper and T-cytotoxic subsets, have been identified (Horton, 1994) as have the equivalents of MHC class I and class II (Du Pasquier *et al.*, 1989). In addition, *Xenopus* possesses three types of antibody molecules: IgM, IgY, a likely precursor of mammalian IgG and IgE, and IgX, a possible equivalent of mammalian IgA (Du Pasquier *et al.*, 1989).

A second advantage of studying *Xenopus* lies in the ability to remove the thymus during the initial stages of histogenesis in the early larval development of the animal (Horton and Manning, 1972). These early-thymectomised (Tx) animals develop into healthy adults in which virtually no functional T cells can be identified (Horton *et al.*, 1998a). The many similarities between CTL and NK cells can sometimes make it difficult to attribute cytotoxic responses to one cell type or the other and it is therefore advantageous to be able to remove T cells. Tx *Xenopus* thus offer a unique environment in which to study NK cells free of any T cell complications. In addition, the physical removal of the thymus also means that Tx *Xenopus* are not hindered by any of the congenital defects associated with athymic nude rodents (Horton *et al.*, 1998a).

Thirdly, the identification of features which are conserved in the immune systems of vertebrates underlines their fundamental importance in immunology, as shown by the discovery of MHC, CTL and B cells in many lower vertebrates (reviewed in Horton and Ratcliffe, 1996). In the case of NK cell receptors, the studies of rodents and humans have drawn a rather confusing picture involving many different receptor families, some of which do not appear to be shared between the different species. By characterising the receptors found in the NK cells of lower vertebrates, it may be possible to identify which ones are conserved in all NK cells. The conservation of such

receptors throughout the evolution of vertebrates would certainly indicate their fundamental importance in NK cell function.

Finally, the study of comparative immunology offers insights into the evolution of the immune system. As mentioned above, it is thought that NK cells are the evolutionary forerunners of CTL. By studying the immune systems of more primitive species it may be possible to compile evidence for a common lymphoid progenitor.

1.3 Phage Display Technology

1.3.1 The Uses of Specific Antibodies

Specific antibodies to cell surface antigens have proven invaluable tools for the study of NK cells. When used in conjunction with flow cytometry they provide accurate quantification of NK cell numbers in different lymphoid organs. Application of specific antibodies to immunocytochemical staining of tissue sections allows the identification of the specific distribution of NK cells in various organs. Using magnetic cell sorting or flow cytometric cell sorting they allow purification of NK cells from mixed lymphoid populations, enabling functional and molecular studies to be performed on purified populations of cells. Furthermore, if they are specific for an important receptor on the NK cell surface, they permit isolation of that protein which can lead to cloning of the relevant gene. In some cases the antibodies even have agonist or antagonist properties which allow detailed analysis of activatory or inhibitory signals.

Up to now, these antibodies have been produced using classical hybridoma technology, whereby B-cells taken from an animal immunised with NK cell antigens are fused *in vitro* to myeloma cells using polyethylene glycol. These hybridoma's can be cultured *in vitro* and cloned by limiting dilution. Because each hybridoma results from the fusion of a single plasma cell to a myeloma cell, it will only secrete monospecific antibodies.

In recent years an alternative technology, known as phage display, has emerged which is also capable of isolating monospecific antibodies to antigens of choice.

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1.3.2 Phage Display

1.3.2.1 The Bacteriophages

Bacteriophages, or 'phages' are a diverse family of viruses which infect bacteria. They do so because they are unable to replicate independently and require the bacterial replication machinery for this purpose. The filamentous bacteriophage M13, the vector of choice in phage display, is shaped like a long fibre about 6nm in diameter and 900-1900nm in length (Prescott *et al.*, 1990). It specifically infects male (F+) *E.coli* cells by attaching to the tip of the F pilus. Following replication, progeny phage are extruded through the pili, leaving the host cell intact (Prescott *et al.*, 1990).

1.3.2.2 The Concept of Phage Display

The human B cell population of a healthy individual expresses approximately 10^{12} different antibodies, each with a distinct specificity for antigen (reviewed in Rolink and Melchers, 1993). This diversity is a result of variation in the amino acid sequence of complementarity determining regions (CDR's) within the variable heavy (VH) and variable light (VL) chains of the antibody molecule, which combine to form the antigen binding site. Using the polymerase chain reaction (PCR) it is possible to amplify DNA sequences which encode the VH and VL regions and express them in bacteria, whereupon they retain the ability to bind antigen (Skerra and Plückthun, 1988).

By cloning large numbers of VH and VL regions and recombining them at random, a diverse library of antibody fragments can be generated. In phage display, this library is usually expressed on the surface of the filamentous bacteriophage M13 by fusion to either gene III or gene VIII, which encode phage coat proteins (reviewed in Winter *et al.*, 1994). In this way, each recombinant phage is like a monoclonal antibody as it expresses a single antibody fragment with a distinct specificity for antigen. Phage antibody libraries can be panned against antigen and those individual phage which bind the antigen can be specifically eluted and re-amplified for further panning. Over successive rounds of panning the original library becomes enriched for those phage with a higher affinity for the antigen. Once individual phage antibodies have been isolated which show specificity for a given antigen, it is possible to increase their binding affinity by site-directed mutation to levels comparable with hybridoma-derived antibodies (Schier *et al.*, 1996).

1.3.2.3 The Advantages and Disadvantages of Phage Display

The most significant feature of phage display is that each recombinant phage is a replicating unit containing the DNA sequence which encodes its surface antibody. This direct link between DNA sequence and protein function enables vast phage libraries, containing as many as 10^{10} different specificities, to be screened by successive rounds of panning against antigen in as little as two weeks (Griffiths and Duncan, 1998). Classical hybridoma technology, on the other hand, usually allows the screening of a few hundred clones over the course of several months (Harlow and Lane, 1988).

The immunisation stage prior to hybridoma production imposes a certain degree of selection pressure on the B cell population. For instance, B cells will not be stimulated to differentiate into antibody-producing plasma cells if an antigen is not immunogenic. Alternatively, B cells will be deleted if they produce antibodies to conserved epitopes which are expressed in the animal's own tissues. This places some constraint on the specificities which are obtainable through the hybridoma technique. Such selection pressures do not exist in the phage display system. By artificially recombining different VH and VL regions or rearranging sequences within the CDR's of the VH and VL chains it is possible to generate novel binding specificities *in vitro* (Nissim *et al.*, 1994). This may offer a significant advantage when trying to raise antibodies to non-immunogenic, conserved or rare antigens.

The ability to manipulate antibody fragments *in vitro* offers other advantages. As mentioned earlier, it is possible to increase the binding affinity of selected clones by site-directed mutagenesis. Production of soluble antibody fragments from bacteria can also be easily achieved and it is possible to introduce peptide tags which enable simple purification, detection or dimerisation of fragments (reviewed in Krebber *et al.*, 1997). DNA encoding these fragments can also be stored indefinitely at -80°C in a stable form which can be revived without any loss of function. Conversely, viable hybridoma cells can be difficult to revive after freezing and can also accumulate mutations which eventually interfere with the production of functional antibodies (Harlow and Lane, 1988).

Despite the many advantages of phage display, it should be stressed that it is still a developing technique. Many of the reports of successful and rapid isolation of specific phage antibodies have used highly purified, 'known' proteins as antigen whereas in reality many researchers wish to raise antibodies to 'unknown' antigens in their native conformation, such as cell surface proteins. Currently phage display is relatively poor

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at raising antibodies to such antigens due to difficulties in the selection process. However, a great deal of research is being made into improving selection strategies and tailoring phage display to the task of raising useful antibodies to novel, cellular antigens (reviewed in Griffiths and Duncan, 1998).

1.4 Aims and Objectives

The aim of this project was to investigate NK cells in the amphibian *Xenopus laevis* by three strategies:

1. The optimisation of protocols to construct and screen phage display libraries against whole, live *Xenopus* cells with a view to raising phage antibodies to NK-enriched lymphocyte preparations from Tx *Xenopus*.

2. Using hybridoma-derived antibodies recently raised to cell surface determinants on *Xenopus* NK cells (Horton *et al.*, manuscript submitted) it was intended that molecular characterisation of NK cell receptors could be conducted using Western blotting and immunoprecipitation. Subsequently, it was envisaged that sequence information could be gained from the purified protein with a view to designing degenerate PCR primers and cloning the relevant gene(s). Attempts could also be made at isolating the relevant gene(s) by immunoscreening cDNA expression libraries cloned from *Xenopus* lymphoid organs. Any sequence information gained could be entered into BLAST search algorithms to try and find homology with NK proteins in other species.

3. By designing degenerate PCR primers to conserved sequences found in mammalian NK receptors, attempts were to be made to isolate *Xenopus* homologues of these NK-related genes.

CHAPTER 2

Generation of Phage Display Antibodies to the *Xenopus* Tumour Cell Line B₃B₇

A trial run was conducted using the *Xenopus* tumour cell line B₃B₇ to evaluate the ability of phage display to produce specific antibodies to cellular epitopes. The intention of this work was to assess the performance of phage antibodies and soluble antibody fragments in different immunological assays such as flow cytometry and Western blotting.

2.1 Introduction

2.1.1 Phage Display Libraries

Antibody libraries cloned from both naïve and immunised animals have been used successfully in the isolation of specific phage antibodies (Huse *et al.*, 1989; Vaughan *et al.*, 1996). Of these, the immune libraries have the advantage that the majority of cloned VH and VL regions encode antibodies against the immunogen which means that relatively small (10^5 clone) libraries can be panned successfully (Huse *et al.*, 1989; Clackson *et al.*, 1991). These VH and VL regions have also undergone affinity maturation during immunisation which in turn increases the affinity of the phage-displayed antibodies (Griffiths and Duncan, 1998). For these reasons it was decided that libraries cloned from immunised mice would be used in this study.

The PCR primers required to amplify all known mouse VH and VL regions have been published (Krebber *et al.*, 1997). These primer sequences were obtained by combining previously published primers sets which were designed to amplify mouse VH and VL regions (Kettlebrough *et al.*, 1993; Ørum *et al.*, 1993; Zhou *et al.*, 1994). The optimised set incorporates all VH and VL sequences found in a comprehensive collection of mouse immunoglobulin data (Kabat *et al.*, 1991).

The most frequently used phage display vectors are phagemids, which are bacterial plasmids containing a bacteriophage origin of replication. Antibody fragments can be cloned into phagemids directly upstream of a sequence encoding an M13 phage coat protein, usually coat protein III (cpIII). This allows expression of antibody fragments

as fusions to M13 coat proteins in a suitable *E.coli* host cell (Hoogenboom *et al.*, 1991). Wild type, also known as helper, M13 bacteriophage can infect and replicate in the same host cell which allows incorporation of the fusion protein into the coat of the progeny phage. By fusing antibody fragments to cpIII, up to five copies of the antibody will be expressed on the surface of the recombinant phage particle (Barbas *et al.*, 1991). Because the phagemid DNA also contains an M13 origin of replication it can also be packaged inside each recombinant phage. Upon re-infection of *E.coli*, recombinant phage are unable to propagate and the phagemid DNA reverts to replication as a plasmid. An overview of the production of recombinant M13 phage particles is shown in Figure 2.1.

Although complicated in theory, the use of phagemid vectors has been preferred in recent years for their higher cloning efficiency compared with direct cloning into phage vectors (Huse *et al.*, 1989; McCafferty *et al.*, 1990). In this way, phagemids facilitate the construction of larger, more diverse libraries (Griffiths and Duncan, 1998).

The two types of antibody fragment used in phage display are single chain variable fragments (scFv's) and Fab fragments. ScFv's are formed by linking VH and VL regions together to form a single polypeptide, whereas Fab fragments consist of larger portions of heavy and light chains which are expressed separately and associate in the bacterial periplasm (Winter and Milstein, 1991). The smaller size of scFv fragments makes them easier to express in bacteria leading to greater stability of libraries during bacterial culture. The phage display system used in this study (Krebber *et al.*, 1997) utilises scFv fragments fused to cpIII of bacteriophage M13 for expression on the phage surface.

2.1.2 Phage Display Panning Strategies

The *Xenopus* lymphoid cell line B₃B₇ was derived from an animal which had developed a tumour of the thymus (Du Pasquier and Robert, 1992). Its lack of class I MHC expression (Du Pasquier *et al.*, 1995) has made it an excellent target for assaying natural killer cell activity in *Xenopus* (Horton *et al.*, 1996). For this reason, and because it can be continuously cultured *in vitro*, B₃B₇ cells were chosen to test the efficacy of phage display panning for raising specific antibodies.

As mentioned in Chapter 1, the majority of successful phage panning strategies have been conducted using purified protein antigens coated onto plastic tubes (Marks *et al.*, 1991). The main drawback of this approach is that only antigens which have already

been characterised by other methods may be used for panning. In addition, phage antibodies raised in this way often fail to bind native protein (Griffiths and Duncan, 1998). In order to try and raise phage antibodies to novel proteins in their native conformation, this study used whole B_3B_7 cells to immunise the mice from which the phage display library was cloned. This library was then panned on whole, live B_3B_7 cells and flow cytometry was used for the detection of bound phage. Similar methods have been described elsewhere which have been successful in raising phage antibodies to novel cellular epitopes (De Kruif et al., 1995; Watters et al., 1997).

2.2 Materials and Methods

2.2.1 General Materials and Methods

Except where indicated, all chemicals and biological reagents were obtained from Sigma Chemical Company Ltd or BDH Ltd and were 'Analar' or the best grade available.

2.2.1.1 Buffers

Amphibian phosphate buffered saline (APBS)	0.11M NaCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4 (pH7.6)
DNA loading buffer	0.25% bromophenol blue, 40% (w/v) sucrose in ddH_2O
FACS buffer	APBS + 0.1% bovine serum albumin (BSA) + 0.1% NaN_3
Phosphate buffered saline (PBS)	0.14M NaCl, 6mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.6mM KCl (pH7.4)
Protein loading buffer	50mM Tris HCl (pH6.8), 100mM dithiothreitol (DTT), 2% sodium dodecyl sulphate (SDS), 0.1% bromophenol blue, 10% glycerol
SDS-PAGE running buffer	0.25M Tris, 0.5M Glycine, 1% SDS (pH8.3)
TAE	40mM Tris acetate, 1mM Ethylenediaminetetraacetic acid (EDTA) (pH7.7)

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| TBS | 10mM Tris HCl (pH7.5), 150mM NaCl |

2.2.1.2 Sterilisation Procedure

All glassware, plasticware and other equipment required for aseptic work was autoclaved for 15 minutes at 120°C, 15 psi. Solutions were either autoclaved or filter sterilised through 0.2μm filters before use.

2.2.1.3 General DNA Manipulations

Quantification of DNA or RNA solutions

DNA or RNA solutions were diluted with nuclease-free water (Promega) and the absorbance at 260nm measured on a Beckman DU7500 spectrophotometer. An A_{260} of 1.0 is equivalent to 50μg/ml of double stranded DNA or 40μg/ml of RNA.

For very dilute DNA solutions, quantification was achieved by visual comparison to DNA of known concentration after agarose gel electrophoresis, staining with ethidium bromide and exposure to UV light. Standards of known concentration were derived from a dilution series of linearised plasmid pUC19.

Restriction digestion of DNA

Restriction digests were normally performed in a volume of 50μl. The DNA to be digested was adjusted to approximately 1μg/10μl before addition of 0.1 volumes of the manufacturer's 10x digestion buffer and 1-2 units of restriction enzyme per 10μl volume. Samples were incubated at 37°C for 2-3 hours, with the exception of the restriction enzyme SfiI which required incubation at 50°C for 2-3 hours.

Agarose gel electrophoresis

For separation of DNA fragments according to size, agarose gel electrophoresis was employed. 0.5g (1%) or 1g (2%) agarose (Gibco) was dissolved in 50ml TAE buffer by heating in a microwave for 1 minute. Ethidium bromide was added to a final concentration of 0.5μg/ml before pouring the molten agarose into gel formers and allowing to set. Gels were immersed in TAE buffer containing 0.5μg/ml ethidium bromide in electrophoresis tanks. 0.2 volumes DNA sample loading buffer were added

to each sample prior to loading into the wells. Gels were electrophoresed for 2 hours at 10V/cm and DNA bands visualised under long wave UV light (320nm).

Polyacrylamide gel electrophoresis (PAGE)

In some cases, DNA samples were separated by PAGE. 1mm thick gels were cast using the Bio-Rad Mini-Protean II protein gel apparatus. All glass plates used for casting gels were cleaned with 100% ethanol prior to use. A 10% separating and 4% stacking gel were prepared as follows:

	10% Separating	4% Stacking
30% Acrylamide (29.2% acrylamide, 0.2% bisacrylamide)	3.33ml	0.67ml
Distilled water	6.42ml	4.20ml
50x TAE buffer	0.20ml	0.10ml
10% ammonium persulphate	0.05ml	0.025ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.005ml	0.005ml
	Total: 10ml	Total: 5ml

3.8ml of the separating gel mixture was pipetted into the gel casting apparatus, overlaid with 200µl isopropanol and left for 1 hour to set. The isopropanol was poured off and 1ml of the stacking gel mixture was pipetted on top of the separating gel and the comb inserted. After 1 hour, the cast gel was placed into the electrophoresis tank and immersed in TAE buffer. 0.2 volumes DNA sample loading buffer was added to each sample prior to loading into the wells. Gels were electrophoresed for 1.5 hours at 20V/cm then soaked in TAE buffer containing 0.5µg/ml ethidium bromide for 30 minutes and DNA bands were visualised under long wave UV light (320nm).

Purification of DNA from agarose gels using silica fines

The DNA band of interest was excised from a 1% agarose gel using a straight-edged razor blade. The excised piece of agarose was placed in an eppendorf tube, weighed, and 3 volumes of sodium iodide stock solution (6M sodium iodide, 0.1M sodium sulphite in ddH$_2$O) were added. The tube was incubated at 50°C for 5 minutes to allow the agarose to melt. 10µl silica fines (50% w/v in ddH$_2$O), generously donated by Prof. N. Robinson of Newcastle University, was added and the tube incubated for 15 minutes at 50°C, mixing every 3 minutes to keep the silica fines in suspension. The

silica fines were pelleted by spinning at 15000rpm for 5 seconds, the supernatant was removed and replaced with 500μl 70% ethanol. This wash step was repeated before removing as much 70% ethanol as possible by pipetting and allowing the rest to evaporate for 2 minutes at RT. The silica fines were eluted by resuspending in 10μl ddH$_2$O and incubating for 5 minutes at 50°C. After spinning at 15000rpm for 5 minutes, the supernatant containing the DNA was transferred to another tube and stored at -20°C.

Preparation of plasmid DNA

Small scale purification of plasmid DNA from 1.5ml overnight cultures of *E.coli* was carried out using the Genie DNA Extraction Kit (Helena Biosciences). The cultures were spun in eppendorfs for 30 seconds at 15000rpm to pellet the *E.coli* cells which were then resuspended in 150μl Solution I (50mM glucose, 10mM EDTA (pH8.0), 25mM Tris HCl (pH8.0). 150μl Solution II (0.2M NaOH, 2% (w/v) SDS) was added to lyse the cells before addition of Solution III (3M Potassium acetate, 0.1% (v/v) glacial acetic acid). The tubes were spun at 15000rpm for 5 minutes to pellet the precipitated protein and genomic DNA. The supernatant was added to 200μl DNA Binding Resin and the plasmid DNA captured by spinning at 15000rpm for 30 seconds in the columns provided. Captured DNA was washed once in 500μl 70% ethanol before being eluted in 50μl ddH$_2$O and stored at -20°C.

Ligation reactions

DNA fragments with compatible cohesive ends produced by restriction enzyme digestion were ligated together using T4 DNA ligase (MBI Fermentas). A molar ratio of vector to insert DNA of 3:1 was used in each ligation in a total volume of 10μl. Added to each reaction were 0.1 volumes of 10x ligation buffer (Boehringer Mannheim) and 4 units T4 DNA ligase. Ligation reactions were incubated for 3-4 hours at 14°C.

2.2.1.4 General Protein Manipulations

SDS-PAGE

Denatured proteins were separated according to size by SDS-PAGE. 1mm thick gels were cast and electrophoresed using the Bio-Rad Mini-Protean II protein gel apparatus.

All glass plates used for casting gels were cleaned with 100% ethanol prior to use. A 10% separating and 4% stacking gel were prepared as follows:

	10% Separating	4% Stacking
30% Acrylamide	3.33ml	0.67ml
(29.2% acrylamide, 0.2% bisacrylamide)		
Distilled water	3.61ml	2.80ml
1.5M Tris HCl (pH8.8)	2.50ml	-
0.5M Tris HCl (pH6.8)	-	1.25ml
2% SDS	0.50ml	0.25ml
10% ammonium persulphate	0.05ml	0.025ml
TEMED	0.005ml	0.005ml
	Total: 10ml	Total: 5ml

3.8ml of the separating gel mixture was pipetted into the gel casting apparatus, overlaid with 200μl isopropanol and left for 1 hour to set. The isopropanol was poured off and 1ml of the stacking gel mixture was pipetted on top of the separating gel and the comb inserted. After 1 hour, the cast gel was placed into the electrophoresis tank and immersed in SDS gel running buffer. 0.2 volume protein sample loading buffer was added to each sample which was then boiled for 3 minutes to denature the protein. Standard protein size markers were included for comparison and all samples were loaded into wells and the gel electrophoresed at 200V for 45 minutes.

Coomassie staining of protein gels

Following electrophoresis, gels were incubated for 1 hour at RT in 20ml protein stain solution (0.25g Coomassie blue, 45ml methanol, 45ml distilled water, 10ml glacial acetic acid) on a rocker platform. Gels were then transferred to 20ml de-stain solution (45ml methanol, 45ml distilled water, 10ml glacial acetic acid) and incubated for several hours, or until the background was clear and protein bands were visible.

Western blotting

Transfer of proteins from polyacrylamide gels to nitrocellulose was carried out using the Bio-Rad Trans-Blot® Electrophoretic Transfer cell. Following separation of proteins by SDS-PAGE, gels were placed in 50ml transfer buffer (25mM Tris, 192mM glycine, 20% methanol) and soaked for 15 minutes to remove SDS. The gel was placed on top of a piece of nitrocellulose (Schleicher and Schuell) and sandwiched between 4

sheets of 3MM paper (Whatman). The gel sandwich was placed between two fibre pads (Bio-Rad) before assembling in the transfer cell and immersing in an electrophoresis tank filled with transfer buffer. 30V was applied to the transfer cell overnight. To check for transfer of proteins, nitrocellulose filters were stained in 20ml Ponceau red stain (0.2% Ponceau S, 3% trichloroacetic acid in distilled water) and de-stained in 20ml 5% acetic acid in distilled water.

2.2.2 Phage Display Library Construction

2.2.2.1 Bacterial Strains

The strains of *Escherichia coli* used for recombinant antibody expression were as follows:-

XL1-Blue   *endA*1, *hsd*R17 (r_k-m_k+), *sup*E44, *thi*-1, λ-, *rec*A1, *gyr*A96, *rel*A1, Δ(*lac*), [F' *pro*AB, lacI^q, lacZΔM15, Tn10 (Tet^r)]

JM83   *thi*Δ, *lac*-, *pro*AB, *xyl*, *ara*, *mtl*, *rpsL* (ϕ80 *lacZ* ΔM15), λ-

2.2.2.2 Bacterial Growth Media

The media used for growing bacteria were made up as follows:-

2xYT medium:

> 16g   Trypticase peptone (Becton-Dickinson)
> 10g   Yeast extract (Oxoid)
> 5g   NaCl
> Made up to 1 litre with distilled water
> (For 2xYT agar, 15g agar (Gibco) was added)
> (For 2xYT soft-agar, 7.5g agar (Gibco) was added)
> This solution was autoclaved before use.

NE (non-expression) medium:

> 2xYT broth or 2xYT agar was made up, autoclaved and allowed to cool before the addition of 0.05 volumes of 20% glucose and 25μg/ml chloramphenicol

LE (low expression) medium:

> 2xYT broth or 2xYT agar was made up, autoclaved and allowed to cool before the addition of 0.05 volumes of 20% glucose, 25μg/ml chloramphenicol and 0.5mM isopropyl-ß-D-thiogalactoside (IPTG) (Melford laboratories)

Expression medium:

> 2xYT broth or 2xYT agar was made up, autoclaved and allowed to cool before the addition of 25μg/ml chloramphenicol.

2.2.2.3 Immunisation of Mice with *Xenopus* B₃B₇ Tumour Cells

Two Balb/C mice were each given two intra-peritoneal injections of 1×10^7 B$_3$B$_7$ tumour cells in 0.25ml PBS over a twenty day period. After the second injection, a test bleed was performed on the mice to confirm the reactivity of the serum to B$_3$B$_7$ cells by flow cytometry (data not shown). Two weeks after the second injection, the mice were killed by cervical dislocation and the spleens were dissected out.

2.2.2.4 Isolation of Total RNA from the Spleen of Immunised Mice

All tubes in contact with RNA were previously treated with 'RNase AWAY' (Molecular Bio-Products, San Diego) to remove RNA degrading enzymes. RNase-free tips (Molecular Bio-Products, San Diego) were used for pipetting all solutions containing RNA.

Splenocytes were flushed from the dissected mouse spleen using a hypodermic needle attached to a syringe containing phosphate buffered saline (PBS). The spleen cells were washed with 50ml PBS and then spun down in a 50ml Falcon tube for 5 minutes at 2000rpm. The supernatant was removed carefully and replaced with 8ml of TRI reagent (Sigma) before vortexing for 3-4 minutes to resuspend the cell pellet. The tube

was left to stand for 10 minutes at RT to lyse the spleen cells. 1.6ml of water saturated chloroform:isoamylalcohol (24:1) was added and the tube shaken for 15 seconds, then left to stand for 15 minutes at RT. The tube was spun for 5 minutes at 5000rpm and the upper aqueous layer, containing the RNA was transferred to eppendorf tubes in 900µl aliquots, taking care to avoid the DNA-containing interface. 600µl of isopropanol was added to each tube and the tubes were mixed and then left to stand for 1.5 hours to allow the RNA to precipitate. The eppendorfs were spun at 12,000g for 15 minutes in a micro-centrifuge and the supernatant discarded carefully before resuspending the RNA pellet in 1ml 70% ethanol. These tubes were again spun at 12,000g for 15 minutes before removing the supernatant carefully and allowing the RNA pellet to dry for 5 minutes at RT. The pellet was resuspended in 200µl nuclease-free water and the amount of RNA was quantified using a UV spectrophotometer, as described in Section 2.2.1.3

2.2.2.5 RT-PCR Amplification of VH and VL DNA Fragments

The VH and VL forward and reverse primer sets used for the amplification of mouse VH and VL regions are described in Appendix 1. Two separate RT-PCR reactions were performed in RNase-free eppendorf tubes to amplify VH and VL fragments:

100ng RNA	5µl
Nuclease-free water	71µl
10x PCR buffer (Boehringer Mannheim)	10µl
10mM dNTP's (Gibco)	2µl
40 units RNase inhibitor (Promega)	1µl
0.1M Dithiothreitol (Gibco)	0.5µl
10µg BSA	5µl
VH or VL reverse primer mix	2µl

The tube was heated to 65°C for 15 minutes, then placed onto ice before addition of the following components:

5 units Taq polymerase (Boehringer Mannheim)	1µl
200 units SuperScript II Reverse Transcriptase (Gibco)	1µl
VH or VL forward primer mix	2µl

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

48°C	1 hour	1 cycle	[Reverse transcriptase reaction]
92°C	3 minutes	1 cycle	[Denaturation]
92°C	1 minute		[Denaturation]
63°C	30 seconds		[Annealing]
58°C	50 seconds	7 cycles	[Annealing]
72°C	1 minute		[Elongation]
92°C	1 minute		[Denaturation]
63°C	30 seconds	30 cycles	[Annealing]
72°C	1 minute		[Elongation]

2.2.2.6 Splicing by Overlap Extension (SOE)-PCR to Join VH and VL DNA

The following components were added to an eppendorf tube:-

10ng VH DNA	2μl
10ng VL DNA	2μl
10x PCR buffer (Boehringer Mannheim)	5μl
10mM dNTP's (Gibco)	1μl
5 units Taq polymerase (Boehringer Mannheim)	1μl
ddH$_2$O	37μl

The tubes were overlaid with mineral oil and incubated in a Perkin Elmer thermal cycler as follows:

92°C	3 minutes	1 cycle	[Denaturation]
92°C	1 minute		[Denaturation]
63°C	30 seconds		[Annealing]
58°C	50 seconds	2 cycles	[Annealing]
72°C	1 minute		[Elongation]

The primers required to amplify the full length scFv fragments (see Appendix 1) were then added:

ScFor	1μl
ScBack	1μl

The remainder of the SOE-PCR reaction was then carried out:

92°C	1 minute		[Denaturation]
63°C	30 seconds	23 cycles	[Annealing]
72°C	1 minute		[Elongation]

2.2.2.7 Ligation and Transformation Reactions

The scFv insert and pAK100 phage display vector (see Appendix 2) were both digested with SfiI for 2-3 hours at 50°C and then purified from agarose using silica fines. Ligation reactions were performed as described in Section 2.2.1.3. The reaction volume was then made up to 50µl with ddH$_2$O and 500µl n-butanol was added to precipitate the DNA. The tube was vortexed for 5 seconds and then spun at 15000rpm for 10minutes. The supernatant was removed and the DNA pellet resuspended in 15µl water.

The precipitated ligation reaction was added to 40µl electrocompetent XL1-Blue *E.coli* in an electroporation cuvette (Flowgen). A Bio-Rad Gene Pulser™ was used to apply 10000 volts/cm to the cuvette for approximately 4.5 milliseconds and the bacteria were then transferred to 200µl 2xYT medium and incubated for 1 hour at 37°C. Aliquots of this were then plated onto NE agar and incubated overnight at 37°C. Transformants were collected into 2xYT medium and stored at -80°C in 40% glycerol.

2.2.2.8 RFLP Analysis of scFv Sequences

scFv sequences were amplified by PCR from individual colonies of bacteria harbouring the scFv-containing phagemid pAK100. Colony PCR's were performed in 20µl volumes, made up as follows:

10x PCR buffer (Boehringer Mannheim)	2µl
10mM dNTP's (Gibco)	0.5µl
ScFor	0.5µl
ScBack	0.5µl
Taq polymerase (Boehringer Mannheim)	0.2µl
ddH$_2$O	16.3µl

A nichrome wire was sterilised by passing through a flame, then touched on a bacterial colony and used to transfer bacteria to the PCR reaction tube. The PCR reaction was overlaid with mineral oil and subjected to the following cycles in a Perkin Elmer thermal cycler.

94°C	1 minute			[Denaturation]
52°C	1 minute	} 30 cycles		[Annealing]
68°C	2 minute			[Elongation]
68°C	7 minutes	1 cycle		[Elongation]

HaeIII digestion was carried out on 10μl of the completed PCR reaction in a total volume of 20μl (as described in Section 2.2.1.3). Digested samples were analysed by agarose gel electrophoresis or PAGE.

2.2.2.9 Rescue and Titration of Phage-scFv's

The phagemid-scFv library which had been transformed into *E.coli* strain XL1-Blue was retrieved from storage at -80°C and grown at 37°C in 50ml NE medium to an optical density at 550nm (OD_{550}) of 0.5 (determined by spectrophotometry). 10^{11} VCSM13 helper bacteriophage (Stratagene) and 25μl 1M IPTG were added and the culture incubated at 37°C without agitation for 15 minutes. A further 100ml LE medium was added and the culture shaken for 10 hours at 30°C. 2 hours after infection, 30μg/ml kanamycin was added. Following incubation, bacteria were pelleted by centrifugation at 5000rpm for 10 minutes and the supernatant removed to fresh tubes. 1/6 volume of 20% polyethylene glycol (PEG) 8000, 2.5M NaCl was added and tubes left to stand for 15 minutes at RT to allow phage particles to precipitate. Phage were pelleted by centrifugation at 5000rpm for 10 minutes and resuspended in 15ml APBS. 1/6 volume of 20% PEG 8000, 2.5M NaCl was added and the tubes left to stand for 15 minutes at RT, before spinning at 5000rpm for 10 minutes. Phage were resuspended in 1ml FACS buffer and spun at 15000rpm for 5 minutes to pellet any debris. The supernatant was transferred to a new tube and stored at 4°C.

A dilution series of recombinant phage particles was titred by incubating briefly with XL1-Blue to allow infection, then plating the XL1-Blue on NE agar and counting the numbers of chloramphenicol-resistant colony forming units (CFU's) which arose from phagemid infection. A 5ml culture of XL1-Blue was grown overnight at 37°C in 2xYT containing 10μg/ml tetracycline. A tenfold dilution series of the phage (down to a dilution of 10^{-11}) was performed in 10μl volumes of water. Each dilution was added to

a 40µl aliquot of the XL1-Blue overnight culture and incubated at 37°C without agitation for 15 minutes. XL1-Blue cells were then plated onto NE agar and incubated overnight at 37°C.

2.2.2.10 Amplification and Titration of Wild-Type (Helper) M13 Phage

5ml 2xYT containing 10µg/ml tetracycline was inoculated with XL1-Blue and incubated overnight at 37°C. 500µl of the overnight culture was added to 17ml 2xYT (plus 10µg/ml tetracycline) and grown to an OD_{550} of 1. 10^{11} VCSM13 helper phage were added and the flask left to stand at 37°C for 30 minutes. The flask was then shaken overnight at 30°C, with 30µg/ml kanamycin added 2 hours after the addition of helper phage. Following overnight culture, the XL1-Blue cells were pelleted by spinning at 5000rpm for 10 minutes and the supernatant transferred to a fresh tube and heated to 65°C for 15 minutes. The supernatant was then distributed in 1ml aliquots to sterile eppendorf tubes and the phage precipitated with 1/6 volume 20% PEG 8000, 2.5M NaCl (as in Section 2.2.2.9). Purified helper phage were then resuspended in FACS buffer and stored at 4°C.

A dilution series of helper phage particles was titred by incubating briefly with XL1-Blue to allow infection, then mixing the XL1-Blue with molten 2xYT soft-agar and plating on 2xYT agar and counting the numbers of plaque forming units (PFU's) which arose from phage infection. A 5ml culture of XL1-Blue was grown overnight at 37°C in 2xYT containing 10µg/ml tetracycline. A tenfold dilution series of the phage (down to a dilution of 10^{-11}) was performed in 10µl volumes of water. Each dilution was added to a 40µl aliquot of the XL1-Blue overnight culture and incubated at 37°C without agitation for 15 minutes. XL1-Blue cells were then added to 3ml molten 2xYT soft-agar (pre-heated to 55°C) and immediately plated on 2xYT agar and incubated overnight at 30°C.

2.2.3 Phage Display Panning on *Xenopus* cells

2.2.3.1 Culture of *Xenopus* Tumour Cell Line B₃B₇

The *Xenopus* tumour cell line B_3B_7 was grown in 50ml tissue culture flasks (Falcon) in 10ml tumour medium in 5% CO_2 at 27°C. The lids of the flasks were only loosely tightened to permit gaseous diffusion. Every 2-3 days the cells were subcultured by removing 1ml to a new flask containing 8ml fresh tumour medium. Tumour medium

was prepared by diluting 400ml serum-free medium to amphibian strength with distilled water. The medium was supplemented with 8% supernatant from the *Xenopus* A6 kidney cell line (Rafferty, 1969), 2% decomplemented foetal calf serum (FCS) (First Link) and 200μg/ml kanamycin (Gibco). Serum-free medium contains 500ml Iscove's medium (Gibco), 5ml 100x non-essential amino acids (Gibco), 50μg/ml penicillin/streptomycin (Gibco), 5μg/ml insulin, 50mM mercaptoethanol and 1.5ml 10% Primatone (Roche).

2.2.3.2 Preparation of *Xenopus* Spleen Lymphocytes and Red Blood Cells

Outbred *Xenopus laevis* were used for these studies and were bred and reared under standard conditions (Horton and Manning, 1972). Prior to dissection, animals were heavily anaesthetised in 3-amino-benzoic acid ethyl ester (MS222). Spleens were dissected out with forceps and micro-scissors and placed in 3.5cm petri dishes (Costar) in 3ml FACS buffer. The spleens were teased apart to release splenocytes into the FACS buffer which was then transferred to a 15ml Falcon centrifuge tube (Becton Dickinson). After pipetting to break up clumps of cells, debris was allowed to settle and the supernatant carefully pipetted on top of 3ml 1.077 density Ficoll (Amersham-Pharmacia) in a fresh 15ml tube. This tube was centrifuged at 4°C for 5 minutes at 250g and lymphocytes removed from a tight band above the Ficoll layer to a new 15ml tube. The red blood cells (RBC's) which had pelleted below the Ficoll layer were also transferred to a new 15ml tube. Both cell populations were resuspended in 5ml FACS buffer and centrifuged at 4°C for 10 minutes at 300g to pellet the cells. After removal of the supernatant, the cells were resuspended in 1ml FACS buffer and a 10μl aliquot pipetted into a haemocytometer for counting cell numbers. Cells were either used directly or transferred to 24 well plates (Becton Dickinson) in 1ml tumour medium and incubated at 27°C in 5% CO_2 for up to 3 days.

2.2.3.3 Panning the Phage-ScFv Library by Subtractive Isolation

1×10^{10} of the 'anti-B_3B_7' phage-scFv library and 1×10^{10} wild type phage (control) in 1ml FACS buffer were each added to eppendorf tubes containing 1×10^6 RBC's and incubated for 2 hours at 4°C with rotation. The cells were spun down at 3,400 rpm for 2 minutes and the supernatants were transferred to tubes containing 1×10^6 B_3B_7 cells and incubated for 2 hours at 4°C with rotation. After incubation, 100μl was removed for flow cytometric analysis (also called fluorescence-activated cell sorting (FACS) analysis; see Section 2.2.3.6). The remaining 900μl was spun down at 3,400 rpm for 1

min and the supernatant removed and replaced with 1ml FACS buffer. This wash step was repeated a further 6 times to remove unbound phage.

Bound phage were eluted by adding 100µl 0.1M glycine/HCl pH2.2 to the pelleted cells and incubating at RT for 10 minutes. Cell debris was then spun down at 6,900 rpm for 1 min and the supernatant neutralised with 100µl 10mM Tris HCl pH8.0. 2µl was removed at this point to test for elution titres (Section 2.2.3.4). The remainder of the eluted phage was added to 500µl of overnight cultured XL1-Blue and incubated at 37°C for 30 minutes without shaking. The XL1-Blue were then added to 50ml of NE medium and grown at 37°C to an OD^{550} of 0.5 before preparation of phage as described in Section 2.2.2.9. The same protocol was employed in all subsequent rounds of panning.

2.2.3.4 Testing Enrichment by Elution titres

The 1:100 dilution of eluted recombinant phage and eluted wild type phage (control) were further diluted in 3 tenfold dilutions down to 10^{-6} in 10µl volumes. The phage were titred by infection of XL1-Blue and counting subsequent CFU's for recombinant phage (as described in Section 2.2.2.9) and by counting PFU's for wild type phage (as in Section 2.2.2.10).

2.2.3.5 Testing Enrichment by RFLP Analysis

Individual scFv sequences were amplified by PCR from colonies of phagemid-transformed bacteria after each round of panning (as in Section 2.2.2.8). HaeIII digestion and analysis by agarose gel electrophoresis or PAGE were also performed as described in Section 2.2.2.8.

2.2.3.6 Testing Enrichment by Flow Cytometric (FACS) Analysis

For flow cytometric analysis, the 100µl aliquots of B_3B_7 cells removed from the recombinant library phage and wild type phage panning steps (Section 2.2.3.3) were each washed three times in 1ml of fresh FACS buffer, with spins performed at 300g for 10 minutes at 4°C. The 2 aliquots of cells were resuspended in 400µl of FACS buffer and each transferred to 2 wells of a 96-well microtitre plate (Becton Dickinson). The following is an overview of the antibody incubations performed in separate wells prior to FACS analysis:-

B₃B₇ cells plus library phage	rabbit anti-M13 antibody	FITC conjugated goat anti-rabbit Ig antibody
B₃B₇ cells plus library phage	no rabbit anti-M13 antibody	FITC conjugated goat anti-rabbit Ig antibody
B₃B₇ cells plus wild type phage	rabbit anti-M13 antibody	FITC conjugated goat anti-rabbit Ig antibody
B₃B₇ cells plus wild type phage	no rabbit anti-M13 antibody	FITC conjugated goat anti-rabbit Ig antibody

Both the detecting antibodies were adsorbed with a 1:20 dilution of filter-sterilised *Xenopus* serum prior to incubation with B_3B_7 cells. The rabbit anti-M13 antibody was used at a working dilution of 1:1000 and applied to the cells in a 50μl volume before incubating on ice for 30 minutes. The cells were then washed three times in 200μl FACS buffer, with spins performed at 300g for 10 minutes at 4°C. The FITC conjugated goat anti-rabbit Ig antibody was diluted to 1:160 and applied to the cells in a 50μl volume before incubating on ice in the dark for 30 minutes. The cells were then washed three times in 200μl FACS buffer, with spins performed at 300g for 10 minutes at 4°C, before resuspending in 500μl FACS buffer for flow cytometric analysis.

Flow cytometry was performed using a Coulter Epics® XL-MCL flow cytometer in conjunction with the Epics® XL-MCL and EXPO software for data analysis. FITC fluorescence on cells was detected by the fluorescence 1 (FL1) sensor, which detects light with a wavelength of between 505 and 545nm. Background levels of fluorescence were set at 2% using the control sample in which the anti-M13 antibody was omitted.

2.2.4 Manipulations of Individual Phage Antibodies

2.2.4.1 Isolation and Rescue of Individual Phage Antibodies

For growing up individual phage antibodies, a colony of XL1-Blue *E.coli* bearing the phagemid-scFv of interest was used to inoculate 50ml NE medium and the culture was grown at 37°C until the OD_{550} reached 0.5. The recombinant phage-scFv's were then grown up, rescued, purified and titred as in Section 2.2.2.9.

2.2.4.2 Single and Dual Stain FACS Analysis

The protocol for labelling cells with phage for FACS analysis was the same for B_3B_7 cells, splenocytes and red blood cells. In each case, 5×10^5 cells were incubated with 1×10^{10} phage-scFv's for 1 hour on ice. The method for labelling with antibodies and for FACS analysis was then exactly as described in Section 2.2.3.6.

For dual stain analysis, the cells were first labelled with phage as described above, washed three times in FACS buffer and transferred to a 96 well plate. The rabbit anti-M13 antibody, adsorbed on 1:20 *Xenopus* serum and then diluted 1:1000, was applied to the cells in a 50μl volume as before except that included in this 50μl volume was the co-staining mouse mAb 8E4, at a dilution of 1:30, or 2B1 at a dilution of 1:15 (For details of anti-*Xenopus* mAb's see Appendix 3). The cells were washed three times in FACS buffer and the goat anti-rabbit Ig FITC, adsorbed on *Xenopus* serum and diluted to 1:160, was then added and the cells incubated on ice in the dark for 30 minutes. The cells were washed three times in FACS buffer containing 1:100 filter-sterilised normal rabbit serum before detection of the co-staining antibody with phycoerythrin (PE)-conjugated rabbit anti-mouse Ig antibody (DAKO), adsorbed on *Xenopus* serum and diluted 1:20. Three further washes in FACS buffer were carried out before resuspending the cells in 500μl FACS buffer and analysing on the flow cytometer.

The FITC fluorescence was detected by the FL1 sensor and the PE fluorescence was detected by the FL2 sensor which detects light at 560 to 590nm. The control for FITC fluorescence was the same as described above (Section 2.2.3.6). The control for PE fluorescence was to run a sample in which the co-staining mouse mAb had been omitted, but the PE-conjugated rabbit anti-mouse Ig included.

2.2.4.3 Western Blotting of Phage Particles to Detect scFv Fusion Proteins

10^{10} phage particles in 20μl APBS were added to 4μl protein sample loading buffer and boiled for 3 minutes to denature the protein. Samples were subjected to SDS-PAGE and transferred to nitrocellulose as described in Section 2.2.1.4. All the following manipulations of the nitrocellulose filters were carried out at RT on a rocker platform. After staining with Ponceau S to confirm protein transfer (as in Section 2.2.1.4), the protein molecular weight size markers were marked in pencil and the Ponceau stain washed off by incubating the nitrocellulose in 50ml TBS for 10 minutes. The filter was then transferred to 50ml blocking solution (5% non-fat dried Marvel milk, 0.02% Tween 20 in TBS) for a further 10 minutes. This solution was then poured

off and replaced with a further 50ml blocking solution and left to incubate for 2 hours. Following blocking, the filters were incubated for 2 hours in 10mls blocking solution supplemented with 1µg/ml anti-FLAG mAb (Kodak), which detects a short peptide incorporated into the scFv protein. The filters were then washed three times in 20ml blocking buffer before transferring to 10mls blocking solution containing a 1:20000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. After two hours incubation, filters were washed three times in TBS and then transferred to a 10ml solution containing 100mM Tris HCl (pH8.5), 1.25mM luminol, 0.2mM p-coumaric acid and 0.1% hydrogen peroxide for 1 minute in a dark room. Filters were blotted to remove excess moisture then placed on 3MM paper, covered with cling film and exposed to blue-sensitive X-ray film (Fujifilm) inside a film cassette for 5 minutes. Films were developed using a Compact X4 X-ray film processor (X-Ograph).

2.2.4.4 Use of Individual Phage Antibodies to Probe Western Blots of B_3B_7 Cell and Spleen Lymphocyte Lysates

1×10^7 B_3B_7 cells and 1×10^7 spleen lymphocytes were washed twice in APBS before being resuspended in 100µl lysis buffer (1%NP-40, 150mM NaCl, 50mM Tris HCl (pH8.0), 1mM $MgCl_2$, 1mM phenylmethane sulphonyl fluoride (PMSF), 1µg/ml leupeptin and 1µg/ml pepstatin A). The cells were lysed on ice for 30 minutes then spun at 15000rpm for 3 minutes to remove insoluble material. 20µl aliquots of the supernatant were added to 4µl protein sample loading buffer and the samples subjected to SDS-PAGE and transferred to nitrocellulose (as in Section 2.2.1.4). Nitrocellulose filters were probed using the same conditions as those described in Section 2.2.4.3 with the following exceptions. The primary antibody was either the phage antibody or wild type M13 phage at a concentration of 10^{11} phage per ml of blocking buffer. Phage were detected using a mouse anti-M13 antibody (1:1000), followed by an HRP-conjugated goat anti-mouse IgG antibody (1:20000).

2.2.5 Manipulation of Soluble Antibody Fragments

2.2.5.1 Production of Soluble ScFv's From *E.coli* Strain JM83

DNA encoding the single chain variable fragment (scFv) was isolated from the phagemid vector pAK100 following restriction digestion with the enzyme SfiI and gel purification of the DNA (Section 2.2.1.3). The scFv was cloned into the expression vector pAK400 (see Appendix 2), which had also been digested with SfiI, using the

ligation protocol described in Section 2.2.1.3. Ligation reactions were transformed into the non-suppressor *E.coli* strain JM83. Transformed colonies were grown at 30°C in 50ml expression medium to an OD_{600} of 1.5. The cells were pelleted by centrifugation at 6000 rpm for 20 minutes at 4°C and the supernatant removed. The cells were resuspended in 1ml 30mM Tris HCl (pH8.0), 20% sucrose, transferred to an eppendorf tube and shaken on ice for 10 minutes. Centrifugation at 15000rpm for 10 minutes was used to pellet the cells again and the supernatant was discarded. The cells were resuspended in 600µl 5mM $MgSO_4$ and shaken on ice for 10 minutes. The tube was spun at 15000rpm for 10 minutes and the supernatant (the osmotic shock fluid containing scFv proteins) was stored at 4°C.

2.2.5.2 Purification of Soluble ScFv's using the Ni-NTA Spin Kit (Qiagen)

The purification of scFv's was permitted by the incorporation of a sequence encoding a polyhistidine tag sequence at the 3' end of the scFv DNA (see Appendix 2). The purification was performed using Ni-NTA spin columns (Qiagen) using the method enclosed with the kit. Briefly, spin columns were equilibrated by adding 600µl wash buffer (50mM NaH_2PO_4 (pH8.0), 300mM NaCl, 50mM imidazole), spinning at 2000rpm for 2 minutes at 4°C and discarding the excess liquid. 600µl osmotic shock fluid (described in Section 2.2.5.1) was added, the column was spun again, using the same spin conditions, and the flow-through was saved for analysis. The column was washed twice with wash buffer and the flow-through was saved each time for analysis. Finally, the scFv's were eluted by adding 200µl elution buffer (50mM NaH_2PO_4 (pH8.0), 300mM NaCl, 250mM imidazole), spinning again and transferring the eluate to a fresh tube. Purified scFv protein was verified by SDS-PAGE analysis and then stored at -20°C.

2.2.5.3 FACS Analysis of Soluble ScFv's

For analysis of soluble scFv labelling of *Xenopus* cells, $3x10^5$ cells (either B_3B_7 or spleen lymphocytes) were incubated with 1µg/ml soluble scFv in 50µl FACS buffer on ice for 30 minutes. The cells were washed three times in 200µl FACS buffer before adding 50µl FITC conjugated rabbit anti-mouse Ig antibody (DAKO), adsorbed on *Xenopus* serum and diluted 1:20, and incubating on ice in the dark for 30 minutes. Cells were washed three times in 200µl FACS buffer before resuspending in 500µl FACS and analysing on the flow cytometer.

2.3 Results

2.3.1 Construction of the α-B$_3$B$_7$ Phage Display Library

2.3.1.1 Amplification of the VH and VL Fragments by RT-PCR

Following immunisation with B$_3$B$_7$ cells, RNA was isolated from the spleens of the mice as described in Section 2.2.2.4 and two separate RT-PCR reactions were set up for the amplification of VH and VL DNA (Section 2.2.2.5). A tenth of each completed RT-PCR reaction was analysed by agarose gel electrophoresis (see Figure 2.2). The presence of DNA bands at the predicted sizes of 386-440bp for VH and 375-402bp for VL (Krebber *et al.*, 1997) indicated that RT-PCR reactions were successful. VH and VL bands were excised from agarose gels and the DNA purified using silica fines, as described in Section 2.2.1.3.

2.3.1.2 Joining of the VH and VL Regions by PCR

The primers designed by Krebber *et al* incorporate short complementary sequences into the VH and VL fragments which allow them to be joined using splicing by overlap extension (SOE)-PCR. Using VH and VL DNA purified from agarose, an SOE-PCR reaction was set up in a 100µl volume (As described in Section 2.2.2.6). After completion of the reaction, 10µl was removed for electrophoretic analysis (Figure 2.3). A band of around 800bp was seen which indicated that pairs of VH and VL DNA fragments had been joined together to form scFv-encoding DNA fragments. The band corresponding to the scFv DNA was excised from the gel and purified using silica fines.

2.3.1.3 Cloning of the scFv DNA into the Phagemid Vector pAK100

The phagemid vector pAK100, which was used for the cloning of scFv sequences, contains a chloramphenicol resistance gene that enables selection of bacteria which harbour the phagemid (Krebber *et al.*, 1997). The pAK100 vector also has two different restriction sites which are both recognised by the restriction endonuclease SfiI. These two SfiI restriction sites are also incorporated into the SOE-PCR primers, which permits directional cloning of scFv's into pAK100 (Krebber *et al.*, 1997).

Separate SfiI restriction digests were performed on the pAK100 vector and scFv inserts and the digested DNA was gel purified using silica fines. A ligation reaction was set

up with a molar ratio of vector to insert of 3:1. Control reactions were also carried out with vector alone to account for vector religation. Ligation mixtures were precipitated to remove buffer salts and electroporated into *E.coli* strain XL1-blue (see Section 2.2.2.7). Around 1000 chloramphenicol-resistant transformants resulted from a ligation reaction containing 100ng of vector and 25ng of insert. Less than 5 transformants per 100ng of vectors were due to vector religation.

By performing a series of ligation reactions, a library of approximately 1×10^5 scFv's was transformed into *E.coli* and stored in glycerol at -80°C.

2.3.1.4 Diversity of the scFv Library

In order to verify the presence of scFv sequences in the transformant *E.coli*, ten individual colonies were used in separate 20μl 'colony-PCR' reactions to re-amplify their scFv sequences (Section 2.2.2.8). 10μl of each reaction was examined by electrophoresis (Figure 2.4a). In addition, the diversity of the scFv sequences was assessed by digesting the other 10μl of PCR product with the frequent-cutting restriction endonuclease HaeIII. The digested DNA was separated on a 2% agarose gel to allow analysis of restriction fragment length polymorphisms (RFLP's) (see Figure 2.4b). All ten randomly picked colonies contained scFv inserts and all ten gave different RFLP profiles suggesting that the library contained a diverse repertoire of scFv sequences.

2.3.2 Panning the α-B_3B_7 Library

Prior to panning, the phagemid-scFv library in *E.coli* was expressed on the surface of recombinant phage by infecting the *E.coli* with helper phage in a 50ml overnight culture. Following purification, the recombinant phage library had a titre of 5×10^{12} CFU/ml.

To remove non-specific phage-scFv's which might otherwise be enriched during panning, a method known as subtractive isolation was used (Van Ewjik *et al.*, 1997). This involved pre-incubation of the recombinant phage with a population of adsorber cells, in this case *Xenopus* erythrocytes, before each round of panning on B_3B_7 cells. Following incubation with phage, the B_3B_7 cells were washed extensively to remove non-specific phage before eluting the specifically bound phage in a solution of low pH. Eluted phage were used to re-infect *E.coli* strain XL1-blue and consequently cultured and converted to phage-scFv's for further rounds of panning.

Three different strategies were used to test the effectiveness of panning in enriching the phage library for binders to B_3B_7 cells. Firstly, the phage eluted from B_3B_7 cells after each round of panning was titred to check for any increase due to selection of binding phage. Secondly, RFLP analysis was used on scFv's from various stages of panning to observe whether certain scFv sequences were being selected over others. Thirdly, flow cytometry was used during each round of panning to analyse the B_3B_7 cells for phage bound to the cell surface.

2.3.2.1 Testing for Enrichment by Elution Titres

After each round of panning, an aliquot of the eluted phage was serially diluted and each dilution was titred. As a control for phage sticking non-specifically to cells, wild type M13 phage were panned in parallel to recombinant phage-scFv's and their elution titres were checked for comparison. The results (Table 2.1) show that over 3 rounds of panning there is an increase in the number of recombinant phage eluted from the cells whereas the elution titre of the wild type phage did not increase during panning. This suggest that the increase observed with the recombinant phage is due to an enrichment of the initial phage library for phage scFv's with specificity for B_3B_7 cells.

2.3.2.2 Testing for Enrichment using RFLP Analysis of ScFv Sequences

RFLP analysis was performed on twelve randomly isolated scFv sequences after each round of panning (see Figure 2.5). After one round of panning, minimal selection of scFv's is apparent as all 12 RFLP patterns are different. However, after a second round of panning, one particular RFLP pattern was recurrent in 8 of the 12 samples (lanes 5 to 12) and after a further round of panning this pattern was present in all 12 lanes. This suggested that one scFv was being strongly selected by panning for its ability to bind B_3B_7 cells.

2.3.2.3 Testing for Enrichment by Flow Cytometry

During each round of panning, an aliquot of B_3B_7 cells which had been incubated with recombinant phage was analysed by flow cytometry for the presence of phage bound to the cell surface. The cells were incubated with a rabbit anti-M13 antibody, followed by a FITC-conjugated anti-rabbit Ig antibody. As a control for background fluorescence, an aliquot of B_3B_7 cells was also incubated with wild type phage before addition of the same anti-M13 and anti-rabbit FITC antibodies. The results (Figure

2.6) show that recombinant phage which had been panned three times on B_3B_7 cells were able to bind approximately 90% of the B_3B_7 cells whereas wild type phage showed only background levels of fluorescence.

2.3.3 Analysis of Individual Phage Antibodies

2.3.3.1 Flow Cytometric Testing of 5 Individual Phage Antibodies for their Ability to Bind B_3B_7 Cells

Following the success of panning, it was decided that individual panned phage were to be tested separately for their ability to bind B_3B_7 cells. Using the RFLP profiles obtained after 2 rounds of panning (Figure 2.5b), five phage expressing different scFv sequences were prepared for flow cytometric analysis. The five phage antibodies corresponding to the scFv sequences in lanes 1-5 of Figure 2.5b were designated XLB-1 to XLB-5 respectively (for *Xenopus laevis* B_3B_7). Flow cytometric analysis (Figure 2.7) showed that phage antibodies XLB-1 to XLB-4 were poor at binding B_3B_7 cells whereas XLB-5, which was strongly selected during panning, bound around 90% of B_3B_7 cells.

2.3.3.2 Verification of ScFv Expression on Phage XLB-5 by Western Blotting

In order to confirm that phage XLB-5 was expressing surface scFv, 10^{10} phage particles were subjected to SDS-PAGE and then blotted to nitrocellulose. As a control, the same number of wild type phage particles were included in the Western blot. ScFv's were detected by virtue of the fact that they express a four amino-acid 'FLAG' sequence which is bound by an anti-FLAG mouse mAb. This mAb was detected by an HRP-conjugated goat anti-mouse IgG antibody, followed by chemiluminescent detection of positive bands. The result shown in Figure 2.8 confirms that recombinant phage XLB-5 does indeed express the ScFv fusion to cpIII which is detected at 48 kDa by the anti-FLAG mAb. Also detectable are copies of the soluble scFv at 28kDa which are not fused to cpIII.

2.3.3.3 FACS Analysis of Phage XLB-5 on Different *Xenopus* Cell Types

The specificity of phage XLB-5 was tested by labelling *Xenopus* spleen lymphocytes and *Xenopus* red blood cells and analysing by flow cytometry. The histograms (Figure 2.9) show that XLB-5 phage bound a distinct sub-population of both red blood cells (60% labelled with XLB-5) and spleen lymphocytes (53% XLB-5 positive).

2.3.3.4 Dual Stain Analysis of Phage XLB-5 on *Xenopus* Splenocytes

Dual stain analysis of spleen lymphocytes was conducted using XLB-5 phage in conjunction with the mouse mAb's 2B1 and 8E4. 2B1 is an anti-*Xenopus* CD5 antibody which binds T-cells and 8E4 is an anti-*Xenopus* IgM antibody which binds B-cells. Figure 2.10 shows the dual-stain histograms which were performed on spleen lymphocytes from two separate animals. XLB-5 phage appears to bind a large and distinct subset, equivalent to 80%, of CD5-positive *Xenopus* T-cells and a distinct sub-population, around 67%, of IgM-positive *Xenopus* B-cells.

2.3.3.5 Probing Western Blots of B_3B_7 and Spleen Lymphocyte Lysates with Phage XLB-5

In order to identify the antigen bound by phage antibody XLB-5, Western blots of B_3B_7 and spleen cell lysates were probed with phage antibody XLB-5, and wild type phage as a control. Detection was achieved using a mouse anti-M13 antibody, followed by an HRP-conjugated goat anti-mouse IgG antibody and chemiluminescent identification of protein bands. The result (Figure 2.11) shows that no specific protein band was identified by XLB-5, whereas the wild type phage displays some non-specific background staining of *Xenopus* proteins.

2.3.4 Production and Analysis of Soluble XLB-5 ScFv

2.3.4.1 Purification of Soluble XLB-5 (sXLB-5) ScFv Fragments

sXLB-5 was purified from *E.coli* JM83 cells using osmotic shock followed by His-tag purification using the Ni-NTA kit (Qiagen) as described in Sections 2.2.5.1 and 2.2.5.2. The purity of sXLB-5 was confirmed by SDS-PAGE (Figure 2.12) which shows that the scFv had been successfully isolated from a complex mixture of *E.coli* proteins.

2.3.4.2 FACS Analysis of sXLB-5 on B_3B_7 Cells and Splenic Lymphocytes

sXLB-5 was tested for its ability to bind B_3B_7 cells and splenic lymphocytes by flow cytometry (Section 2.2.5.3). Figure 2.13 shows that sXLB-5 is unable to bind B_3B_7 cells but is able to bind a small population (9%) of splenic lymphocytes.

2.4 Discussion

2.4.1 The Efficiency of Phage Display in Generating Antibodies to B₃B₇ Cells

The objective of the work detailed in this Chapter was to perform a 'trial run' of generating phage display antibodies to *Xenopus* cells, assessing whether it was possible to efficiently select phage antibodies which bound cellular epitopes displayed on B_3B_7 *Xenopus* tumour cells. Using a method of subtractive isolation to remove general anti-*Xenopus* phage antibodies, 3 rounds of panning were performed on B_3B_7 cells. Evidence from elution titres, RFLP and FACS analysis strongly suggested that the original phage library, consisting of around 10^5 clones, became enriched for phage antibodies capable of binding to B_3B_7 cells. When five phage antibodies were isolated from the panned library and tested individually for their ability to bind B_3B_7 cells, one of them, XLB-5, showed strong binding. The trial run was thus successful in raising a phage antibody which was able to bind to a surface epitope on the target cell. Furthermore, the scFv fusion to phage cpIII was detected in Western blots of phage XLB-5, suggesting that phage were binding through surface scFv molecules.

Despite the success of panning in raising a single phage antibody XLB-5, it is often desirable to produce several antibodies to the target cell. The strong selection of XLB-5 during 3 rounds of panning appears to have prevented selection of other, possibly lower affinity, phage antibodies to B_3B_7. The selection of higher affinity clones such as XLB-5 may have been favoured by the stringency of the wash conditions as every round of panning on B_3B_7 cells included 7 x 1ml wash steps to remove non-specifically bound phage prior to elution. Watters *et al* have reported that reducing the number of wash steps to 4 increases the number of specifically bound phage eluted during each round of panning. However, they also show in the same paper that using only 4 wash steps also increases the number of non-specifically bound phage which are eluted (Watters *et al.*, 1997). Another study (De Bruin *et al.*, 1999) reports that non-specifically eluted phage-scFv's can become enriched during amplification of the phage library between panning rounds if the encoded scFv offers growth advantages to the *E.coli* host. This could occur if, for example, a phagemid encodes an scFv which is not full-length. It was therefore decided that the more stringent wash conditions should continue to be used for future panning experiments. In addition the higher affinity phage antibodies selected by more stringent panning are likely to be of greater use when screening by FACS analysis for antibodies which identify small sub-populations of cells. It should be noted, however, that phage antibodies which produce

relatively poor results in FACS analysis can display more convincing binding in other assays, such as immunocytochemistry (Palmer et al., 1997).

One way to circumvent the problem of selecting only a small number of high affinity phage antibodies would be to screen a large number of phage antibodies, ie 50 to 100, after only 2 or 3 rounds of panning for their ability to bind the target cell. In addition to flow cytometric analysis, other assays, such as immunocytochemistry, could be used to increase the chance of detecting useful, lower affinity, phage antibodies. However, as the panning on B_3B_7 cells was only intended as a trial run of phage display panning on *Xenopus* cells and due to the time-consuming nature of screening many phage antibodies, these modifications were not tested.

2.4.2 Further Characterisation of Phage Antibody XLB-5

Further flow cytometric testing of XLB-5 on *Xenopus* splenic lymphocytes and red blood cells suggested that XLB-5 bound a specific sub-population of both these cell types. These FACS traces are indicative of specific binding as they feature distinct negative and positive peaks of fluorescence. In addition, XLB-5 could be used in dual stain analysis, in conjunction with mouse mAb's 2B1 and 8E4, which identify *Xenopus* T cells and B cells respectively. The dual-stain traces further confirm that XLB-5 is capable of highlighting a sub-population of *Xenopus* cells in the same way as hybridoma-derived mAb's to cell-surface structures.

In order to probe the molecular nature of the antigen bound by XLB-5, Western blots were performed on both B_3B_7 cells and spleen lymphocyte lysates, using XLB-5 as the primary detecting antibody. Although phage antibodies have been used successfully in such assays (Nissim *et al.*, 1994), XLB-5 was unable to detect any specific protein bands. There is a strong possibility that this was due to the fact that XLB-5 was raised against cell surface proteins in their native conformation. When the cell lysates were denatured, subjected to SDS-PAGE and blotted to nitrocellulose, the proteins were no longer in their native conformation and thus may have been unrecognisable to XLB-5. This problem may have been countered by immunoprecipitation of the XLB-5 antigen from native lysates of B_3B_7 protein, but the time consuming nature of such an experiment prevented it from being carried out.

The production and purification of soluble XLB-5 scFv was easily achieved after sub-cloning of the scFv DNA into the high expression plasmid vector pAK400 (See Appendix 2). Surprisingly, the soluble, purified XLB-5 scFv was incapable of binding B_3B_7 cells and bound only ~10% of *Xenopus* spleen lymphocytes instead of the ~70%

45

bound by XLB-5 phage. The only possible explanation for this change in binding specificity is that the soluble scFv molecule adopted a different conformation to the scFv/cpIII fusion protein expressed on the phage surface.

The single and dual-stain FACS traces of XLB-5 phage on *Xenopus* spleen lymphocytes suggest an intriguing pattern of binding to subsets of both T-cells and B-cells. In both cases, XLB-5 appears to label 60 to 80% of cells. Unfortunately, XLB-5 also appears to bind to approximately 60% of red blood cells which suggests that it is not binding to a cell surface marker of major immunological interest. However, the ability of XLB-5 to differentiate red blood cells, which are generally regarded as a fairly homogenous population, into 2 subsets is interesting and may be linked to the fact that amphibian red blood cells are nucleated and not perhaps as homogenous as mammalian RBC's.

Figure 2.1 An Overview of the Strategy for Production of Recombinant M13 Phage Expressing Surface ScFv's

These diagrams illustrate the stages involved in the production of recombinant phage from an *E.coli* host containing a single scFv-encoding phagemid. The production of a library of recombinant phage would require a mixed population of *E.coli* host cells, each harbouring a different scFv-encoding phagemid. The diagrams are not drawn to scale.
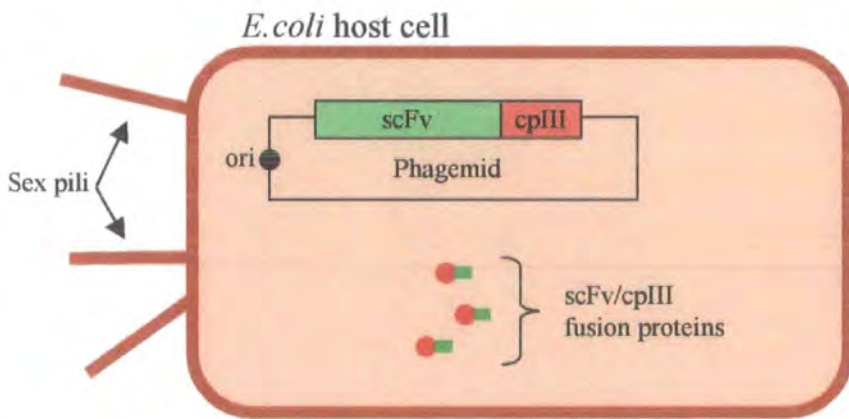
A. The phagemid, containing the scFv DNA fused to the cpIII gene and also the phage origin of replication (ori), exists as a plasmid inside a male *E.coli* host with sex pili. The phagemid-containing host is only capable of producing the scFv/cpIII fusion proteins at this stage.
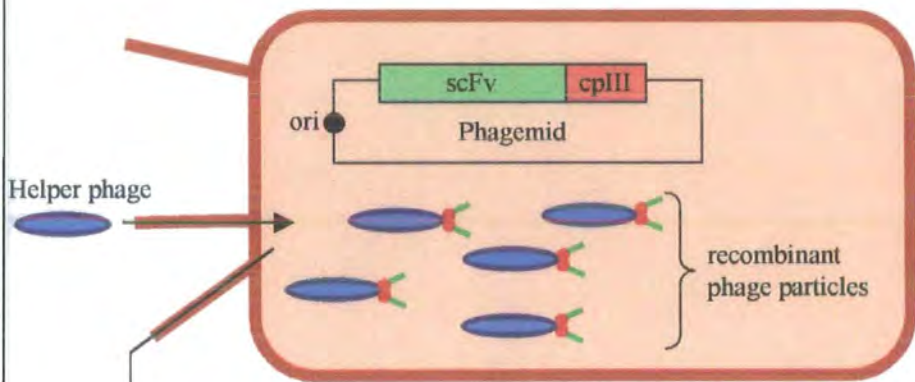
B. Upon infection of the host cell via the sex pili, the helper phage replicate and incorporate scFv/cpIII fusion proteins encoded by the phagemid. The resulting recombinant phage particles also incorporate the phagemid DNA due to the presence of the ori sequence. Recombinant phage are then extruded through the sex pili. N.B. It should be noted that wild type phage (lacking surface scFv) are extruded along with the recombinant phage.
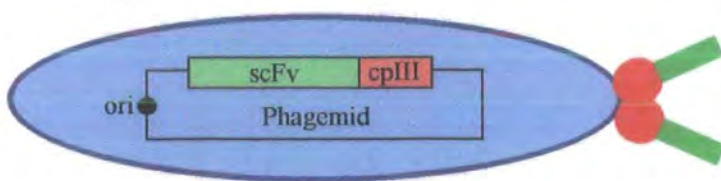
C. The recombinant phage particle contains the phagemid DNA encoding the scFv/cpIII which it expresses on its surface. Upon re-infection of *E.coli*, the phagemid DNA reverts to the plasmid form, shown in part A.

Figure 2.2   VH and VL RT-PCR's

1% agarose gel showing amplification of variable heavy (VH) and variable light (VL) chain DNA by RT-PCR. Template RNA was obtained from the spleens of mice immunised with B_3B_7 cells. Lane 1 contains λPst1 DNA markers.
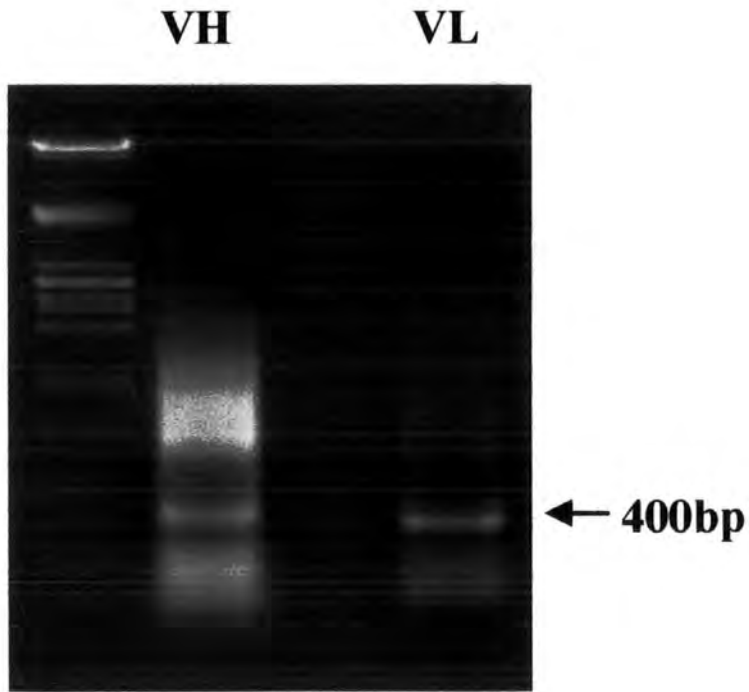
Figure 2.3   SOE-PCR's

1% agarose gel showing the ability of SOE-PCR to join VH and VL DNA
fragments together to form scFv-encoding DNA fragments. VH and VL DNA
are included for size comparison. Lane 1 contains λPstI DNA size markers.
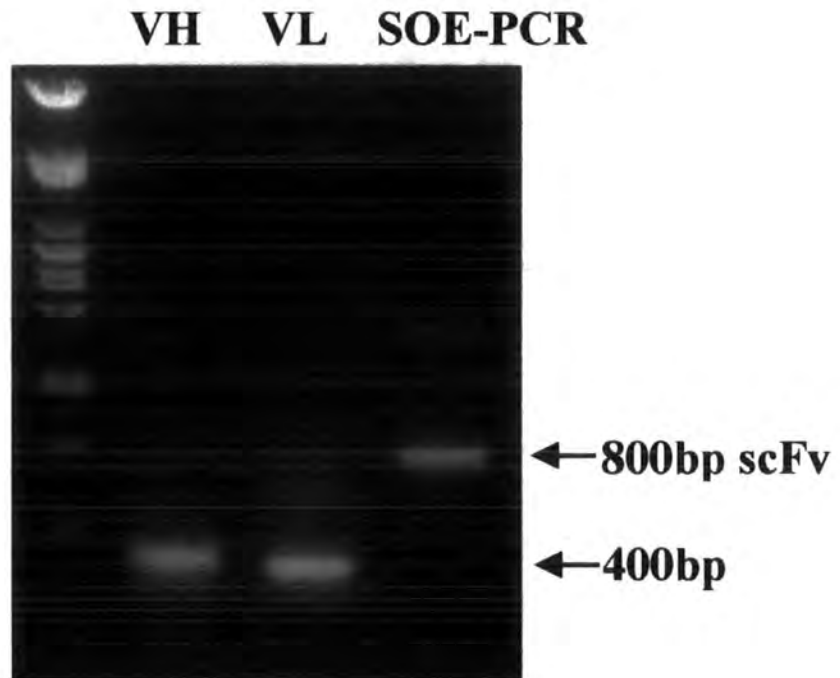
Figure 2.4   Diversity of ScFv Sequences in Library by RFLP Analysis

10 scFv sequences were reamplified from the α-B_3B_7 phage library by PCR and analysed by gel electrophoresis (A). Following HaeIII digestion, scFv fragments were run on a 2% agarose gel for analysis of RFLP's (B). Marker in lane 1 of both gels is λPstI DNA.

A.
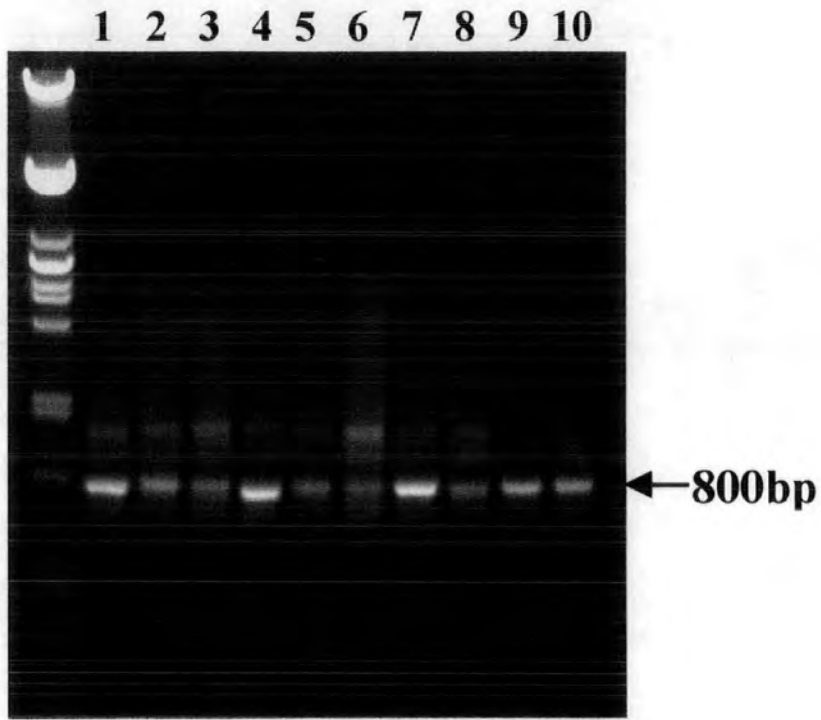
B.
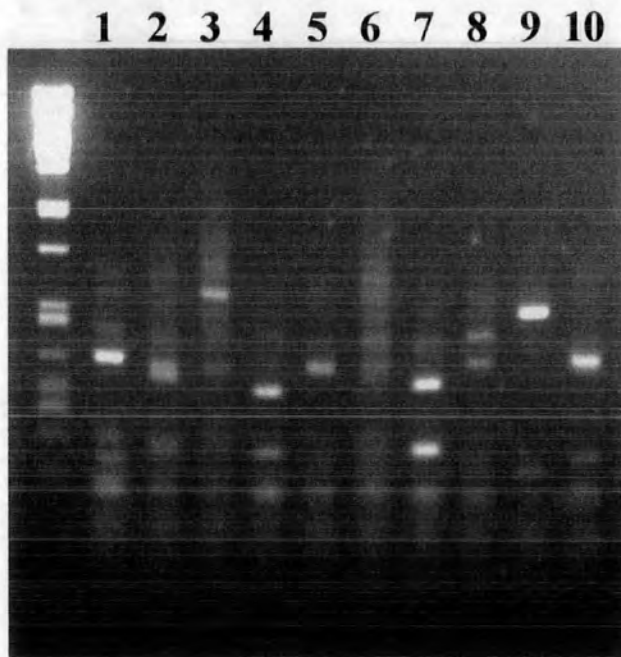
Table 2.1   Enrichment of Phage Binders during Panning on B_3B_7 as assessed by Elution Titres

The number of phage eluted from B_3B_7 cells was recorded after each round of panning. Wild type (helper) phage were panned and titred in parallel as a control. Phage numbers are expressed as the total number of plaque forming units (PFU's) or colony forming units (CFU's) eluted from the cells.

	Elution titre: wild type phage (PFU's)	Elution titre: recombinant phage (CFU's)
Round 1	~100	~200
Round 2	~300	~2000
Round 3	~200	~100,000

Figure 2.5   Enrichment of Phage Binders as determined by RFLP Analysis of ScFv Sequences

After each round of panning, 12 scFv sequences were amplified and digested with HaeIII before separation on 2% agarose gels. M indicates λPstI DNA.

A. Round 1

M 1 2 3 4 5 6 M   M 7 8 9 10 11 12 M

B. Round 2

M 1 2 3 4 5 6 M   M 7 8 9 10 11 12 M

C. Round 3

M 1 2 3 4 5 6 M   M 7 8 9 10 11 12 M
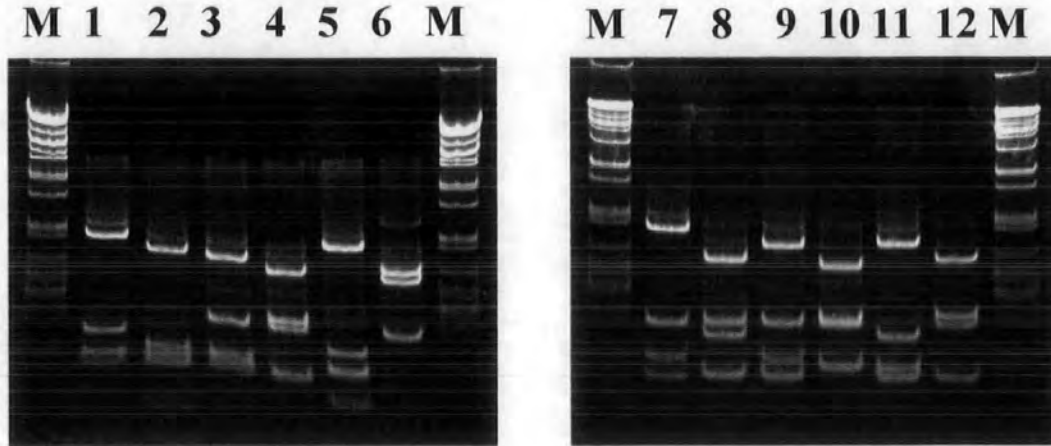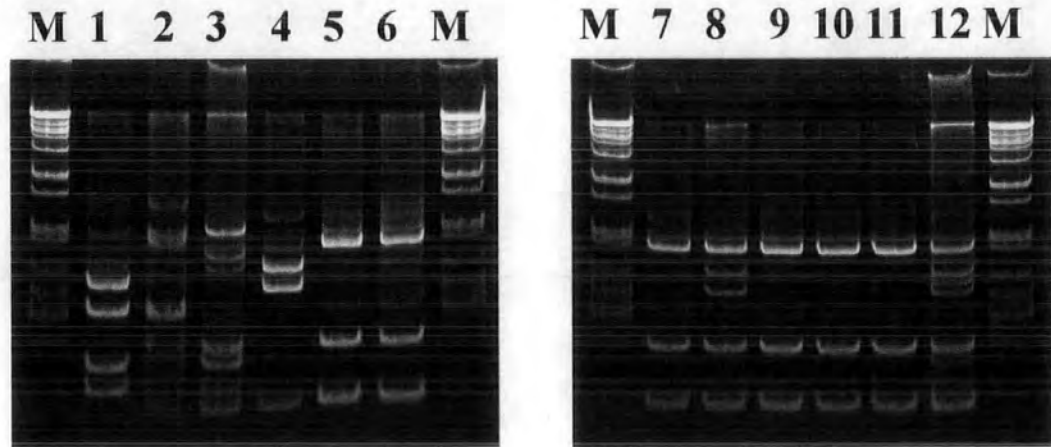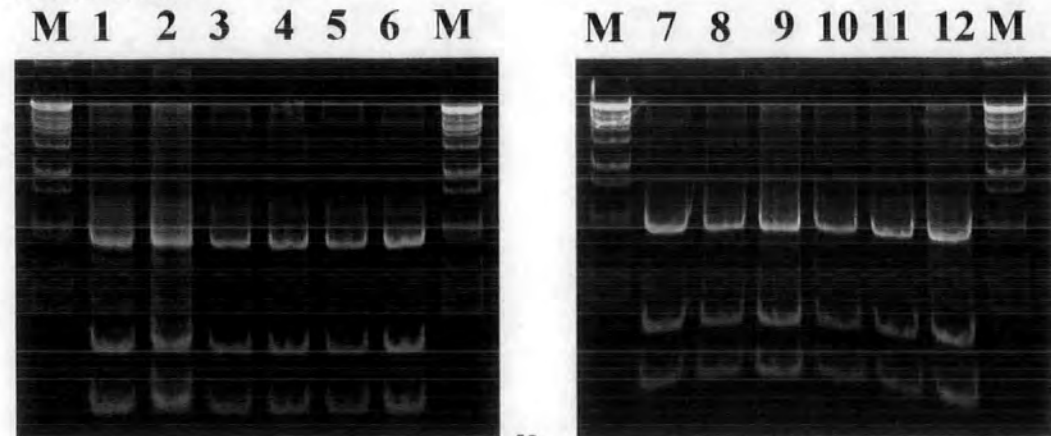
Figure 2.6   Flow Cytometric Analysis of Phage during Panning

During each round of panning, both wild type (WT) and recombinant phage were tested by flow cytometry for their ability to bind B_3B_7 cells. Phage were detected by a rabbit anti-M13 antibody followed by a FITC-conjugated anti-rabbit Ig antibody. The percentage of positively stained cells is indicated in the top-right corner of each histogram.
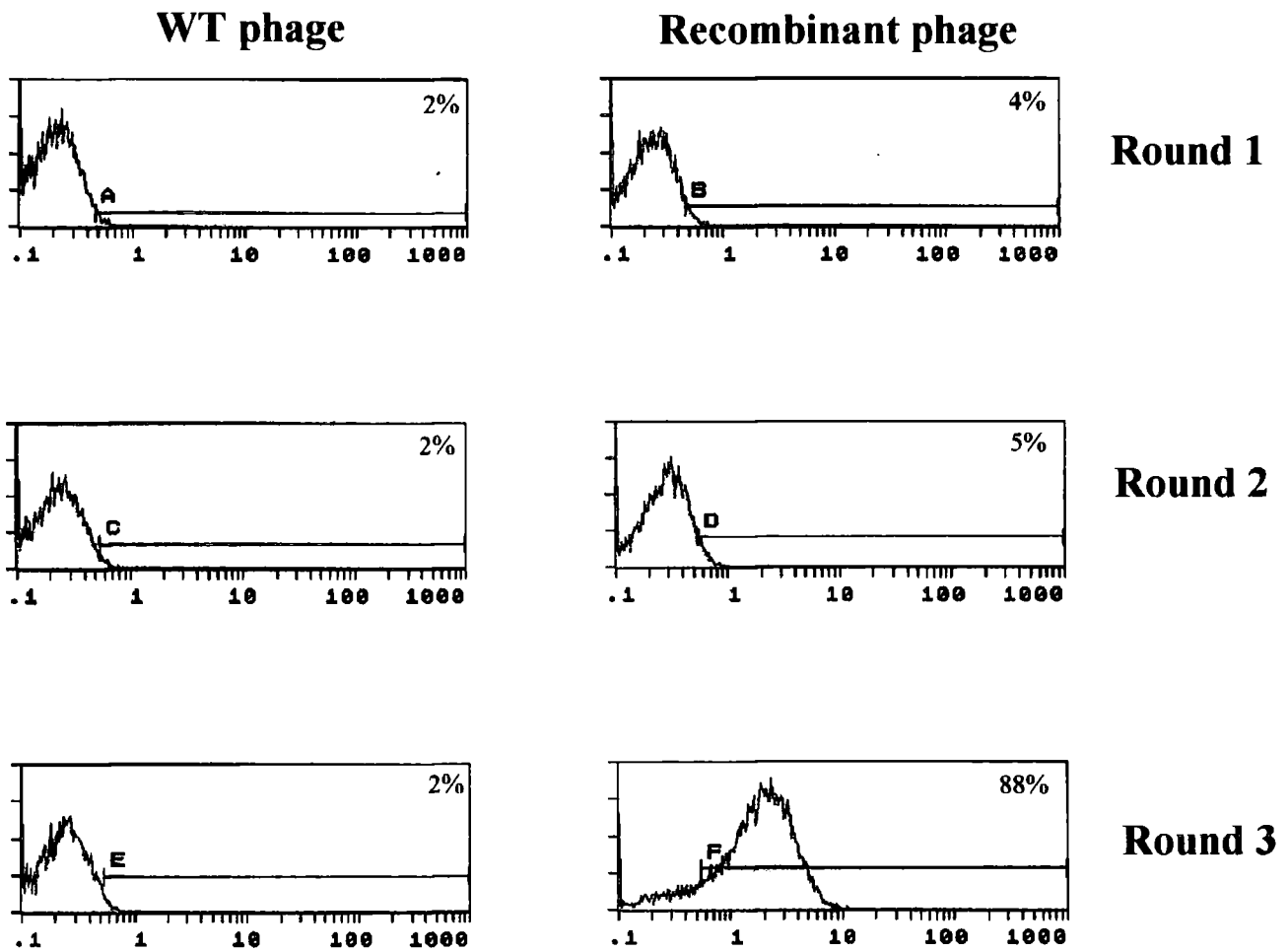
Figure 2.7   Flow cytometric Analysis of Five Individual Phage
Antibodies following Panning

Five different recombinant phage, isolated from the library which had been panned twice on B_3B_7 cells, were tested individually by flow cytometry for their ability to bind B_3B_7. Phage were detected by a rabbit anti-M13 antibody followed by a FITC-conjugated anti-rabbit antibody.
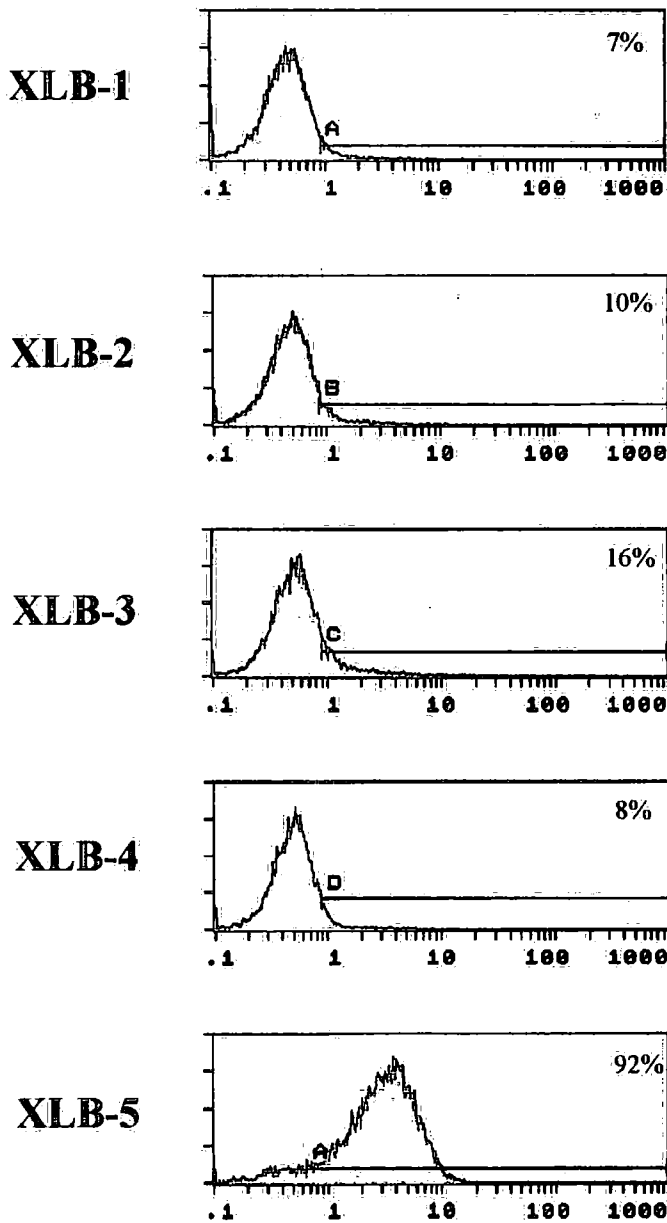
Figure 2.8   Confirmation of ScFv/cpIII Fusion Protein on Phage XLB-5 by Western Blotting

10^{10} wild type (WT) and 10^{10} XLB-5 phage particles were separated by SDS-PAGE and blotted to nitrocellulose. Blots were probed with a mouse mAb to a FLAG epitope expressed by the scFv, followed by an anti-mouse Ig antibody conjugated to horseradish peroxidase (HRP). Positive bands were detected by chemiluminescence.
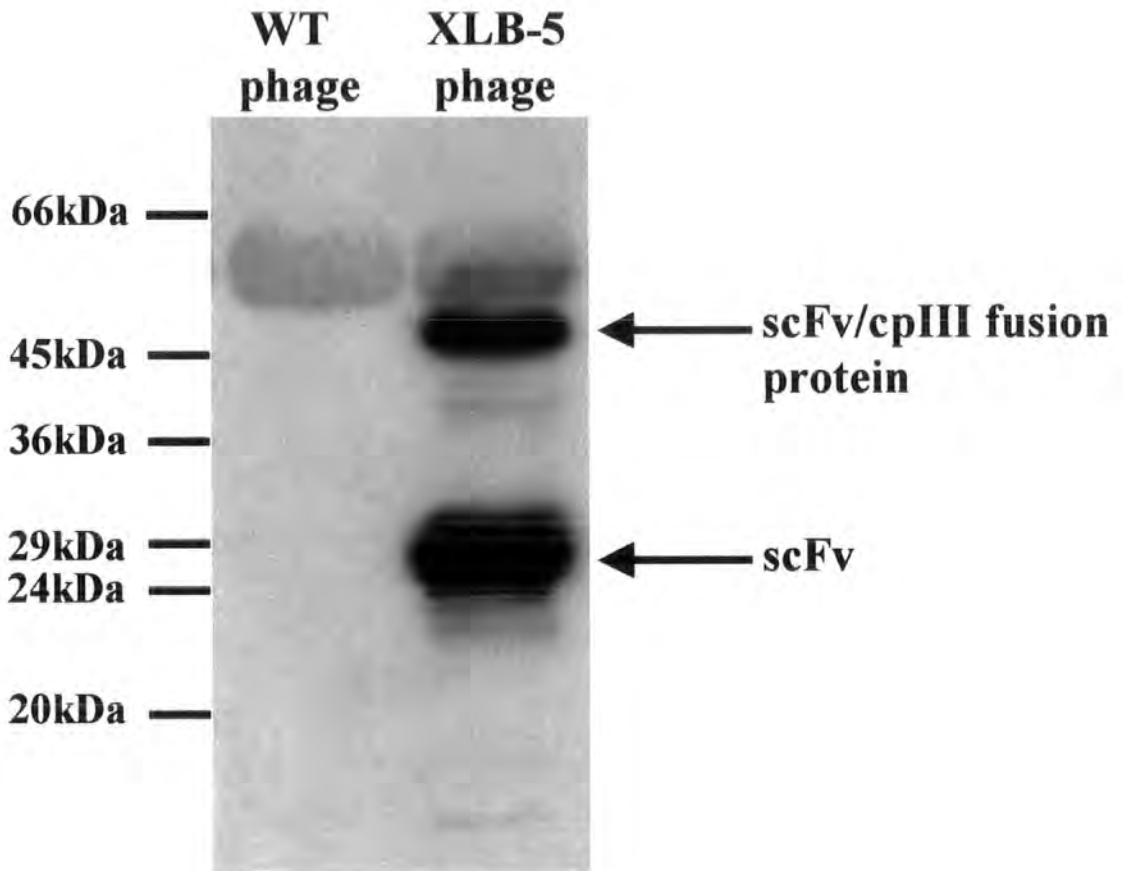
Figure 2.9   Flow Cytometric Testing of XLB-5 on *Xenopus* Red Blood Cells and Splenic Lymphocytes

The specificity of XLB-5 was tested using flow cytometry on different *Xenopus* cell types. The first histogram (A) shows XLB-5 staining of red blood cells (RBC's) and the second (B) shows XLB-5 staining of splenic lymphocytes.

A.

RBC's

XLB-5

60%

.1   1   10   100   1000

B.

Spleen lymphocytes

XLB-5

53%

.1   1   10   100   1000
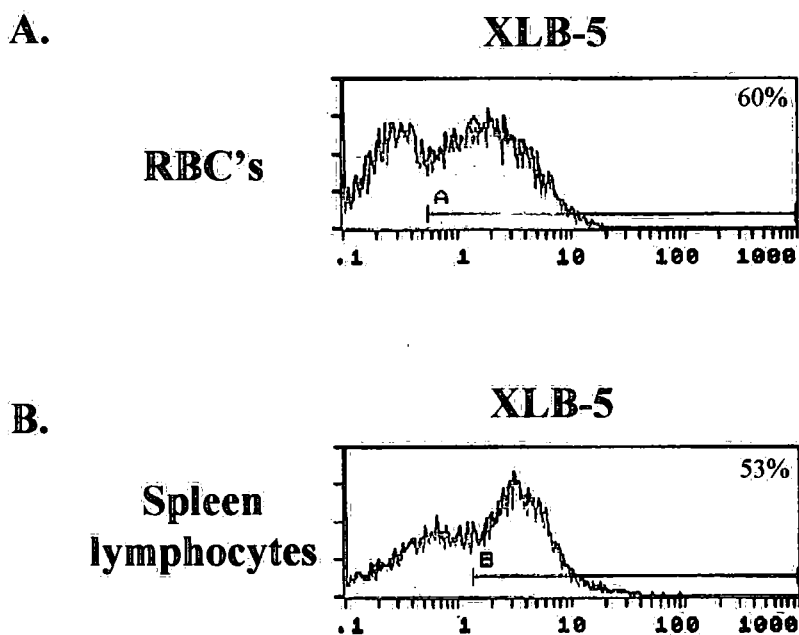
Figure 2.10   Dual Stain Testing of XLB-5 Phage on Splenocytes

Spleen cells were dual-labelled by using XLB-5 (detected by FITC) in conjunction with phycoerythrin (PE) labelling of 2 antibodies to different *Xenopus* lymphocyte populations. The co-staining antibodies used were 2B1, an anti-*Xenopus* CD5 antibody which binds T-cells, and 8E4, an anti-*Xenopus* IgM antibody which binds B-cells. The XLB-5 (FITC) fluorescence is measured on the X-axis of the histogram, while the PE fluorescence is shown on the Y-axis. The results shown on these histograms are gated for spleen lymphocytes.

Spleen lymphocytes

14%   57%

2B1 (α-CD5)

21%

XLB-5

Spleen lymphocytes

5%   10%

8E4 (α-IgM)

53%
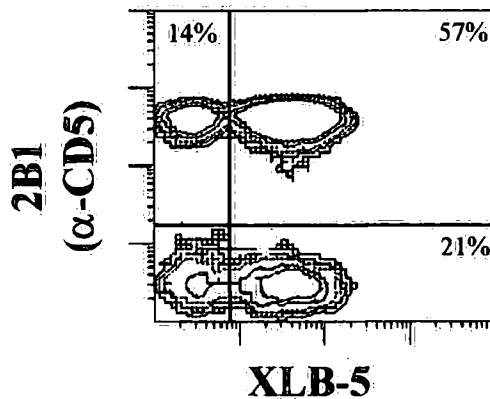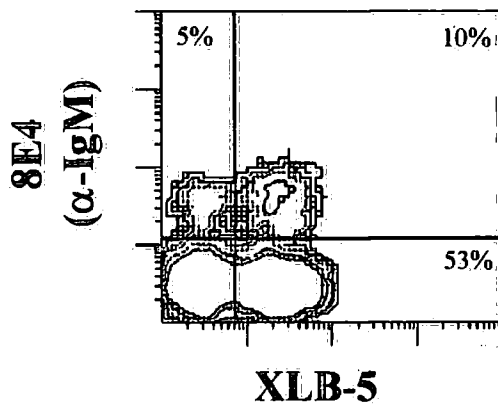
XLB-5

Figure 2.11   XLB-5 Phage as a Reagent in a Western Blot

Protein lysates from B_3B_7 cells and *Xenopus* spleen lymphocytes were separated by SDS-PAGE and transferred to nitrocellulose before being probed with XLB-5 phage (10^{11} per ml). Wild type (WT) phage (10^{11} per ml) were used in parallel as a control for non-specific binding. Bound phage were detected by a rabbit anti-M13 antibody, followed by an HRP-conjugated anti-rabbit antibody. HRP activity was detected using chemiluminescence.
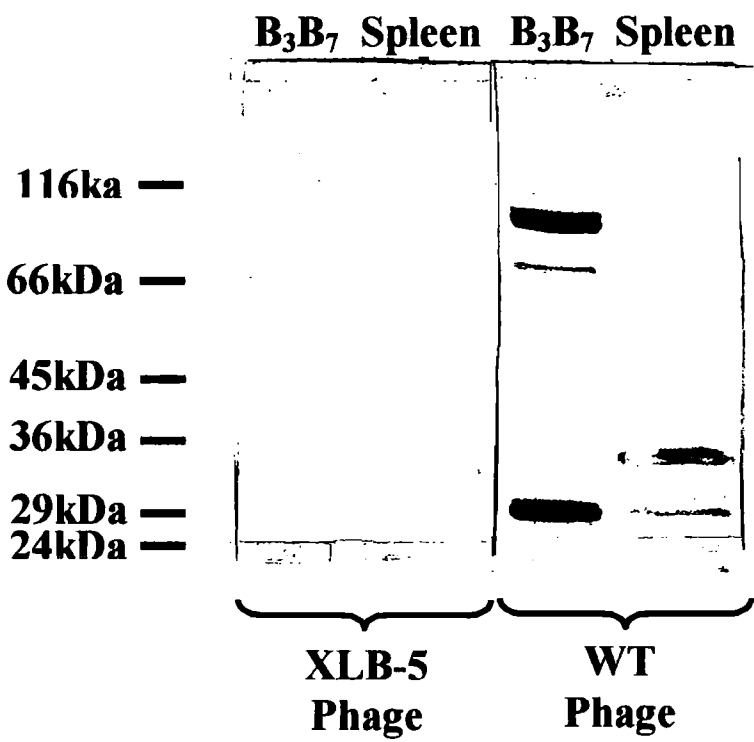
The WT phage control blot shown here is also displayed in Figure 3.14 as the experiments were performed at the same time.

Figure 2.12   His-tag Purification of Soluble XLB-5 (sXLB-5)

Soluble XLB-5 scFv fragments, containing poly-histidine tag sequences, were purified from the periplasm of JM83 *E.coli* following osmotic shock of the cells. Ni-NTA spin columns (Qiagen) were used to specifically bind the poly-histidine tag of the scFv fragments. Non-specifically bound proteins were washed away before elution of the scFv fragments. The following were analysed by SDS-PAGE:

M   Protein molecular weight markers
1   Osmotic shock fluid
2   Wash fluid
3   Eluent containing scFv fragments
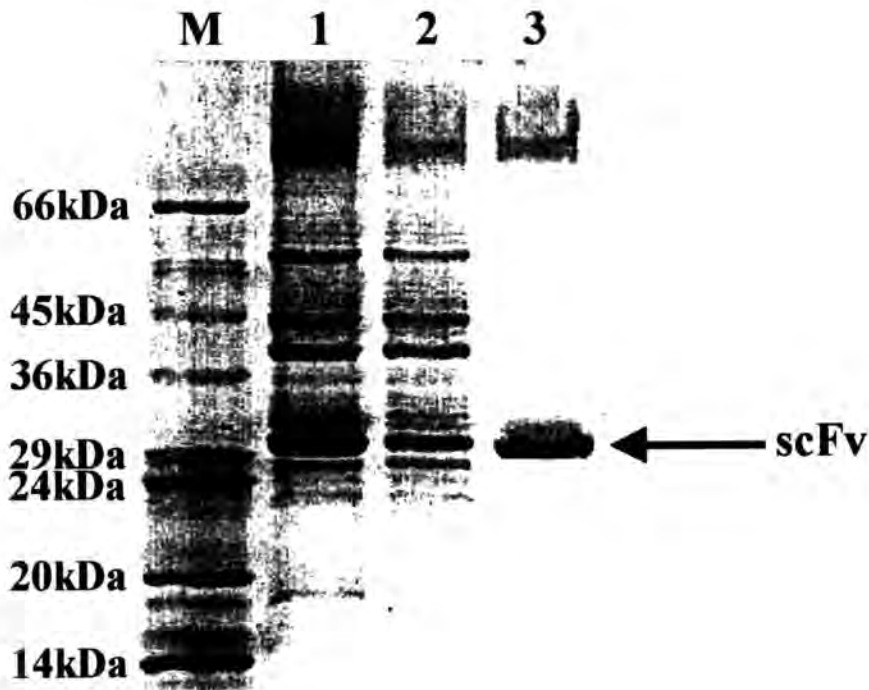
Figure 2.13 Flow cytometric testing of sXLB-5 on B₃B₇ cells and *Xenopus* spleen lymphocytes

Purified, soluble XLB-5 scFv's were tested by flow cytometry for their ability to bind B_3B_7 cells and spleen lymphocytes. ScFv's were detected by a FITC-conjugated anti-mouse Ig antibody.

sXLB-5

B_3B_7

sXLB-5

Spleen lymphocytes
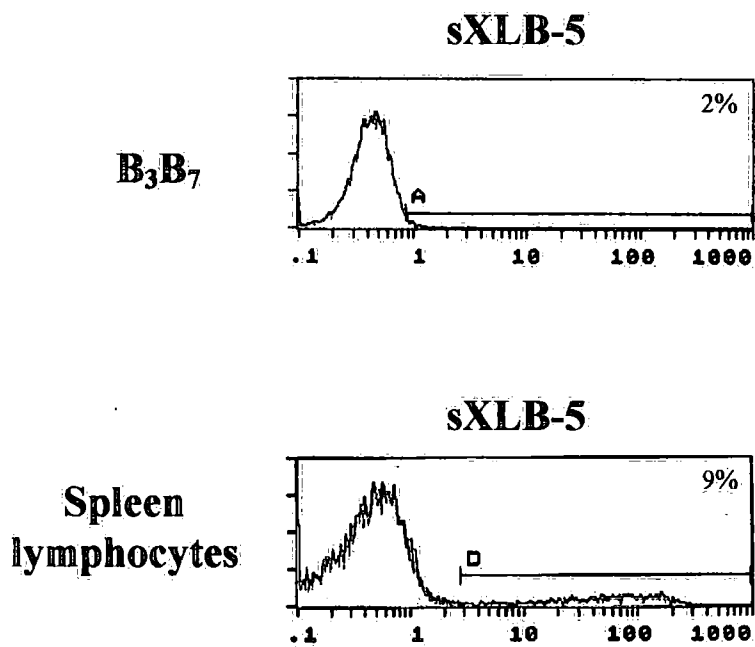
CHAPTER 3

Attempts to Generate Phage Display Antibodies to *Xenopus* NK-like Cells

Having shown in the previous Chapter that phage display technology is capable of raising specific phage antibodies to cellular epitopes on *Xenopus* B_3B_7 cells, a similar panning protocol was used to try and raise antibodies to *Xenopus* NK-like cells. ScFv libraries were cloned from mice immunised with NK-enriched lymphocyte populations from thymectomised (Tx) *Xenopus*. Following panning to select phage binding to NK-enriched splenocytes, phage antibodies of interest were isolated by flow cytometric screening on *Xenopus* splenocytes.

3.1 Introduction

3.1.1 NK-enriched Populations from Tx *Xenopus*

In order to raise cell specific antibodies by hybridoma or phage display technology, it is useful to have pure preparations of these cells for the immunisation and screening stages. In the case of *Xenopus* NK cells, a degree of purification from total spleen mixtures is possible by depletion of other cell types. Firstly the problem of T-cell contamination can be removed by using spleen cells from Tx animals, which have been shown to lack functional T-cells (Horton *et al.*, 1998b). In addition, immunomagnetic cell separation can be used to remove B cells and thrombocytes from the Tx spleen. The remaining cells, although not a pure *Xenopus* NK population, are highly enriched for NK cells. This has been verified by demonstrating effective spontaneous cytotoxicity of similar NK-enriched populations towards the MHC-deficient, allogeneic tumour target B_3B_7 (Horton *et al.*, 1998, Horton, T.L., pers. comm.).

3.1.2 Phage Display Panning on *Xenopus* NK-enriched Cells

The phage display panning protocol used in Chapter 2 was shown to be successful in selecting phage antibodies which bound whole, live cells. Flow cytometric testing of phage antibody XLB-5 on B_3B_7 cells revealed a convincing and consistent binding ability, which was comparable with that of hybridoma-derived mAb's. As discussed in the previous Chapter, the B_3B_7 panning protocol was thus successful in selecting phage

antibodies which bound with a high enough affinity to be useful in flow cytometric analysis. As a result, the protocol for panning on NK-enriched populations in this Chapter is based on the B_3B_7 panning strategy.

The ability of phage antibody XLB-5 to bind not only B_3B_7 cells but also red blood cells, T-cells and B-cells suggested that it was binding to a common epitope expressed on many *Xenopus* cell types. To favour the production of phage antibodies to more specific epitopes on *Xenopus* cells, it is necessary to improve the negative selection step of the panning process so that non-specific phage antibodies are more effectively removed from the library.

Another aspect of the trial run conducted on B_3B_7 cells which requires improvement is the screening stage. Due to the limited number of antibodies screened after panning, only one phage antibody was isolated which showed strong binding to the target cell. By screening a larger number of phage antibodies from the panned library, it is hoped that several useful phage antibodies will be isolated. In addition, it will be necessary to optimise the VH and VL RT-PCR reactions in order to increase the size and diversity of the starter library. A further improvement is the incorporation of a proof-reading DNA polymerase enzyme into the PCR reactions to ensure the faithful copying of mouse VH and VL sequences and thus increase the percentage of scFv's in the starter library which encode functional proteins.

3.2 Materials and Methods

3.2.1 General Materials and Methods

All the general materials and methods used in this Chapter are described in Section 2.2.1, with the following exception:

3.2.1.1 Thymectomy Operations on *Xenopus laevis* Animals

Thymectomy operations were carried out by microcautery on 5 to 7 day old tadpoles by Dr J.D. Horton (as in Horton and Manning, 1972). When adult thymectomised animals were taken for dissection, the thymic region was examined to verify that no regeneration of the thymus had occurred.

3.2.2 Phage Display Library Construction

The methods for phage display library construction were as described in Section 2.2.2, with the following exceptions:

3.2.2.1 Preparation of NK-enriched Cells from Tx *Xenopus* for Immunisation

Splenic lymphocytes were isolated from Tx animals, as described in Section 2.2.3.2., and incubated with B cell-specific mAb's, including the anti-IgM mAb 8E4 (see Appendix 3 for details) together with a cocktail of anti-light chain mAb's (Hsu et al., 1991) on ice for 30 minutes. Following 3 washes with FACS medium, 5×10^6 cells in 80µl Hank's balanced salt solution (HBSS)(Gibco), diluted to amphibian strength and supplemented with 1% foetal calf serum, were added to 20µl of MACS microbeads conjugated with goat anti-mouse IgG (Miltenyi Biotec). The cells and beads were incubated for 15 minutes at 4°C, and the mixture passed over a prewashed Mini-MACS separation column placed in a separation unit (magnet). The non-adherent splenocytes, depleted of B cells, eluted from the column were then incubated with the thrombocyte-specific mAb XLH-1 (See Appendix 3 for details). Following three washes with FACS medium, the thrombocytes were removed by magnetic sorting using goat anti-mouse IgM microbeads.

3.2.2.2 Immunisation of Mice with *Xenopus* 'NK-enriched' Cells

Two Balb/C mice were given three injections of 8×10^5 NK-enriched cells, the first in complete Freund's adjuvant and the second and third in incomplete Freund's adjuvant, into the foot pad over a 10 day period. The mice were then dissected and the spleens were removed for RNA isolation. At the same time, the mouse sera were tested to confirm reactivity to *Xenopus* splenocytes.

3.2.2.3 RT-PCR Amplification of VH and VL Fragments

The amplification of VH and VL fragments from RNA was performed in a two-step reaction, with the first step being a general cDNA amplification using an oligo dT primer (Gibco) to reverse transcribe all messenger RNA's containing a poly-A tail, instead of the protocol described in Chapter 2 which used the reverse VH and VL primers at this stage. The second step was the specific amplification of VH and VL regions by PCR, using the VH and VL-specific primers listed in Appendix 1.

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The cDNA amplification was performed in an RNase-free eppendorf tube as follows:

400ng RNA	20μl
Nuclease-free water (Promega)	65μl
10x Expand™ buffer (Boehringer Mannheim)	10μl
10mM dNTP's (Gibco)	2μl
40 units RNase inhibitor (Promega)	1μl
Oligo dT$^{12-18}$ primer (Gibco)	2μl

The tube was heated to 65°C for 15 minutes and then placed on ice before the addition of 1μl (200 units) SuperScript II reverse transcriptase (Gibco). The tube was then incubated at 48°C for 1 hour.

VH and VL fragments were amplified in separate eppendorf tubes as follows:

cDNA from first reaction	10μl
ddH2O	31.5μl
10x Expand™ buffer (Boehringer Mannheim)	5μl
10mM dNTP's (Gibco)	1μl
VH or VL forward primer mix	1μl
VH or VL reverse primer mix	1μl
Expand™ Polymerase Mixture (Boehringer)	0.5μl

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

92°C	3 minutes	1 cycle	[Denaturation]
92°C	1 minute	⎫	[Denaturation]
50°C	1 minute	⎬ 7 cycles	[Annealing]
72°C	1 minute	⎭	[Elongation]
92°C	1 minute	⎫	[Denaturation]
63°C	30 seconds	⎬ 30 cycles	[Annealing]
72°C	1 minute	⎭	[Elongation]

3.2.2.4 SOE-PCR

The following components were added to an eppendorf tube:-

10ng VH DNA	2µl
10ng VL DNA	2µl
10x Expand™ buffer (Boehringer Mannheim)	5µl
10mM dNTP's (Gibco)	1µl
Expand™ Polymerase Mixture (Boehringer)	1µl
ddH2O	37µl

The tubes were overlaid with mineral oil and incubated in a Perkin Elmer thermal cycler as follows:

92°C	3 minutes	1 cycle	[Denaturation]
92°C	1 minute		[Denaturation]
63°C	30 seconds	2 cycles	[Annealing]
58°C	50 seconds		[Annealing]
72°C	1 minute		[Elongation]

The primers required to amplify the full length scFv fragments (see Appendix 1) were then added:

ScFor	1µl
ScBack	1µl

The remainder of the SOE-PCR reaction was then carried out:

92°C	1 minute		[Denaturation]
63°C	30 seconds	23 cycles	[Annealing]
72°C	1 minute		[Elongation]

3.2.3 Phage Display Panning on Tx *Xenopus* Spleen Lymphocytes

Panning on Tx *Xenopus* splenic lymphocytes and FACS analysis during panning was carried out as described in Section 2.2.3, with the following exceptions:

3.2.3.1 Negative Panning on *Xenopus* Red Blood Cells

1×10^{10} of the phage-scFv library and 1×10^{10} wild type phage (control) in 1ml FACS buffer were each added to eppendorf tubes containing 1×10^6 red blood cells and incubated for 1 hour at 4°C with rotation. The cells were centrifuged at 3,400 rpm for 2 minutes and the supernatants were transferred to tubes containing another 1×10^6 red blood cells and incubated for a further hour at 4°C with rotation. Following this second incubation, the red blood cells were pelleted by centrifugation at 3,400 rpm for 2 minutes and the phage supernatant was removed and saved for positive panning.

3.2.3.2 Positive Panning on Tx Lymphocytes

The cells used for positive panning were Tx splenic lymphocytes, which had been passed over Ficoll to remove red blood cells (As in Section 2.2.3.2). Due to the cost of multiple magnetic cell separations, removal of B cells and thrombocytes was not carried out on these cells during each round of positive panning. The phage supernatant which had been negatively panned on red blood cells was transferred to an eppendorf tube containing 1×10^6 Tx lymphocytes and the tube incubated for 2 hours at 4°C with rotation.

3.2.4 Manipulations of Individual Phage Antibodies

3.2.4.1 RFLP Analysis of scFv Sequences

Following 3 rounds of panning, 48 individual scFv sequences were amplified by PCR and their RFLP profiles analysed as described in Section 2.2.2.8.

3.2.4.2 Isolation and Rescue of Individual Phage Antibodies

Following identification of 12 different scFv sequences by RFLP analysis, the 12 respective phagemid-containing colonies of *E.coli* strain XL1-Blue were grown up in 50ml NE medium. The cultures were infected with M13 helper phage and the recombinant phage-scFv's rescued and titred as in Section 2.2.2.9

3.2.4.3 Identification of cpIII/scFv Fusion Proteins by Western Blotting

Recombinant phage were subjected to SDS-PAGE, transferred to nitrocellulose and probed for the presence of cpIII/scFv fusion proteins as described in Section 2.2.4.3. Instead of using the anti-FLAG mAb (Kodak), filters were probed directly with the HRP-conjugated goat anti-mouse IgG antibody (1:20000) and bands detected by chemiluminescence.

3.2.4.4 Production of Soluble scFv's from *E.coli* Strain JM83

The cloning of scFv sequences into pAK400, transformation of *E.coli* strain JM83 and subsequent production of soluble scFv protein was carried out exactly as described in Section 2.2.5.1. Instead of purifying the scFv proteins using Ni-NTA spin columns (Qiagen), as described in Section 2.2.5.2, osmotic shock supernatants were used directly.

3.2.4.5 Preparation of Lymphocytes from *Xenopus* Thymus, Liver and Peripheral Blood

Prior to dissection, animals were heavily anaesthetised in 3-amino-benzoic acid ethyl ester (MS222). Thymuses and livers were exposed by removing the overlying skin and dissected out with forceps and micro-scissors and placed in 3.5cm petri dishes (Costar) in 3ml FACS buffer. The organs were teased apart to release cells into the FACS buffer which was then transferred to a 15ml Falcon centrifuge tube (Becton Dickinson). Peripheral blood was drawn off from the heart with a pipette and placed into a tube containing 3ml heparinised FACS buffer to prevent coagulation of red blood cells. Cell suspensions from thymus, liver and peripheral blood were then carefully pipetted on top of 3ml 1.077 density Ficoll (Amersham-Pharmacia) in a fresh 15ml tube. This tube was centrifuged at 4°C for 5 minutes at 250g and the lymphocytes removed from a tight band above the Ficoll layer to a new 15ml tube. The cells were resuspended in 5ml FACS buffer and centrifuged at 4°C for 10 minutes at 300g to pellet the cells. After removal of the supernatant, the cells were resuspended in 1ml FACS buffer and a 10μl aliquot pipetted into a haemocytometer for counting cell numbers. Cells were either used directly or transferred to 24 well plates (Becton Dickinson) in 1ml tumour medium and incubated at 27°C in 5% CO_2 for up to 3 days.

3.2.4.6 Culture of B₃B₇ Thymus Tumour and A6 Kidney Tumour Cell Lines

Both the B_3B_7 and A6 tumour cell lines were cultured in tumour medium in a 5% CO_2 atmosphere as summarised in Section 2.2.3.1.

3.2.4.7 Use of Soluble scFv's in Single and Dual Stain FACS Analysis

Single stain FACS analysis with soluble scFv's was carried out exactly as described in Section 2.2.5.3 with gates set on lymphoid cell populations except in the analysis of RBC's or tumour cells. Background levels of FITC fluorescence were set at 2% using a control sample in which soluble scFv was omitted.

For dual stain analysis, the cells were first labelled with scFv and rabbit anti-mouse Ig FITC antibody (DAKO) as described above. The cells were washed three times in FACS buffer containing 1:100 filter-sterilised normal mouse serum before incubating in the dark on ice for 20 minutes with 50µl PE-conjugated mouse mAb. The PE-conjugated mouse mAb's used and their working dilutions are listed in Appendix 3. As a control for PE fluorescence, a sample was incubated with PE-conjugated mouse immunoglobulins (DAKO) and used to set the background fluorescence marker to 2%. Three further washes in FACS buffer were carried out before resuspending the cells in 500µl FACS buffer and analysing on the flow cytometer.

In one dual stain experiment, using the anti-NK mAb 1F8 (described in Chapter 4), cells were first labelled with soluble scFv and rabbit anti-mouse Ig PE antibody (DAKO). The cells were washed three times in FACS buffer containing 1:100 filter-sterilised normal mouse serum before incubating in the dark on ice for 20 minutes with 50µl FITC-conjugated 1F8, diluted 1:200.

3.2.4.8 Use of Individual Phage Antibodies to Probe Western Blots of B₃B₇ Cells and Spleen Lymphocyte Lysates

Phage antibodies were used to probe lysates of B_3B_7 cells and spleen lymphocytes as described in Section 2.2.4.4.

3.3 Results

3.3.1 Construction of the anti-NK Phage Display Library

3.3.1.1 Amplification of VH and VL Regions by RT-PCR

A two stage RT-PCR reaction was performed on RNA isolated from the spleens of the mice immunised with NK-enriched *Xenopus* lymphocytes (as described in Section 3.2.2.3). Following the initial cDNA amplification, individual PCR reactions were performed using the Expand™ proof-reading DNA polymerase mixture (Boehringer Mannheim) to amplify VH and VL DNA. A tenth of each reaction was examined by agarose gel electrophoresis (Figure 3.1). The bright DNA bands visible at around 400bp in each lane indicated that the VH and VL fragments were more efficiently amplified using this two stage method than the single step method used in Chapter 2 (See Figure 2.2).

3.3.1.2 Joining of VH and VL Regions by SOE-PCR

The gel-purified VH and VL DNA fragments were assembled into scFv-encoding DNA fragments by SOE-PCR, again using the Expand™ proof-reading DNA polymerase mixture (Described in Section 3.2.2.4). A tenth of the reaction was analysed by agarose electrophoresis and a DNA band was visible at around 800bp, indicating that the VH and VL DNA had been assembled correctly (Figure 3.2). DNA bands corresponding to scFv sequences were gel-purified using silica fines (See Section 2.2.1.3).

3.3.1.3 Cloning of the scFv DNA into Phage Display Vector pAK100

ScFv sequences were cloned into the pAK100 phagemid vector and transformed into *E.coli* strain XL1-Blue as described in Section 2.2.2.7. A series of ligation and transformation reactions were performed which resulted in the cloning of approximately 5×10^5 scFv sequences into the pAK100 vector. Following transformation of the scFv-containing vectors into XL1-Blue, the library was stored in 40% glycerol at -80°C.

3.3.1.4 Testing the Diversity of the Library by RFLP Analysis

10 transformed colonies of XL1-Blue containing cloned scFv's were picked to test the diversity of the scFv sequences in the library. ScFv sequences were amplified directly from the bacterial colonies by PCR and digested with the restriction endonuclease HaeIII as described in Section 2.2.2.8. Agarose gel electrophoresis of half the PCR reaction volume confirmed that the ten colonies picked contained scFv DNA (Figure 3.3A). The HaeIII digested scFv DNA was also separated by electrophoresis on agarose (Figure 3.3B) and the profiles show that 9 of the 10 transformants examined contained different scFv sequences.

3.3.2 Panning of the anti-NK Phage Display Library

Following rescue of the recombinant phage-scFv library from *E.coli* using helper phage, two consecutive negative panning steps were carried out on *Xenopus* red blood cells before the positive panning on Tx splenocytes (Described in Section 3.2.3). The Tx spleen cells were washed extensively to remove non-specifically bound phage before elution of bound phage in a solution of low pH. Eluted phage were used to re-infect *E.coli* strain XL1-blue and consequently cultured and converted to phage-scFv's for further rounds of panning.

3.3.2.1 Testing for Enrichment by Flow Cytometry

To test whether panning was enriching the library for phage-scFv's with an ability to bind *Xenopus* spleen cells, flow cytometry was used during each round of panning to detect phage on the surface of cells (As described in Section 2.2.3.6). As a control for non-specific attachment of phage to cells, wild type M13 phage were subjected to the same panning conditions and also tested by flow cytometry. The results (Figure 3.4) show that recombinant phage-scFv's which had been panned three times on Tx spleen were able to bind 94% of the Tx spleen cells whereas wild type phage showed only background levels of fluorescence.

3.3.3 Analysis of Individual Phage Antibodies

3.3.3.1 RFLP Analysis of 48 scFv Sequences from Recombinant Phage after Three Rounds of Panning on Tx Spleen Cells

In order to assess the number of different phage antibodies contained within the panned library, 48 scFv sequences were amplified from randomly isolated phagemids by PCR (As described in Section 2.2.2.8). These scFv sequences were digested with the restriction endonuclease HaeIII and the resulting RFLP profiles examined following agarose gel electrophoresis (See Figure 3.5). Examination of the RFLP patterns allowed segregation of the scFv's into 12 groups which were designated XL-1 to XL-12 (for *Xenopus* lymphocyte). Some recurring patterns were observed, such as XL-4 and XL-6, both of which were present in 10 of the 48 samples tested (21% of samples).

3.3.3.2 Flow Cytometric Testing of 12 Individual Phage Antibodies for their Ability to Bind *Xenopus* Spleen Cells

The RFLP analysis of scFv sequences allowed 12 individual phage antibodies, called XL-1 to XL-12, to be isolated from the panned library. These 12 phage antibodies were screened by flow cytometry for their ability to bind splenocytes from control *Xenopus*. The histograms shown in Figure 3.6 show that each recombinant phage has an ability to adhere to a small percentage (between 6% and 20%) of the total spleen population. However, only the FACS trace corresponding to phage antibody XL-6 shows a separate peak of positive fluorescence to suggest that it is binding specifically to a distinct subset of spleen cells.

3.3.3.3 Verification of ScFv Expression on Phage XL-6 by Western Blotting

In order to confirm that phage XL-6 was expressing surface scFv, 10^{10} XL-6 phage particles were subjected to SDS-PAGE and then blotted to nitrocellulose, as in Section 2.2.4.3. As a control, the same number of wild type phage particles were included in the Western blot. ScFv/cpIII fusion proteins were detected directly using an HRP-conjugated goat anti-mouse IgG antibody, followed by chemiluminescence. The result shown in Figure 3.7 confirms that recombinant phage XL-6 does indeed express the ScFv fusion to cpIII which is detected at 48 kDa.

3.3.3.4 Production of Soluble XL-6 ScFv

After cloning the XL-6 scFv into the expression vector pAK400 and transforming the plasmid into *E.coli* strain JM83, soluble scFv proteins were purified from bacterial cultures by osmotic shock (Described in Section 2.2.5.1). An aliquot of the osmotic shock supernatant was subjected to SDS-PAGE, blotted to nitrocellulose and probed with an HRP-conjugated goat anti-mouse IgG antibody, followed by chemiluminescence. The result shown in Figure 3.8 confirms that soluble XL-6 scFv was produced from the bacteria as a band is present at around 28 kDa.

3.3.3.5 Comparison of Phage XL-6 and Soluble XL-6 Binding to *Xenopus* Spleen Cells by Flow Cytometry

The XL-6 phage and soluble XL-6 scFv were both tested by flow cytometry for their ability to bind control *Xenopus* splenocytes. Figure 3.9 shows that both the phage-bound and the soluble scFv fragments bind to approximately 95% of the total population of splenocytes. The XL-6 phage gives a broader peak of fluorescence than the soluble XL-6 scFv.

3.3.3.6 Testing of Soluble XL-6 on Different Cell Types of Control *Xenopus*

Having confirmed that the soluble XL-6 scFv retains the ability of the phage XL-6 to bind *Xenopus* splenocytes, the specificity of soluble XL-6 was tested by flow cytometry on various cell types of control *Xenopus laevis*. The results in Figure 3.10 show that the XL-6 scFv does not bind to *Xenopus* red blood cells, B_3B_7 thymus tumour cells or the A6 *Xenopus* kidney tumour cell line (Rafferty, 1969). Cells derived from various lymphoid organs of control *Xenopus laevis* were also tested for binding of XL-6 scFv. The traces shown in Figure 3.10 reveal that XL-6 binds 82% of splenocytes, 74% of liver leukocytes, 24% of peripheral blood leukocytes (PBL) and 22% of thymocytes.

3.3.3.7 Dual Stain Testing of Soluble XL-6 on Splenocytes from Control *Xenopus*

Dual stain analysis was carried out using soluble XL-6 in conjunction with PE-conjugated mouse mAb's to *Xenopus* cell surface markers, as described in Section 3.2.4.7. These mAb's and their specificities are listed in Appendix 3. The histograms (Figure 3.11) show that the cells bound by mAb's 2B1 (α-CD5) and D4-3, which are pan T cell markers, are also bound by the XL-6 scFv. The anti-CD8 mAb F17 also co-

stained strongly with XL-6, as did the mAb D12.2, which was originally thought to identify a γδ T cell population (Ibrahim *et al.*, 1991). The anti-μ chain antibody D8, which binds all *Xenopus* B-cells, also co-stained with XL-6 although under half of the B-cells co-expressed the XL-6 antigen.

3.3.3.8 Single and Dual Stain Testing of Soluble XL-6 on Immunomagnetically-Sorted Populations of Splenocytes from Tx *Xenopus*

Splenocytes were taken from a Tx animal and FACS analysis of these cells showed that 38% were bound by XL-6 (Figure 3.12A). Immunomagnetic separation was then performed on the splenocytes, with the anti-μ mAb 8E4, to sort the cells into a non-B and a B cell population (As described in Section 3.2.2.3). Flow cytometric testing of the non-B cell population showed that 14% were XL-6 positive, whereas 42% were positive when tested with the putative anti-thrombocyte mAb XLH-1 (Figure 3.12B). This was taken as indirect evidence that XL-6 did not bind to thrombocytes. A dual stain was also performed on the non-B cells, using XL-6, detected with an anti-mouse PE antibody, followed by the FITC conjugated mAb 1F8 (anti-*Xenopus* NK cell mAb, described in detail in Chapter 4). This dual stain revealed that the XL-6 antigen was expressed on approximately one third of 1F8-labelled *Xenopus* NK cells.

FACS analysis of the enriched B cell population (Figure 3.12C) revealed purity of nearly 90% (86% 8E4-positive). 54% of this enriched B cell population were XL-6 positive. Dual stain analysis using XL-6 and the anti-μ mAb D8 confirmed that the XL-6 antigen was present on over half of the B cells.

3.3.3.9 Dual Stain Testing of Soluble XL-6 on *Xenopus* Thymocytes

Dual stain analysis of thymocytes was carried out using soluble XL-6 in conjunction with PE-conjugated mouse mAb's to *Xenopus* cell surface markers (As described in Section 3.2.4.7). Figure 3.13 shows that the pan T cell markers detected by the mAb's D4-3 and 2B1 (anti-CD5) were expressed on 17% and 54% of thymocytes respectively. XL-6 routinely stained around 20% of thymocytes, the majority of which co-expressed the markers detected by mAb's D4-3 and 2B1. In addition, approximately one quarter of XL-6 positive thymocytes also co-expressed the CD8 antigen recognised by the mAb F17.

3.3.3.10 Probing Western Blots of Control Spleen Cell Lysates with Phage XL-6

In order to identify the antigen bound by phage antibody XL-6, Western blots of spleen cell lysates were probed with phage antibody XL-6 and wild type phage as a control (As in Section 2.2.4.4). Detection was achieved using a mouse anti-M13 antibody, followed by an HRP-conjugated goat anti-mouse IgG antibody and chemiluminescent identification of protein bands. The result (Figure 3.14) shows that no specific protein band was identified by XL-6, whereas the wild type phage displays some non-specific background staining of *Xenopus* proteins. Soluble XL-6 was also used to probe Western blots of *Xenopus* spleen cell lysates but failed to identify any specific proteins (Data not shown).

3.4 Discussion

3.4.1 The Efficiency of Panning on *Xenopus* NK-Enriched Cells

The intention of the phage display panning described in this Chapter was to isolate phage antibodies to *Xenopus* NK cells. The method for cloning the scFv library from an immunised mouse and for panning the library on Tx *Xenopus* spleen cells was based on the protocol used in Chapter 2 to raise phage antibodies to B_3B_7 cells. Evidence from FACS analysis strongly suggested that, through panning, the α-NK phage library of around 5×10^5 scFv clones became enriched for phage antibodies capable of binding to Tx *Xenopus* splenocytes. RFLP analysis of 48 scFv sequences from the panned phage pool revealed 12 different phage scFv's, designated XL-1 to XL-12, which were tested individually for their ability to bind to control *Xenopus* spleen cells. Of the twelve phage, only XL-6 displayed a FACS profile indicating distinct binding to *Xenopus* splenocytes. Further testing of XL-6 phage on spleen cells from a number of control *Xenopus* revealed that it consistently bound between 80% and 95% of splenocytes. The ability of purified soluble XL-6 scFv to bind the same proportion of cells as the phage XL-6 confirmed that the scFv protein, rather than any non-specific interactions of the phage particle, was responsible for binding to *Xenopus* cells.

The ability of XL-6 to bind such a high percentage of splenocytes clearly indicates that it is not specific for *Xenopus* NK cells, which represent less than 10% of splenocytes in control *Xenopus* (See Chapter 4). There are a number of reasons why the panning protocol failed to raise phage antibodies specific to *Xenopus* NK cells. Firstly, the library was derived from a mouse immunised with 'NK-enriched' cells from *Xenopus*

rather than a pure population of *Xenopus* NK cells. This meant that the mouse would have become immunised against epitopes expressed on any other contaminating cell types, and these specificities would have become incorporated into the phage library. In addition, by using whole *Xenopus* cells for injection rather than cell membrane preparations, the mouse would also have become immunised against any intracellular epitopes which were exposed following degradation of the cells. Combined with the relatively small size of the phage library, these factors would have meant that there was a fairly small chance of isolating a phage antibody with a specificity for *Xenopus* NK cells. The library was also panned on mixed cell populations, rather than pure NK cells, which again reduced the chances of enriching the library for NK-specific phage. However, to isolate a pure population of *Xenopus* NK cells for immunisation and panning would have required the use of cell sorting with an anti-NK mAb and such an antibody was not available at the time. In addition, the small number of NK-enriched cells obtainable by immunomagnetic depletion from a Tx spleen meant that the purification of membranes from these cells for immunisation, which would have involved further loss of material, was not considered a viable option. Thus the protocol used in this Chapter was thought to give the best chance of raising NK-specific phage antibodies.

Considering the number of different scFv sequences present in the library after three rounds of panning, it was predicted that more than one of the twelve phage screened would be able to bind *Xenopus* cells. However, only the FACS trace of spleen cells stained with XL-6 phage, which was found to constitute almost a quarter of the panned phage pool by RFLP analysis, showed a distinct peak of fluorescence. It is possible that the other eleven phage antibodies, which had been selected through panning, were low affinity binders and as such did not perform as well in FACS analysis. As mentioned in Chapter 2 they may have performed better in different immunoassays, such as immunocytochemistry. In addition, the screening of a larger number of panned phage may have led to the isolation of other useful, even NK-specific, phage antibodies. However, since performing more than one method of screening on a large number of phage antibodies would have been very time-consuming, it was decided to spend more time investigating the one phage antibody, XL-6, which showed a genuine ability to bind to *Xenopus* cells.

3.4.2 Further Investigation of the Specificity of XL-6 scFv

Using flow cytometric analysis, it was shown that XL-6 scFv did not bind to *Xenopus* red blood cells, the *Xenopus* thymus tumour cell line B_3B_7, or the *Xenopus* kidney

tumour cell line A6. The inability to bind these three cell types reflects the success of negative panning on red blood cells in removing phage which bound general *Xenopus* cell surface epitopes.

Investigation of control spleen and liver leukocytes by flow cytometry revealed that XL-6 typically bound between 75% and 85% of these cells. Dual stain analysis showed that XL-6 co-stained virtually all splenocytes which were labelled with T cell markers. In contrast, not all splenic B cells were XL-6 positive as dual stain analysis with the anti-IgM mAb D8 revealed both XL-6 positive and XL-6 negative B cells. The investigation of immunomagnetically sorted Ig-positive and Ig-negative populations from Tx spleen confirmed that XL-6 co-stained a major population, equivalent to around 50%, of B cells and approximately one third of NK cells. The Ig-negative population from Tx spleen were only 14% XL-6 positive whereas 42% were labelled with the putative anti-thrombocyte mAb XLH-1. Thus XL-6 appears not to label thrombocytes which may explain why PBL, which contains a large proportion of thrombocytes (T.L. Horton, pers. comm.), was only 25% XL-6 positive.

In contrast to the high proportion of peripheral lymphoid cells, especially T cells, that were XL-6 positive, only a small proportion, equivalent to around 20%, of thymic lymphocytes were XL-6 positive. It is of interest to note that XL-6 did not label the thymic tumour cell line B_3B_7. This cell line has been shown to have an immature thymic phenotype as it does not express MHC class II which acts as a differentiation marker of thymocytes and is preferentially expressed on mature medullary thymic cells (Flajnik *et al.*, 1990; Du Pasquier *et al.*, 1995). The immature nature of B_3B_7 cells is further underlined by its expression of a molecule called CTX, which is a known marker of immature, cortical thymocytes (Chrétien *et al.*, 1996). Flow cytometric analysis has also shown that the immature thymic marker CTX is found on between 65% and 80% of thymocytes, implying that the mature medullary population of the thymus is a minor population of between 20% and 35% (Chrétien *et al.*, 1996). Taken together this indirect evidence suggests that XL-6 could be binding to a population of mature thymocytes.

Dual stain FACS analysis of thymocytes revealed that XL-6 also co-stained the majority of D4-3 positive cells in the thymus, which may be of interest as D4-3 also stains mature, peripheral T cell populations (Ibrahim *et al.*, 1991). In addition, the small percentage of CD8 positive thymocytes (detected by mAb F17) which co-stain with XL-6 may represent single positive mature T cells whereas the CD8 positive thymocytes not labelled with XL-6 may be immature, double positive T cells. Also of

interest is the ratio of CD8+ to CD8- cells within the XL-6 labelled thymic population which is similar to the 1:2 ratio of CD8+ to CD8- (putative CD4+) T cells in the periphery (Gravenor *et al.*, 1995). Unfortunately there are currently no markers of CD4+ T cells in *Xenopus* with which to confirm this theory.

There is a distinct possibility that XL-6 identifies an amphibian equivalent of a mammalian CD45 molecule, also known as the leukocyte common antigen. Although the CD45 family as a whole are expressed on all leukocytes, different isoforms are expressed on sub-populations of leukocytes according to differences in cell maturation and function (Poppema *et al.*, 1996; Altin and Sloan, 1997). The specificity of XL-6 for a particular CD45 isoform may explain why, despite being leukocyte specific, it labels all T cells and varying levels of B and NK cells. Interestingly, it has been shown that one isoform of CD45 in mammals, known as CD45RA, identifies a small subset (between 15% and 30%) of thymocytes, which are located in the medulla and are thought to be committed to entering the periphery as mature T cells (Deans *et al.*, 1989, Gillitzer and Pilarski, 1990). This bears a striking similarity with the staining pattern of XL-6 in the thymus which identifies a small population of thymocytes with a 'mature' phenotype.

Studies carried out on leukocyte common antigens in *Xenopus* have used mAb's which appear to identify an epitope common to several isoforms of the putative amphibian CD45 as determined by immunoprecipitation experiments (Ohinata *et al.*, 1989; Smith and Turpen, 1991). One of these studies, using the putative anti-CD45 mAb CL21, showed by flow cytometry that lymphoid cells, but not thrombocytes, from adult spleen were over 90% positive and that an equally high percentage (90%) were stained in the adult thymus (Smith and Turpen, 1991). This contrasts with the much lower level of XL-6 positive cells in the thymus and may reflect the ability of CL21 to bind several isoforms of CD45, whereas XL-6 may be showing specificity for a single isoform.

During these studies that involved many *Xenopus*, it was observed that a minority of animals tested with XL-6 showed insignificant levels of XL-6 positive splenocytes and some showed no binding at all. It is possible that this relates to variability of the extracellular domain of the target antigen and it is of interest to note that mammalian CD45 shows considerable variation in this region due to differential splicing (Poppema *et al.*, 1996).

More definite conclusions as to the nature of the antigen bound by XL-6 were hindered by the failure of XL-6 to identify any protein bands in Western blots. This is probably due to the fact that XL-6 was raised against cell surface proteins in their native conformation. The denatured form of the protein probed in the Western blot may not have been recognisable by XL-6. As such, it would be of great interest in the future to conduct immunoprecipitation experiments with XL-6 on splenocyte and thymocyte lysates.

Figure 3.1   VH and VL RT-PCR's

1% agarose gel showing amplification of variable heavy (VH) and variable light (VL) chain DNA by RT-PCR. Template RNA was obtained from the spleens of mice immunised with an 'NK-enriched' population of *Xenopus* lymphocytes. Lane 1 contains λPst1 DNA markers.
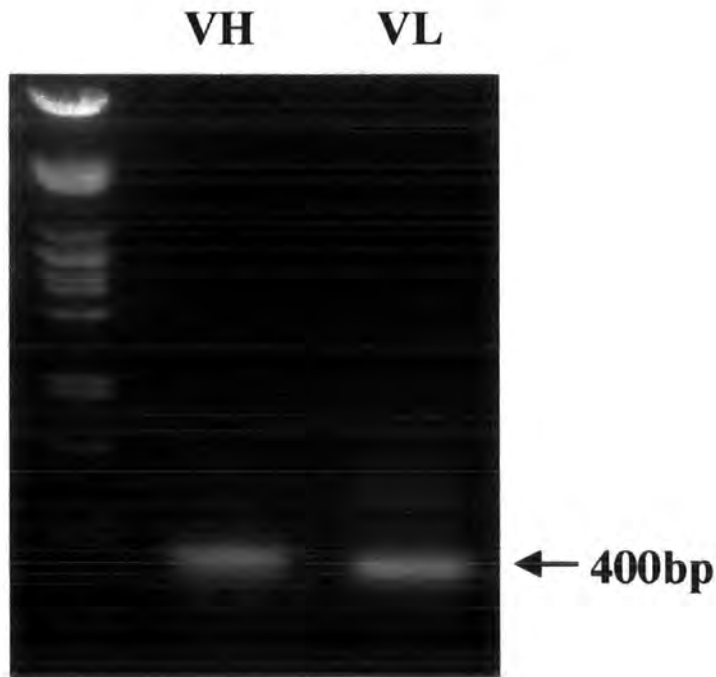
Figure 3.2   Joining of VH and VL Fragments by SOE-PCR

1% agarose gel showing the result of an SOE-PCR reaction on VH and VL DNA fragments amplified from the 'NK-immunised' mouse. Lane 1 contains λPstI DNA size markers.
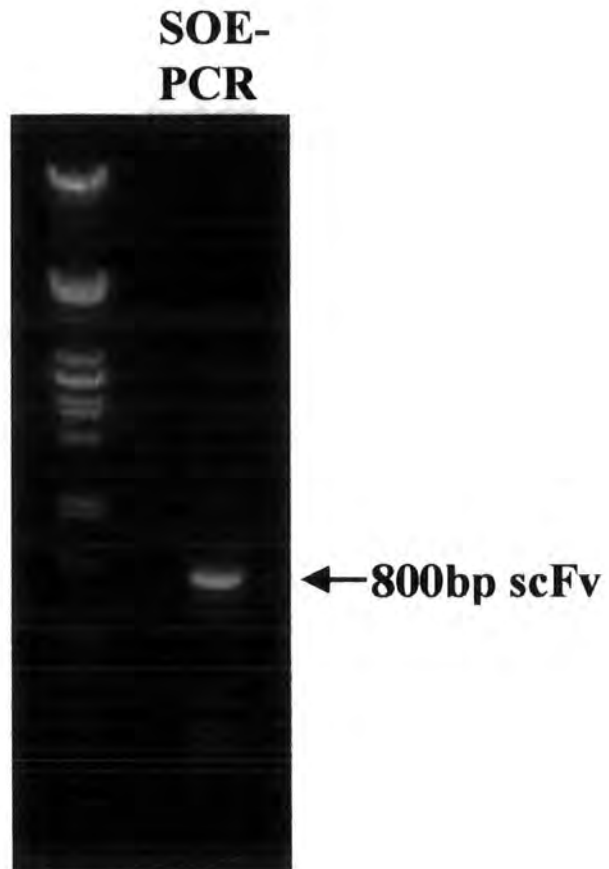
<u>**Figure 3.3**</u>   <u>**Diversity of scFv Sequences in Library by RFLP Analysis**</u>

10 scFv sequences were reamplified from the unpanned phage library by PCR and analysed by gel electrophoresis (A). Following HaeIII digestion, scFv fragments were run on a 2% agarose gel for analysis of RFLP's (B). Marker in lane 1 of both gels is λPstI DNA.

A.

←800bp scFv
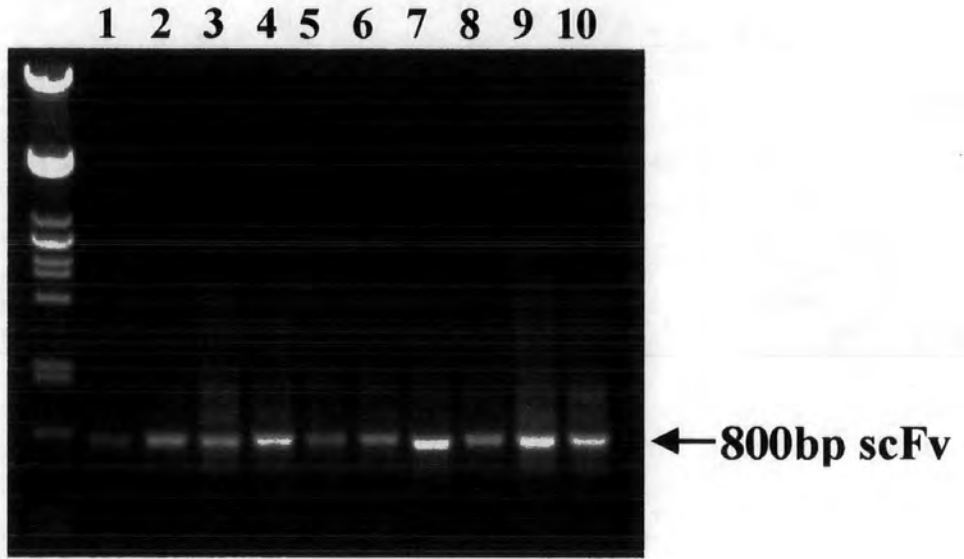
B.
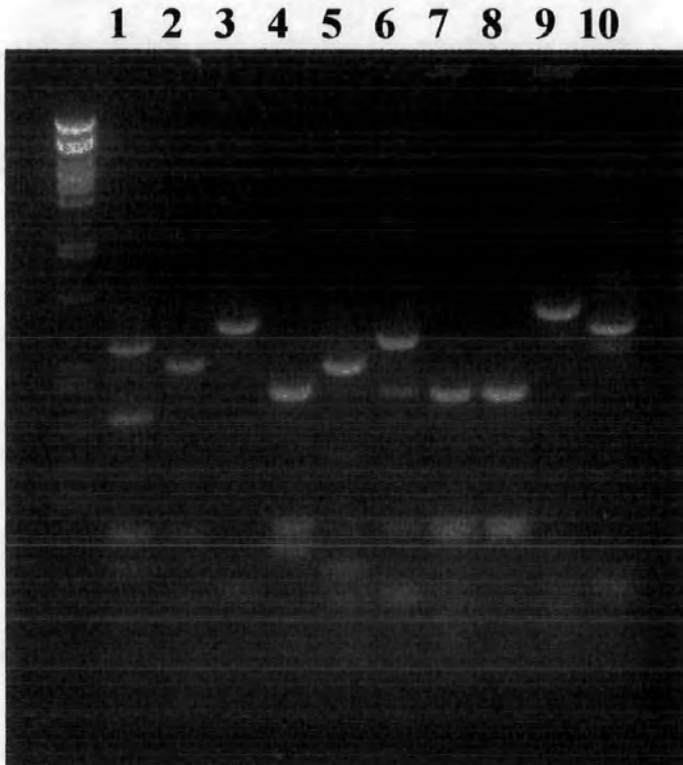
Figure 3.4   Flow Cytometric Analysis of Phage During Panning

During each round of panning, both wild type (WT) and recombinant phage
were tested by flow cytometry for their ability to bind Tx spleen cells. Phage
were detected by a rabbit anti-M13 antibody followed by a FITC-conjugated
anti-rabbit Ig antibody. The percentage of positively stained splenic lymphoid
cells is indicated in the top-right corner of each histogram.
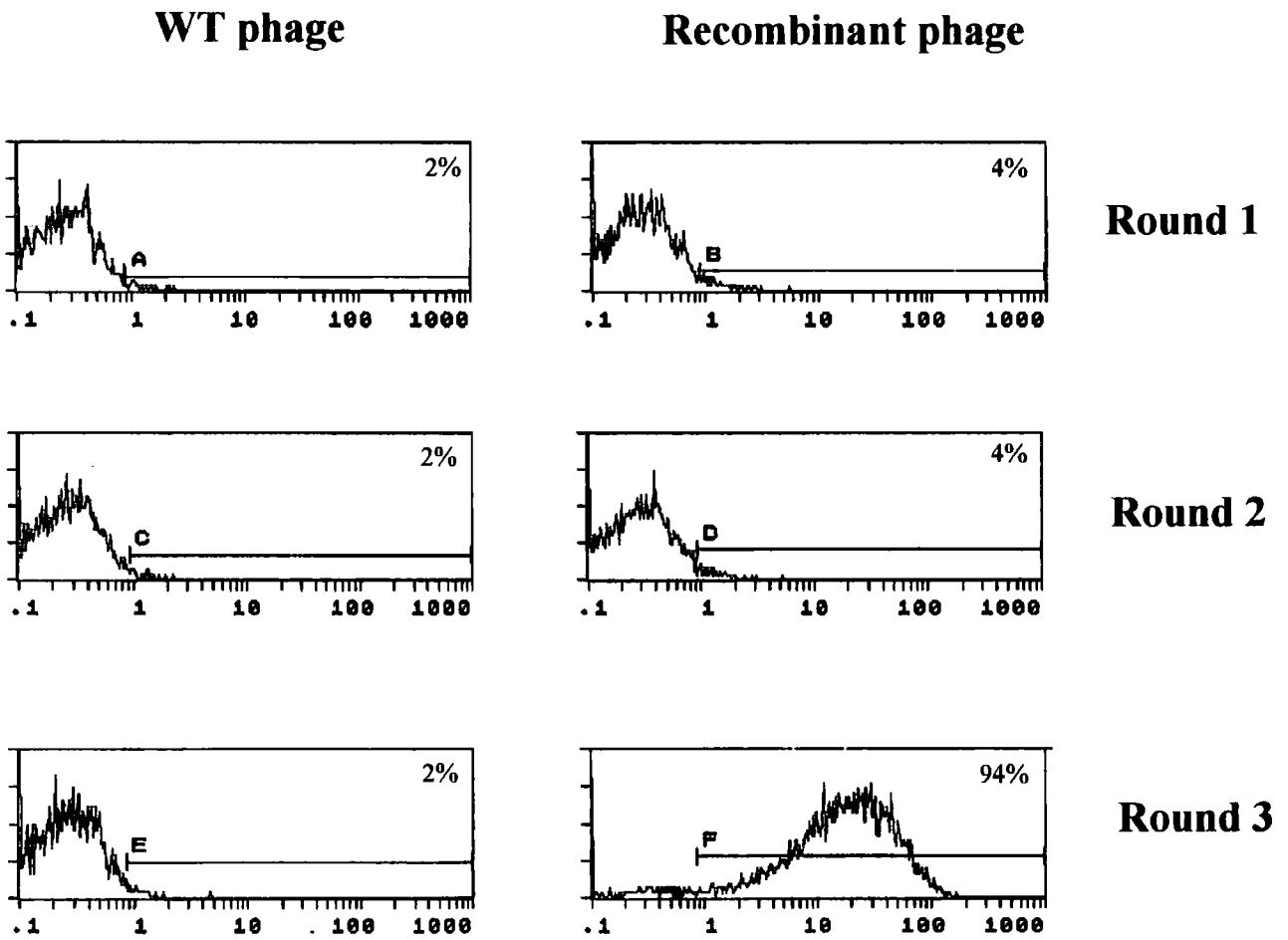
Figure 3.5 RFLP Analysis of Phage scFv Sequences after Three Rounds of Panning

scFv sequences were amplified by PCR and digested with HaeIII before separation on 2% agarose gels. Different RFLP profiles have been designated 1 to12. Lane 1 in each gel is λPstI DNA marker.
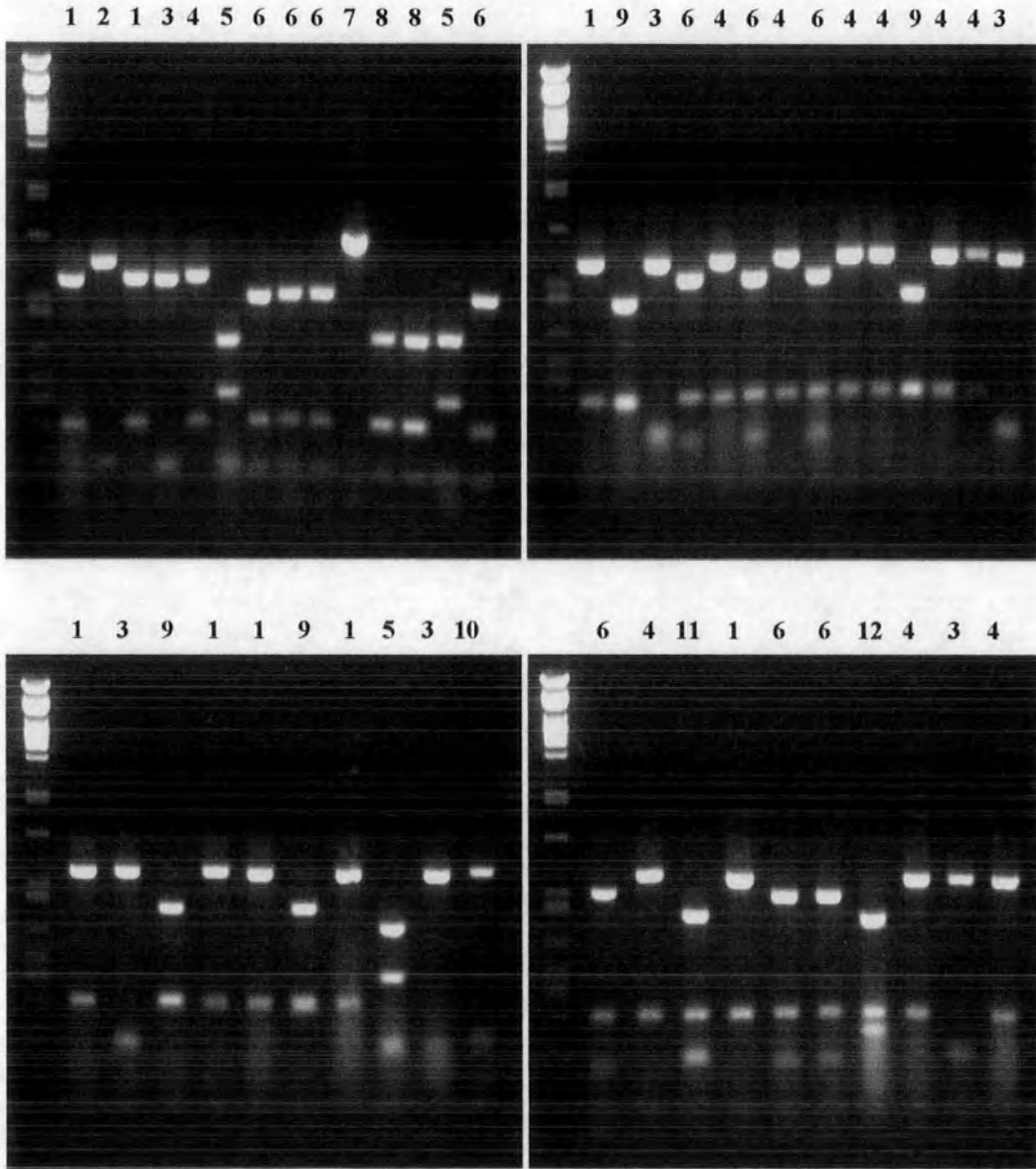
Frequency of each profile:-

1: 7	**2**: 1	**3**: 6	**4**:10	**5**: 3	**6**:10
7: 1	**8**: 2	**9**: 4	**10**: 1	**11**:1	**12**:1

Figure 3.6 Flow Cytometric Analysis of Twelve Individual Phage Antibodies

Twelve different recombinant phage, isolated from the library which had been panned three times, were designated XL-1 to XL-12 and tested individually by flow cytometry for their ability to bind splenocytes from control *Xenopus*. Phage were detected by a rabbit anti-M13 antibody followed by a FITC-conjugated anti-rabbit Ig antibody. Markers for background staining were set at 2% using cells incubated with FITC-conjugated anti-rabbit Ig antibody only.
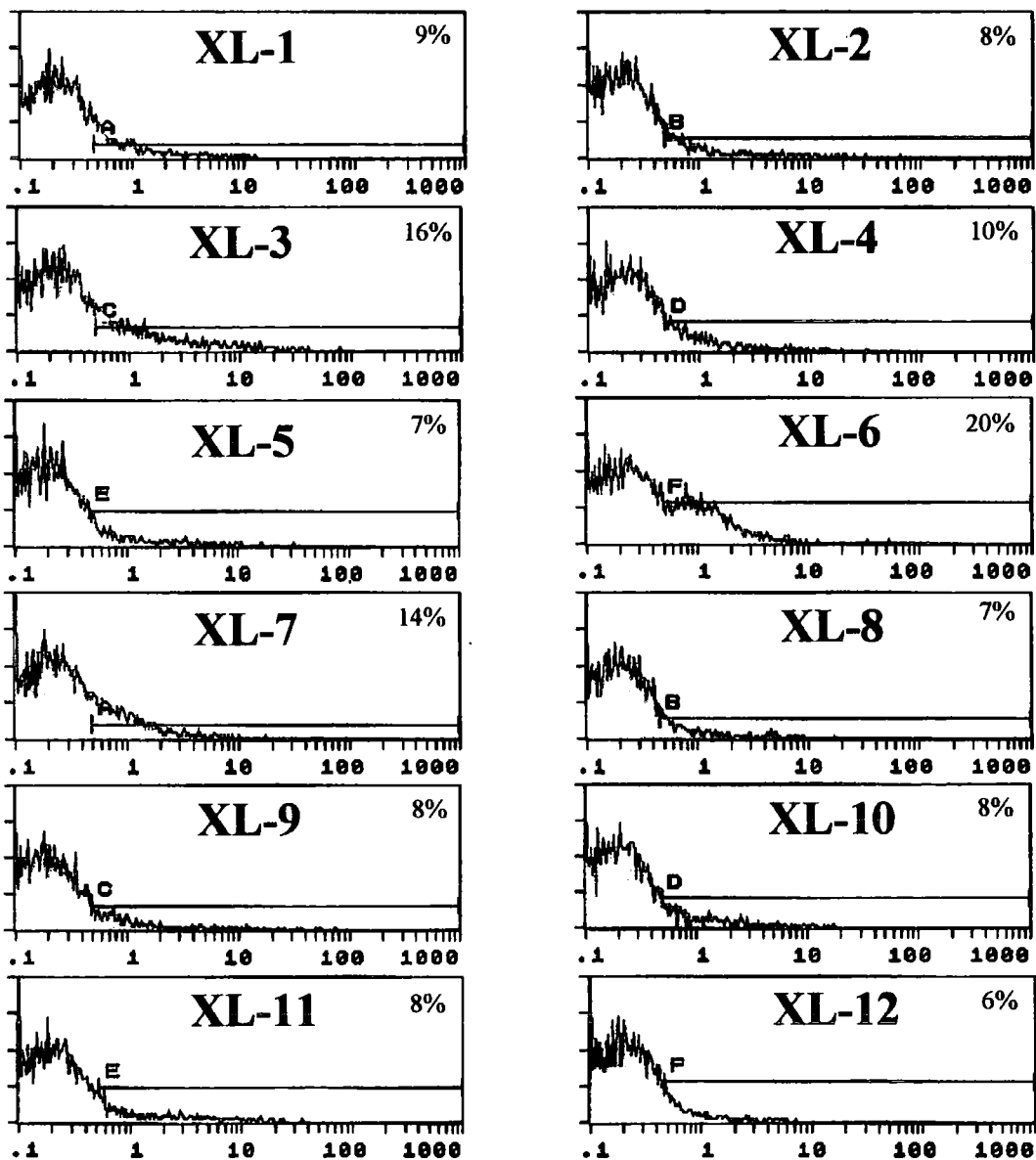
Figure 3.7   Confirmation of scFv on Phage XL-6 by Western Blotting

10^{10} wild type (WT) and 10^{10} XL-6 phage particles were separated by SDS-PAGE and blotted to nitrocellulose. Blots were probed with an HRP-conjugated anti-mouse Ig antibody and HRP activity was detected by chemiluminescence.
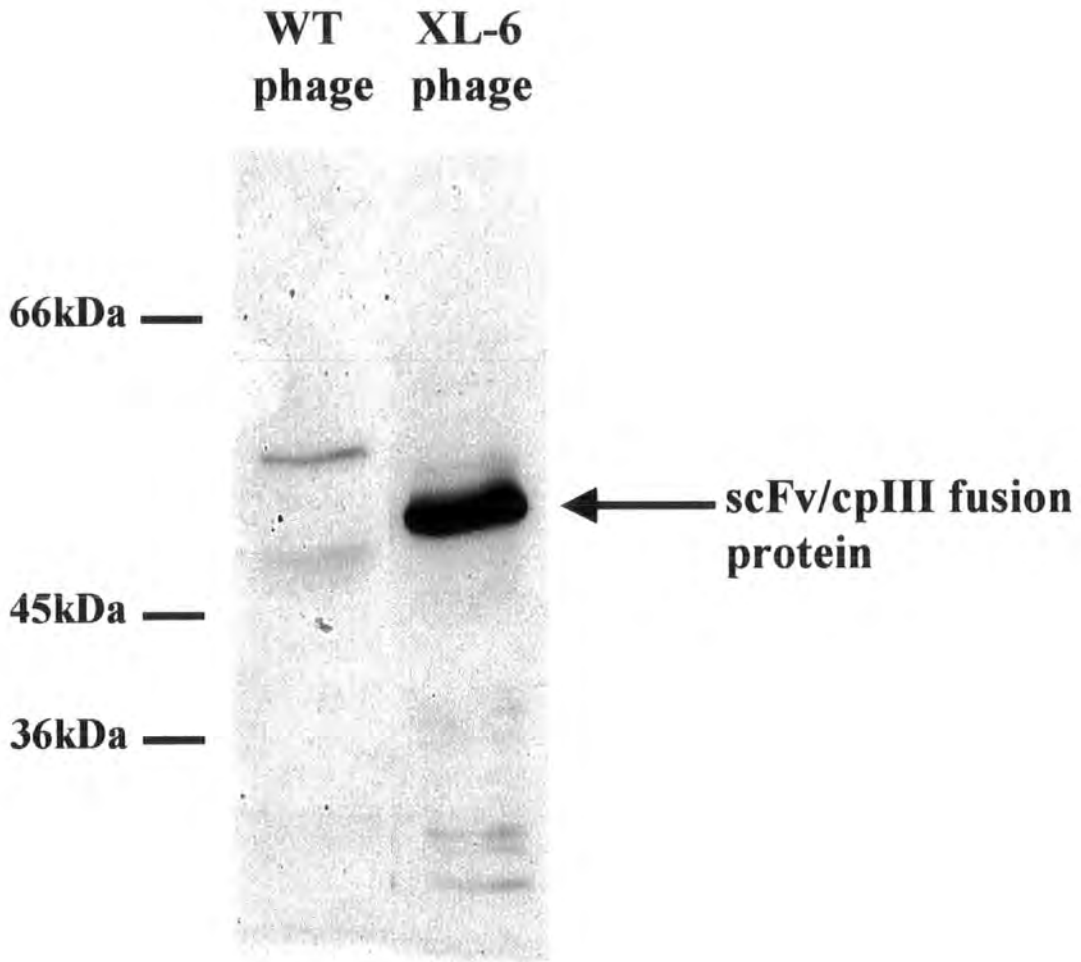
Figure 3.8   Confirmation of Soluble XL-6 scFv Production

Following induction of soluble XL-6 scFv production in *E.coli* strain JM83, an aliquot of osmotic shock fluid was subjected to SDS-PAGE and blotted to nitrocellulose. Blots were then probed with an HRP-conjugated anti-mouse Ig antibody before detection by chemiluminescence.

116kDa —

66kDa —

45kDa —

36kDa —

← ——— XL-6 scFv
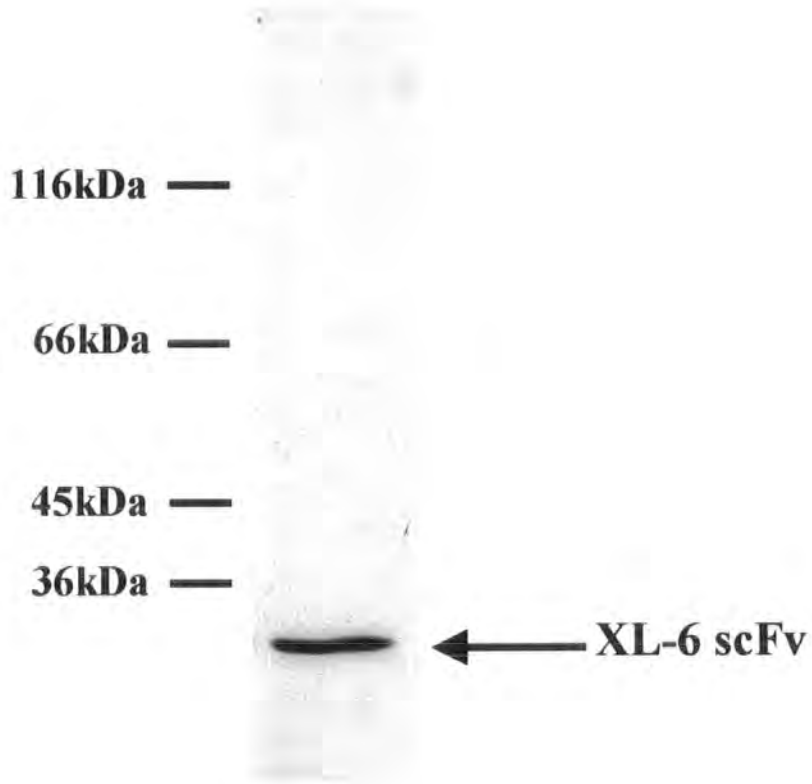
<u>**Figure 3.9 Comparison of Phage and Soluble XL-6 Binding to Control**</u>
<u>*Xenopus* **Spleen Lymphocytes by Flow Cytometry**</u>

Phage expressing surface scFv XL-6 and bacterially produced soluble XL-6
were both tested by flow cytometry for their ability to bind splenocytes from
control *Xenopus*. Phage were detected by a rabbit anti-M13 antibody followed
by a FITC-conjugated anti-rabbit Ig antibody whereas soluble scFv's were
detected directly with a FITC-conjugated anti-mouse Ig antibody.

Phage XL-6
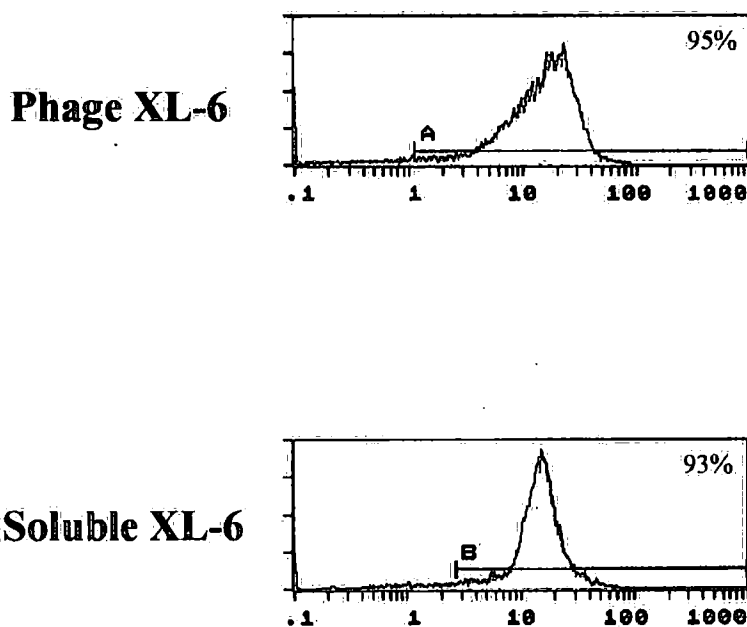
Soluble XL-6

Figure 3.10   Ability of Soluble XL-6 to Bind Diverse Cell Types from *Xenopus laevis*

The specificity of soluble XL-6 was tested by flow cytometry on various lymphoid cells from different organs of *Xenopus laevis*, as well as *Xenopus* peripheral red blood cells and two *Xenopus* tumour cell lines. The histograms shown here are representative of several experiments conducted on one year old animals.

Spleen

Liver

PBL

Thymus

RBC's

B₃B₇ thymus tumour cell line

A6 kidney tumour cell line
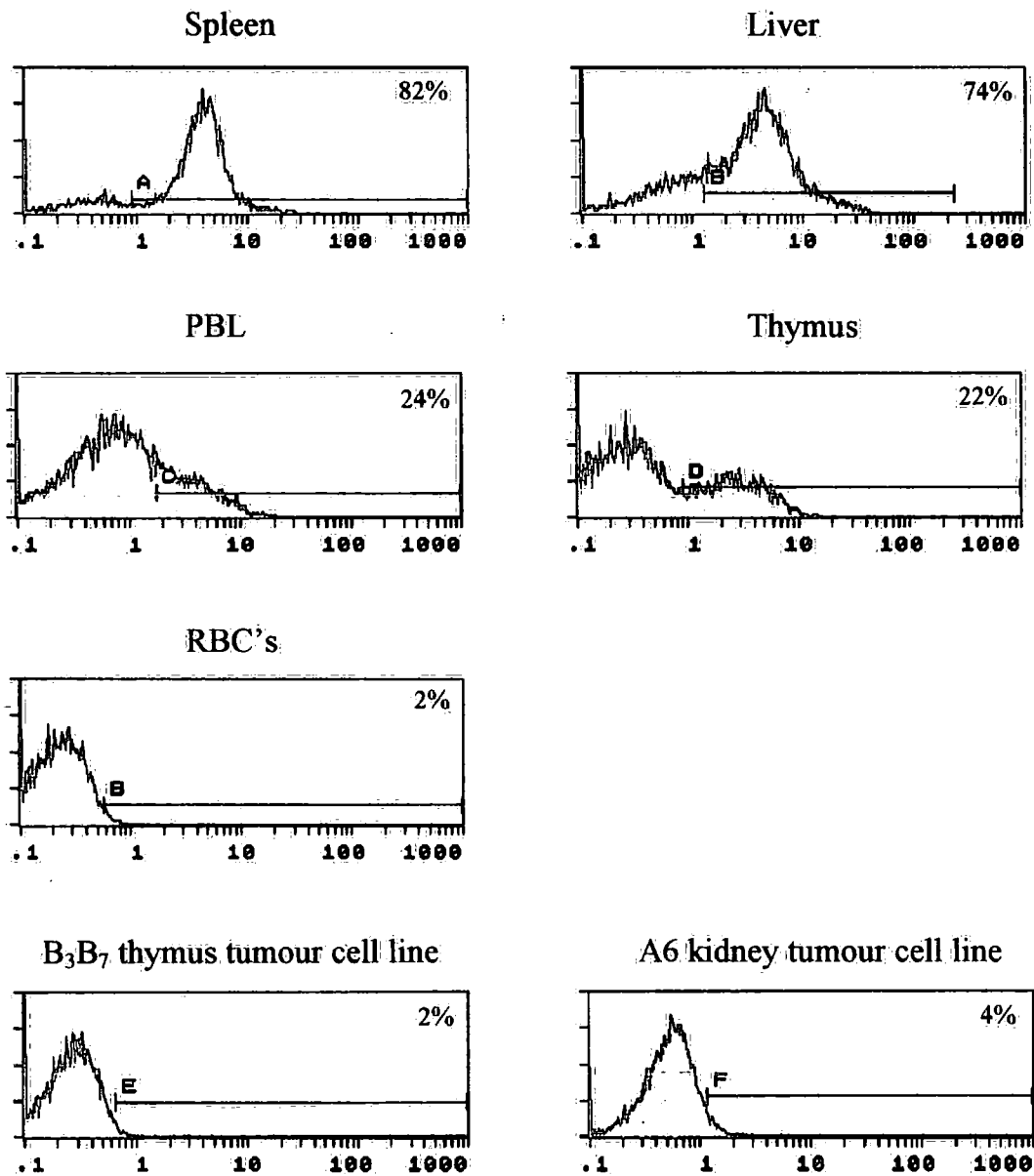
Figure 3.11   Dual Stain Testing of Soluble XL-6 on Control Splenocytes

Spleen cells from control *Xenopus* were dual-labelled by using soluble XL-6 (detected by FITC) in conjunction with phycoerythrin (PE)-conjugated antibodies. The soluble XL-6 (FITC) fluorescence is measured on the X-axis of the histogram, while the PE fluorescence is shown on the Y-axis. The results shown on these histograms are gated for spleen lymphocytes.

2B1 (α-CD5)

2% — 50% — 34%

Soluble XL-6

D 4-3 (Pan T cell)

1% — 52% — 32%

Soluble XL-6

D 8 (α-μ)

9% — 6% — 78%

Soluble XL-6

F 17 (α-CD8)

0% — 24% — 61%

Soluble XL-6

D 12-2

0% — 5% — 70%
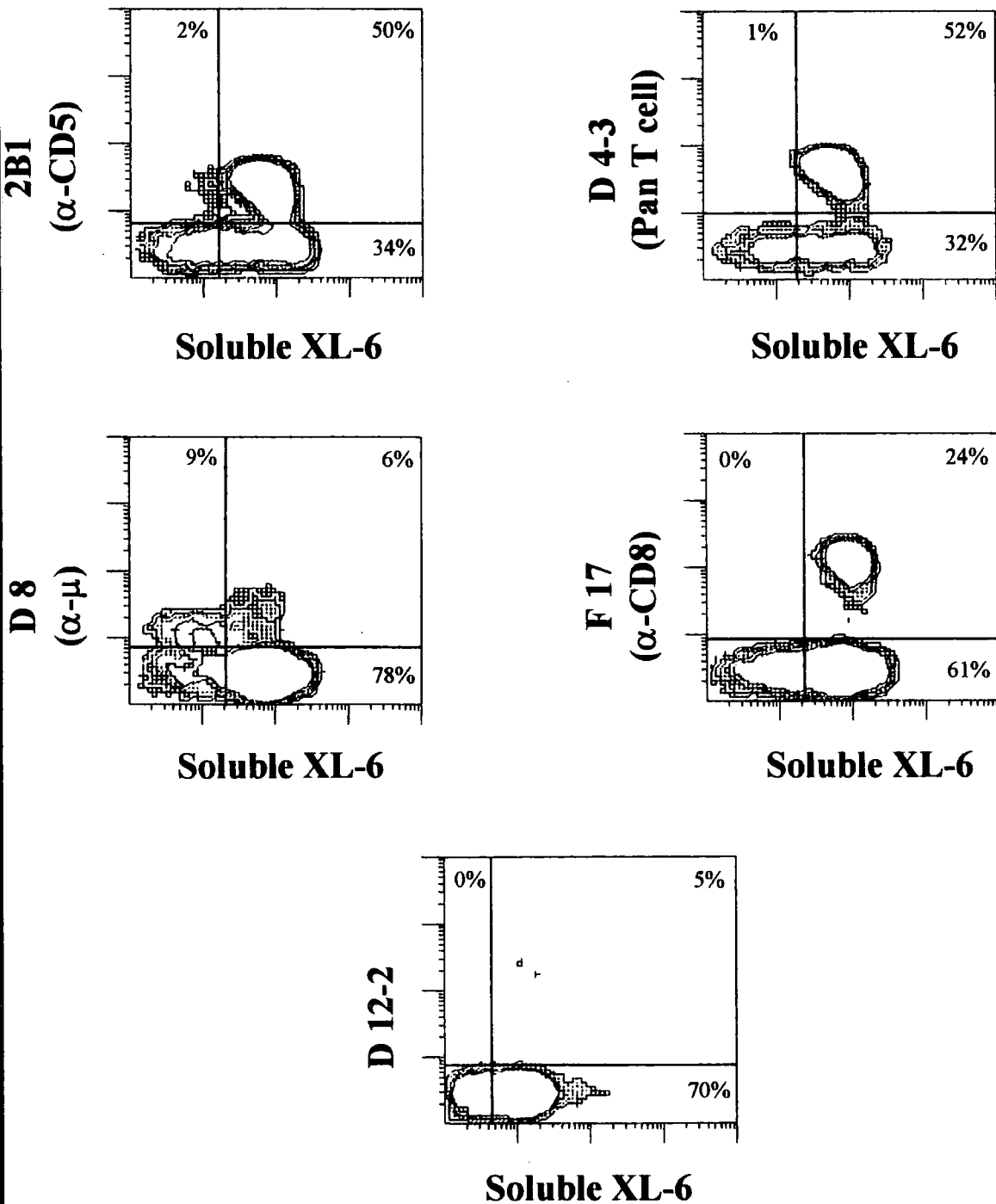
Soluble XL-6

Figure 3.12 Single and Dual Stain Analysis of Immunomagnetically-Sorted Spleen Populations from Tx *Xenopus*

Tx splenocytes were tested by FACS using soluble XL-6 (A) and then immunomagnetically sorted with the B cell-specific mAb 8E4. The non-B cell population was re-tested with XL-6 and the α-thrombocyte mAb XLH-1. This population was also dual stained (B) with the FITC-conjugated α-NK mAb 1F8 after staining with XL-6 (detected by PE-conjugated α-mouse Ig). The B cell-enriched population was re-tested with XL-6 and the α-IgM mAb 8E4, and in a dual stain with XL-6 and the PE-conjugated α-IgM mAb D8 (C).

A. Total Tx splenocytes
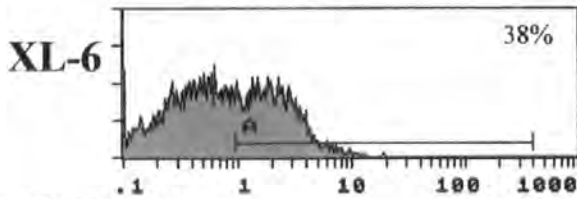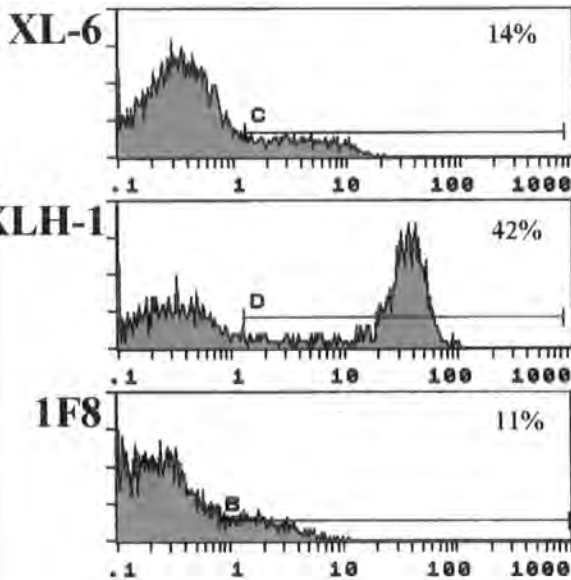
B. Non-B cell population   C. B cell population

90
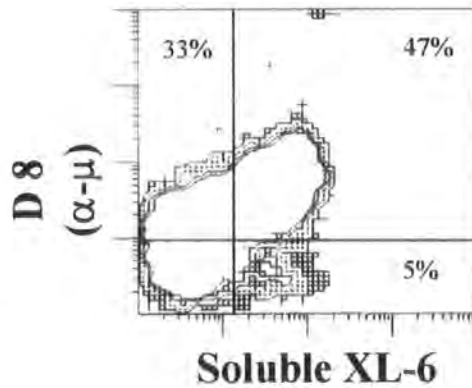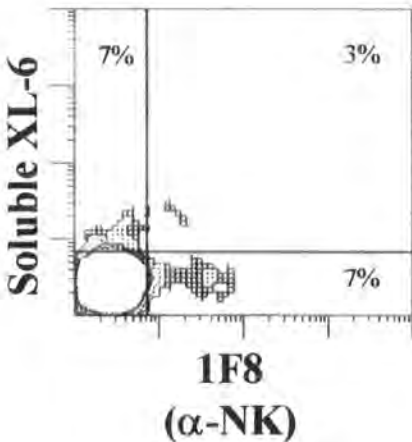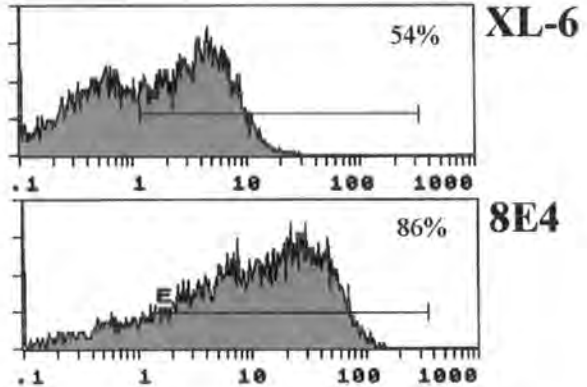
Figure 3.13   Dual Stain Testing of Soluble XL-6 on Thymocytes

Thymocytes from a year-old *Xenopus* were dual-labelled by staining with
soluble XL-6 (detected by FITC-conjugated anti-mouse Ig) in conjunction with
phycoerythrin (PE)-conjugated antibodies. The soluble XL-6 (FITC)
fluorescence is measured on the X-axis of the histogram, while the PE
fluorescence is recorded on the Y-axis. The data shown is representative of
several experiments.
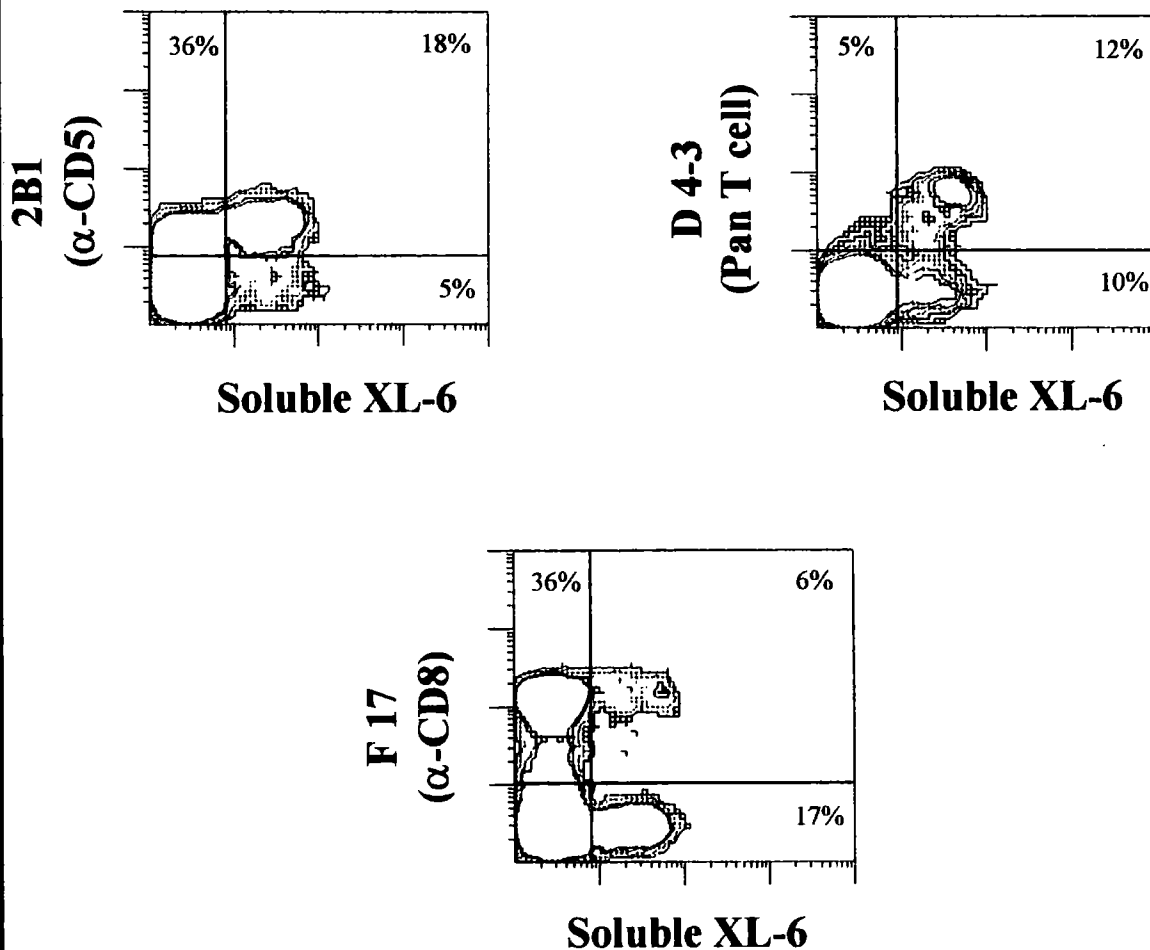
Figure 3.14   XL-6 Phage as a Reagent in a Western Blot

Protein lysates from control *Xenopus* splenocytes were separated by SDS-
PAGE and transferred to nitrocellulose before being probed with XL-6 phage.
Wild type (WT) phage were used in parallel as a control for non-specific
binding. Bound phage were detected by a rabbit anti-M13 antibody, followed
by an HRP-conjugated anti-rabbit antibody. HRP activity was detected using
chemiluminescence.
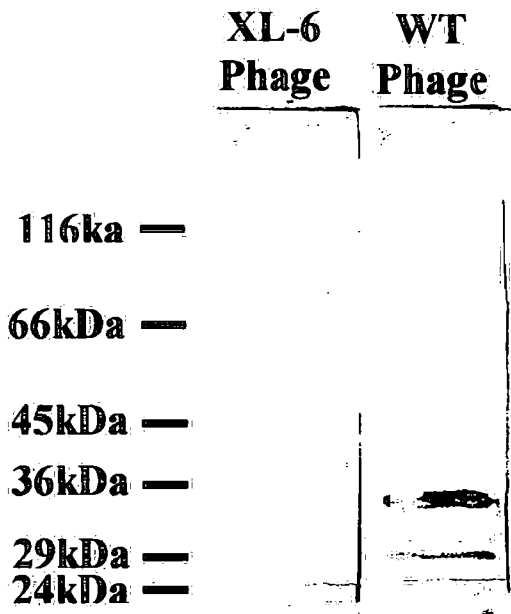
The WT phage control blot shown here is also displayed in Figure 2.11 as
the experiments were performed at the same time.

CHAPTER 4

Use of Hybridoma Technology to Generate Monoclonal Antibodies to *Xenopus* NK Cells

This Chapter details the use of hybridoma technology to isolate monoclonal antibodies to *Xenopus* NK cells. Flow cytometric screening of the hybridoma-derived mAb's was used to highlight those with a potential NK specificity and further investigation of these mAb's on both *Xenopus* and human cells was carried out by flow cytometry. Attempts to define the NK cell surface antigens bound by these mAb's using Western blotting and immunoprecipitation are described.

4.1 Introduction

4.1.1 Natural Cytotoxicity in *Xenopus*

Splenocytes from both control and thymectomised *Xenopus*, when cultured in growth factor supplemented medium (GFM), which contains IL-2 like material (Haynes and Cohen, 1993), are able to lyse the MHC-deficient *Xenopus* thymus tumour cell line B$_3$B$_7$ in chromium release assays (Horton *et al.*, 1996). Furthermore, splenocytes from Tx animals, which have been shown to lack functional T cells, can be further depleted by the immunomagnetic removal of B cells and have been shown to retain their natural cytotoxicity (reviewed in Horton et al., 1998a). Thus a non-T, non-B cell population in *Xenopus* is capable of spontaneous cytotoxicity towards B$_3$B$_7$ tumour cells. The ability to kill tumour targets in a non-MHC dependant fashion strongly suggests that this activity is NK-like.

Despite the evidence for natural cytotoxicity in *Xenopus*, the NK-like cells responsible have yet to be identified by monoclonal antibodies. Chapter 3 described attempts to isolate NK-specific phage antibodies from an scFv library cloned from mice immunised with NK-enriched *Xenopus* cells. This Chapter summarises attempts to isolate NK-specific mAb's from the same immunised mice using the classical hybridoma approach.

4.1.2 The Potential Uses of mAb's to *Xenopus* NK cells

A mAb which identifies NK cells in *Xenopus* would be initially useful in studying the distribution of these cells in various lymphoid organs. Mammalian NK cells are found primarily in the spleen, peripheral blood, liver and gut and it would be of interest to compare the distribution of NK cells in *Xenopus*. In addition, an anti-NK mAb could be used to immunomagnetically purify populations of *Xenopus* NK cells for functional characterisation. The use of such purified NK cells in cytotoxicity assays would be useful in confirming the ability of these cells to lyse the B_3B_7 tumour target.

It would also be of interest to test the mAb on different species to find out if the NK antigen is conserved. Although some mAb's are not able to recognise antigen across species, there are others such as 5C6, which was raised against catfish NCC, which binds specifically to an antigen on human NK cells (Harris *et al.*, 1991).

Perhaps more revealing from an evolutionary perspective would be the molecular characterisation of surface antigens on *Xenopus* NK cells. An NK-specific mAb could be used to identify specific protein antigens through Western blotting or immunoprecipitation. This allows purification of the target protein and consequently derivation of amino acid sequence information. By comparing the *Xenopus* NK protein sequence with those of NK-related proteins from other species it is possible to find out if the *Xenopus* protein is a conserved marker of NK cells or a novel cell surface marker.

4.2 Materials and Methods

4.2.1 General Materials and Methods

All the general materials and methods used in this Chapter are described in Sections 2.2.1. and 3.3.1.

4.2.2 Generation and Initial Screening of Hybridoma's

The generation and initial screening of hybridoma's was carried out by Trudy Horton at Durham University.

4.2.2.1 Preparation of NK-enriched Cells from Tx *Xenopus* for Immunisation

Splenic lymphocytes were isolated from Tx animals, as described in Section 2.2.3.2., and incubated with B cell-specific mAb's, including the anti-IgM mAb 8E4 (see Appendix 3 for details) together with a cocktail of anti-light chain mAb's (Hsu et al., 1991) on ice for 30 minutes. Following 3 washes with FACS medium, 5×10^6 cells in $80 \mu l$ HBSS were added to $20 \mu l$ of MACS microbeads conjugated with goat anti-mouse IgG (Miltenyi Biotec). The cells and beads were incubated for 15 minutes at $4°C$, and the mixture passed over a prewashed Mini-MACS separation column placed in a separation unit (magnet). The non-adherent splenocytes, depleted of B cells, eluted from the column were then incubated with the thrombocyte-specific mAb XLH-1 (See Appendix 3 for details). Following three washes with FACS medium, the thrombocytes were removed by magnetic sorting using goat anti-mouse IgM microbeads.

4.2.2.2 Immunisation of Mice with *Xenopus* 'NK-enriched' Cells

Two Balb/C mice were given three injections of 8×10^5 NK-enriched cells, the first in complete Freund's adjuvant and the second and third in incomplete Freund's adjuvant, into the foot pad over a 10 day period. After 10 days, samples of mouse sera were tested by flow cytometry to confirm reactivity to *Xenopus* splenocytes (Data not shown).

4.2.2.3 Fusion and Selection of Hybridoma's

Following the 10 day immunisation, the mice were dissected and the enlarged popliteal lymph nodes were removed and disrupted to release cells. 8×10^7 lymph node cells were washed twice in PBS and mixed with 1×10^7 P3X63Ag8.653 myeloma cells (ECACC). 0.8ml 50% PEG 1500 (in 1mM NaOH and warmed to $37°C$) was added to the cells and incubated for 5 minutes. The cells were spun down at 300g for 10 minutes at $4°C$ and resuspended in 50ml RPMI medium, supplemented with $50 \mu g/ml$ penicillin/streptomycin (Gibco), 50mM mercaptoethanol and 1ml 50x hypoxanthine, aminopterin and thymidine (HAT) solution (Gibco). The cells were transferred to 96-well plates in $150 \mu l$ aliquots and incubated at $37°C$ in 5% CO_2 for 2 weeks, with one change of medium after 5 days. HAT-selected hybridoma's were grown in RPMI medium, supplemented with 10% foetal calf serum, $50 \mu g/ml$ penicillin/streptomycin (Gibco), 50mM mercaptoethanol and 1ml 50x hypoxanthine and thymidine (HT) solution (Gibco) at $37°C$ in 5% CO_2.

4.2.2.4 Screening of Hybridoma-Derived mAb's on *Xenopus* Splenocytes

Hybridoma supernatants were screened by flow cytometry for their ability to bind splenocytes isolated from control and Tx *Xenopus*. Spleen lymphocytes were isolated from *Xenopus* as described in Section 2.2.3.2 and aliquots of 2×10^5 of these cells were transferred to separate wells in a 96-well plate (Becton Dickinson). 50μl hybridoma supernatant was applied to a well and the cells incubated on ice for 30 minutes. The cells were then spun down at 300g for 10 minutes at 4°C before removing the supernatant and resuspending the cells in 200μl FACS medium. This wash was repeated twice before resuspending the cells in 50μl FITC-conjugated rabbit anti-mouse Ig antibody (DAKO), which had been adsorbed on *Xenopus* serum and diluted 1:20, and incubated on ice in the dark for 30 minutes. The cells were washed three times in 200μl FACS buffer before resuspending in 500μl FACS buffer and analysing on the flow cytometer. As in previous FACS analysis, a sample of cells incubated with FITC-conjugated rabbit anti-mouse Ig antibody only was used to set the fluorescence background marker to 2%. Hybridoma's of interest were cloned by limiting dilution and re-tested by flow cytometry.

4.2.3 Flow Cytometric Analysis of Hybridoma-Derived mAb's on *Xenopus* Cells

4.2.3.1 Preparation of Lymphoid Cells from *Xenopus* Spleen, Liver and Gut

The protocol for preparing lymphoid cells from *Xenopus* spleen and liver are described in Sections 2.2.3.2 and 3.2.4.5 respectively. Preparations of intra-epithelial lymphocytes (IEL) from *Xenopus* gut were carried out according to a protocol devised by Rebecca Stewart in our laboratory which is adapted from a method for isolating IEL from mice (Mosley and Klein, 1992). Animals were anaesthetised as described in Section 2.2.3.2 and the small and large intestines removed and cut longitudinally before placing in a 15ml Falcon centrifuge tube containing 5ml APBS. The tube was inverted several times to loosen any faecal matter and mucus before allowing the intestinal matter to settle. The supernatant was removed and replaced with 5ml APBS and the process repeated twice before transferring the gut tissue to a siliconised conical flask containing 30ml APBS supplemented with 0.1mM EDTA and 0.1mM DTT. A magnetic stirrer was used to agitate the suspension during a 1 hour incubation at RT. Following incubation, the supernatant, containing both lymphoid and epithelial cells, was collected in a 50ml Falcon tube and placed on ice while the gut tissue was resuspended in 30ml APBS and agitated for a further 30 minutes at RT. The second

supernatant was collected and combined with the first before spinning down the cells at 300g for 10 minutes at 4°C and resuspending in 6ml APBS. The cells were then passed through a nylon wool column, which had been made up and sterilised inside a 10ml plastic syringe (Becton Dickinson). The column was hydrated by adding 5ml APBS, before adding the gut cell suspension and collecting the first eluate. A further 10ml APBS was added and the second eluate collected and combined with the first. The purified cells were then layered onto 3ml Ficoll in a 15ml Falcon tube and centrifuged at 250g for 5 minutes. The lymphocytes were removed from a tight band above the Ficoll, washed, counted and incubated in a 5% CO_2 atmosphere as described in Section 2.2.3.2.

4.2.3.2 Single Stain FACS Analysis

Single stain flow cytometric analysis of cells from *Xenopus* spleen, liver and gut was carried out using hybridoma supernatants exactly as described in Section 4.2.2.4.

4.2.3.3 Immunomagnetic Separation of NK Cells from Tx Spleen

Splenic lymphocytes were isolated from Tx animals, as described in Section 2.2.3.2., adjusted to 5×10^6 and incubated with 500µl of the appropriate hybridoma supernatant on ice for 30 minutes. Following 3 washes with FACS medium, 5×10^6 cells in 80µl HBSS/1% FCS were added to 20µl of MACS microbeads conjugated with goat anti-mouse IgG (Miltenyi Biotec). The cells and beads were incubated for 15 minutes at 4°C, and the mixture passed over a prewashed Mini-MACS separation column placed in a separation unit (magnet). The non-adherent splenocytes eluted from the column were collected before removal of the column from the magnetic separation unit to allow collection of the adherent population. Both non-adherent and adherent populations were cultured overnight in tumour medium (As in Section 2.2.3.2) before FACS analysis.

4.2.3.4 Dual Stain FACS Analysis of Immunomagnetically-Separated NK cells

For dual stain analysis, the immunomagnetically-separated NK cells were first labelled with hybridoma supernatant and rabbit anti-mouse Ig FITC antibody (DAKO) as described above. The cells were washed three times in FACS buffer containing 1:100 filter-sterilised normal mouse serum before incubating in the dark on ice for 20 minutes with 50µl PE-conjugated D12, which is a putative marker of γδ T cells in

Xenopus (Ibrahim *et al.*, 1991). Three further washes in FACS buffer were carried out before resuspending the cells in 500μl FACS buffer and analysing on the flow cytometer. Controls for FITC and PE fluorescence were used to set the background markers at 2%, as described in Section 3.2.4.7.

4.2.4 Chromium Release Assays to Test the Cytotoxicity of Immunomagnetically Purified Cells from Tx Spleen

The chromium release assays described in this Section were carried out by Trudy Horton at Durham University. B_3B_7 tumour targets (1×10^6 cells/ml) were incubated overnight, as described in Section 2.2.3.1, in tumour medium supplemented with 100μCi/ml $Na_2[^{51}Cr]O_4$ (Amersham). Following 3 washes in tumour medium, the radiolabelled target cells were incubated for 30 minutes to remove initial spontaneous release of ^{51}Cr, then adjusted to 5×10^5 cells/ml prior to assay. Immunomagnetically sorted populations of Tx spleen cells (described in Section 4.2.3.3), which had been cultured in 25% growth factor rich medium (GFM) derived from concanavalin A-stimulated *Xenopus* splenocytes, were serially diluted in wells of a 96-well plate. To each well was added 100μl ^{51}Cr-labelled targets (5×10^4 cells) and the plates were incubated for 6 hours at 27°C in 5% CO_2. Following incubation, the cells were pelleted by spinning the 96-well plate at 300g for 10 minutes and 100μl supernatant removed and added to 3ml Ecosint scintillation solution (National Diagnostics). Radioactivity was detected in each sample using a Packard Tri-Carb analyser. The percentage of specific ^{51}Cr release was calculated using the formula (E-C/M-C x 100), where E=cpm ^{51}Cr for experimental supernatant, C=cpm for minimum control (ie spontaneous release using 100μl target cells cultured in 100μl medium alone) and M=cpm for maximum control (ie using 100μl target cells in 100μl water, freeze-thawed 3 times).

4.2.5 Flow Cytometric Analysis of Hybridoma-Derived mAb's on Human Peripheral Blood Lymphocytes

The FACS analysis of human PBL described in this section was performed in collaboration with Dr Marco Colonna at the Basel Institute for Immunology in Switzerland. 3ml peripheral blood from a healthy donor was layered onto 3ml density 1.077 Ficoll (Nycomed) and centrifuged at 600g for 20 minutes at 4°C. The lymphocytes were removed from a band above the Ficoll and washed twice in PBS. For dual stain FACS analysis, aliquots of 3×10^5 human PBL were probed with the anti-

Xenopus NK cell mAb 1F8 (detected with a FITC antibody) before the addition of PE-conjugated mAb's to the human cell surface markers CD3, CD4, CD8, CD14, CD16, CD19 or CD56 (Becton-Dickinson). As a control, an aliquot of cells was incubated with FITC-conjugated mouse IgG (Becton-Dickinson), followed by PE-conjugated mouse IgG (Becton-Dickinson) and used to set the background fluorescence parameters on the flow cytometer. Aliquots of 3×10^5 PBL were transferred to individual wells of a 96-well plate and spun down at 300g for 5 minutes at 4°C to pellet the cells before discarding the supernatant. 50µl neat 1F8 hybridoma supernatant was added to each cell pellet and incubated on ice for 30 minutes. The cells were then washed 3 times in PBS before the addition of 50µl FITC conjugated anti-mouse IgG (Becton-Dickinson) and incubation on ice in the dark for 30 minutes. The cells were washed 3 times in PBS before resuspending in 20µl 30% normal mouse serum and incubating on ice for 20 minutes. 1µl of PE-conjugated anti-human CD marker mAb (Becton-Dickinson) was added to the cells and incubated in the dark for 30 minutes on ice before 2 final washes. The cells were resuspended in 500µl PBS and analysed by flow cytometry.

4.2.6 Western Blots of *Xenopus* Cell Lysates

Xenopus lymphoid cells were prepared from spleen, liver and gut of both control and Tx animals as described above. 1×10^7 cells were resuspended in 100µl lysis buffer (1%NP-40, 150mM NaCl, 50mM Tris HCl (pH8.0), 1mM $MgCl_2$, 1mM phenylmethane sulphonyl fluoride (PMSF), 1µg/ml leupeptin and 1µg/ml pepstatin A). The cells were lysed on ice for 30 minutes, then spun at 15000rpm for 3 minutes to remove insoluble material. 20µl aliquots of the supernatant were added to 4µl protein sample loading buffer and the samples subjected to SDS-PAGE and transferred to nitrocellulose (as in Section 2.2.1.4). Nitrocellulose filters were blocked in 5% milk, 0.02% Tween 20 in TBS (pH7.5) using the same conditions as those described in Section 2.2.4.3. 20ml primary antibody (hybridoma supernatant diluted 1:2 in blocking buffer) was added to the nitrocellulose and incubated for 2 hours at RT with gentle rocking. The filter was then washed twice in blocking buffer before incubating for 2 hours at RT in HRP-conjugated goat anti-mouse IgG antibody (diluted 1:20000 in 20ml blocking buffer). Chemiluminescent detection of bands was carried out exactly as described in Section 2.2.4.3.

4.2.7 Immunoprecipitation Reactions Performed on *Xenopus* Cell Lysates

The immunoprecipitation reactions described in this Section were performed in collaboration with Dr Louis Du Pasquier at the Basel Institute for Immunology in Switzerland.

4.2.7.1 Metabolic Labelling of *Xenopus* Cells

Xenopus lymphoid cells were prepared from spleen and gut of both control and Tx animals as described above. Cysteine/methionine-free RPMI medium (Gibco) was made isotonic to frog cells by adding 30% distilled water and supplemented with 10% foetal calf serum and 100μg/ml kanamycin. *Xenopus* cells were resuspended in 4ml of this RPMI medium (6 million cells/ml) in a 50ml Falcon tube and incubated at 27°C in 5% CO_2 for 20 minutes to 'starve' the cells. Following the 20 minute incubation, ^{35}S methionine (Amersham) was added to a final concentration of 0.2mCi/ml and the cells incubated at 27°C in 5% CO_2 overnight, with the lid loosened to allow diffusion of CO_2. Following overnight incubation, the cells were washed twice in 45ml APBS to remove unincorporated ^{35}S methionine.

4.2.7.2 Immunoprecipitation

The metabolically labelled cells were resuspended in 6ml lysis buffer (1%NP-40, 150mM NaCl, 50mM Tris HCl (pH8.0), 1mM $MgCl_2$, 1mM phenylmethane sulphonyl fluoride (PMSF), 1μg/ml leupeptin and 1μg/ml pepstatin A) and incubated on ice for 30 minutes. The lysate was pre-cleared by the addition of 200ul protein A sepharose beads (made up 1:1 (v/v) in 10mM Tris-HCl (pH7.5), 150mM NaCl, 0.2% NP-40, 2mM EDTA). Following incubation for 30 minutes at 4°C with rotation, the protein A beads were pelleted by centrifugation at 5000 rpm for 10 minutes at 4°C and the supernatant divided into 6 eppendorf tubes (equivalent to 4 million radiolabelled cells per tube).

200μl of 6 different hybridoma supernatants (including one negative and one positive control mAb) was added to each tube of pre-cleared lysate. The tubes were capped tightly and incubated for 1.5 hours at 4°C with rotation. After incubation, the tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was transferred to a new eppendorf tube.

The antigen:antibody complexes were captured by the addition of 50μl protein A sepharose beads (made up 1:1 (v/v) in 10mM Tris-HCl (pH7.5), 150mM NaCl, 0.2% NP-40, 2mM EDTA) followed by incubation, with rotation, for 1.5 hours at 4°C. The tubes were then centrifuged at 14,000 rpm for 3 minutes at 4°C to pellet the protein A beads and the supernatant was discarded, before resuspending the beads in 1ml 10mM Tris-HCl (pH7.5), 150mM NaCl, 0.2% NP-40, 2mM EDTA. The beads were washed 2 more times in this buffer before 2 washes in 1ml 10mM Tris-HCl (pH7.5), 500mM NaCl, 0.2% NP-40, 2mM EDTA followed by 2 washes in 1ml 10mM Tris-HCl (pH7.5).

4.2.7.3 SDS-PAGE and Detection of Radiolabelled Proteins

Following the washes, the protein A beads were resuspended in 20ul 1x protein loading buffer and boiled for 5 minutes, followed by centrifugation for 1 minute at 14,000 rpm. The samples were separated by SDS-PAGE (As in Section 2.2.1.4) and the gel fixed in 10% acetic acid, 45% methanol for 30 minutes and then equilibrated with distilled water for 5 minutes. The gel was placed on a piece of 3MM paper (Whatman), wetted with distilled water and covered in cling film prior to vacuum drying in a Bio-Rad Slab Dryer for 2 hours. The dried gel was placed in a film cassette and, in a dark room, a sheet of X-ray film (Fujifilm) placed on top. The closed film cassette was incubated at -70°C overnight before development of the film using a Compact X4 X-ray film processor (X-Ograph). If necessary, a second sheet of X-ray film was exposed to the gel and incubated at -70°C for longer before developing in the same way.

4.3 Results

4.3.1 Flow Cytometric Investigation of mAb's to *Xenopus* NK Cells

4.3.1.1 Flow Cytometric Screening of Hybridoma-Derived mAb's on Control and Tx *Xenopus* Spleen

Following the production of hybridoma's from the mice immunised with *Xenopus* NK-enriched cells, hybridoma supernatants were screened by flow cytometry for their ability to bind both control and Tx splenocytes (As described in Section 4.2.2.4). The histograms in Figure 4.1 show the results for 3 mAbs, designated 1F8, 1G5 and 4D4,

on control and Tx spleen. All three bound between 5% and 7% of control splenocytes and 17% of Tx splenocytes. These figures were consistent with the expected levels of NK cells in the spleen of control and Tx animals.

4.3.1.2 Flow Cytometric Screening of mAb 1F8 on Different Lymphoid Organs from Control and Tx *Xenopus*

Monoclonal antibody 1F8, having been identified as a potential marker of *Xenopus* NK cells was tested by flow cytometry on lymphoid cells prepared from the spleen, liver and gut IEL of control and Tx *Xenopus*. The results in Figure 4.2 show that the levels of 1F8 positive cells in control spleen, liver and gut were respectively 5%, 11% and 25%. In Tx animals, these percentages were more than doubled, as the numbers in spleen, liver and gut were 14%, 30% and 55% respectively.

4.3.1.3 Immunomagnetic Cell Sorting with mAb 1F8 and Dual Stain FACS Analysis

Tx spleen cells were immunomagnetically sorted into 1F8-adherent and non-adherent populations (As described in Section 4.2.3.3). The adherent population were confirmed as being 83% 1F8-positive by FACS analysis and the non-adherent population were shown to be only 5% 1F8-positive (See Figure 4.3). The adherent population were used in a dual stain with 1F8 and the PE-conjugated mAb D12, a putative marker of *Xenopus* γδ T cells (Ibrahim *et al.*, 1991), which demonstrated that D12 labelled around 90% of 1F8-positive cells.

4.3.2 Cytotoxicity Assays Using Immunomagnetically Sorted Cell Populations from Control and Tx Spleen

1F8-adherent and non-adherent populations of cells were prepared from control and Tx spleen, as described in Section 4.2.3.3. These populations, as well as control and Tx spleen samples which had not undergone immunomagnetic separation, were incubated in growth factor rich medium prior to use in chromium release assays to test their cytotoxicity towards the *Xenopus* tumour cell line B_3B_7 (See Section 4.2.4). The graphs in Figure 4.4, which show the results of repeated experiments, clearly demonstrate that 1F8-adherent cells are more effective killers than either the unseparated or 1F8 non-adherent populations. 30% killing of the tumour cells by 1F8-positive cells was possible with an effector:target ratio as low as 3:1. Significantly, the

1F8-negative cells showed very low levels of cytotoxicity and unseparated cells displayed an intermediate level.

4.3.3 Dual Stain FACS Analysis of Human PBL using mAb 1F8

Having verified that mAb 1F8 is a marker of NK cells in *Xenopus*, flow cytometric analysis was used to test the ability of 1F8 to bind human PBL. 1F8 staining was detected using a FITC-conjugated anti-mouse IgG antibody before co-staining the cells with various PE-conjugated mAb's to human CD markers (As described in Section 4.2.5). The results shown in Figure 4.5 demonstrate that mAb 1F8 labelled a subset of human PBL but did not co-stain with the pan T cell marker CD3, or the helper and cytotoxic subsets of T cells identified by the anti-CD4 and anti-CD8 mAb's. Nor did the 1F8-labelled cells co-express CD56, an NK-specific marker, CD16, which labels NK cells, neutrophils and macrophages, or CD14, which is specific for monocytes. However, the pan B cell marker CD19 did co-stain with mAb 1F8.

4.3.4 Western Blots of *Xenopus* Lymphoid Lysates Probed with Anti-NK mAb's

4.3.4.1 A Comparison of mAbs 1F8, 4D4, 1G5 and D12 in Western Blots of Tx *Xenopus* Spleen and Gut Lysates

Western blots of cell lysates from Tx spleen and gut were probed with mAb's 1F8 (IgG2b), 4D4 (IgG2a), 1G5 (IgG2a) and D12 (IgG1), as described in Section 4.2.6. The concentrations of these four mAb's were estimated to be equal from FACS analysis (T.L.Horton, pers. comm.).Figure 4.6 shows that mAb's 1F8 and 4D4 failed to identify any protein bands in the spleen, whereas 1G5, and to a lesser extent D12, both reacted with a doublet at 72 to 74 kDa and a larger band at around 84 kDa. In the gut, all the mAb's detected a doublet at 72 to 74 kDa, with 1F8 and 4D4 showing the weakest staining, D12 slightly stronger staining and 1G5 very strong staining. Tx spleen and gut lysates showed no background staining when probed with secondary antibody alone.

4.3.4.2 Western Blots of *Xenopus* Lymphoid Lysates Probed with mAb 1G5

Cell lysates from control and Tx animals were separated by SDS-PAGE, blotted to nitrocellulose and probed with mAb 1G5, as described in Section 4.2.6. Figure 4.7 shows that probing Western blots of spleen and gut lysates from control *Xenopus* with mAb 1G5 identified a specific doublet of protein bands at 72 to 74 kDa, with much

heavier staining in gut than spleen. The control spleen also exhibited staining of a larger protein band of ~84 kDa. In Tx animals, the doublet at 72 to 74 kDa was more strongly stained than in control animals and was more heavily stained in the gut than in spleen or liver. Both Tx liver and Tx spleen lysates, when probed with 1G5, identified an additional protein band of around 84 kDa. Spleen, liver and gut lysates showed no background staining when probed with secondary antibody alone. The probing of lysates of the tumour cell line B₃B₇ with mAb 1G5 identified no protein bands, whereas probing with XT-1 identified bands at 120 to 130 kDa, corresponding to the XTLA-1 antigen (Nagata, 1986).

4.3.5 Immunoprecipitation Reactions Performed on *Xenopus* Spleen and Gut Cell Lysates with mAb's 1F8, 4D4 and 1G5

Following preparation of lymphoid cells from the spleen and gut of control *Xenopus*, immunoprecipitation reactions were performed with mAb's 1F8, 4D4 and 1G5, as described in Section 4.2.7. Positive control samples immunoprecipitated with mAb 10A9, which identifies IgM heavy (75-80 kDa) and light (25 kDa) chains and mAb TB17, which identifies MHC class I (40-45 kDa) were included for comparison. Also included was a negative control using mAb Gabi, which identifies a 55 kDa molecule called CTX (Chrétien *et al.*, 1996) but which is not expressed in spleen or gut (See Appendix 3 for further details of these mAb's).

The spleen immunoprecipitation results (Figure 4.8) show that bands corresponding to MHC class I (40-45 kDa) and IgM heavy chain (75-80kDa) were isolated using mAb's TB17 and 10A9 respectively. The CTX-specific mAb Gabi shows no specific bands, but mAb's 4D4, 1G5 and 1F8 possibly identify specific protein bands at 55 to 60 kDa. The gut immunoprecipitation reactions with mAb's 4D4, 1G5 and 1F8 show more convincing identification of specific protein bands. As in the spleen, mAb's 4D4 and 1G5 appear to precipitate a protein of ~55 kDa, whereas 1F8 identifies a slightly larger protein of ~60 kDa. The positive control mAb 10A9 appears to precipitate the IgM heavy chain, while the negative control mAb Gabi shows no specific protein bands.

4.4 Discussion

4.4.1 The Overlapping Functions of NK and T cells in *Xenopus laevis*

In the past, the study of NK cells in *Xenopus* has been hindered by the lack of mAb's which specifically bind to NK cell surface antigens. As such, it has proved difficult to

separate the cytotoxic effects of NK cells from those of T cells in control animals. In fact it has been shown, using immunomagnetic separation of control splenocytes to isolate CD5+,CD8+ T cells, CD5+,CD8- (putative CD4+) T cells and CD5-,CD8- non-T cells, that all three *in-vitro* cultured populations kill the tumour target B_3B_7 (Horton *et al.*, 1998b). *In-vivo* studies in *Xenopus*, using transplanted MHC-matched tumour cells, also suggest an anti-tumour role for T cells. These experiments have demonstrated rejection of the tumour cells by adults but not by tadpoles and furthermore that the tumour rejection was abrogated by thymectomy, underlining the importance of T cells (Robert *et al.*, 1995; Robert *et al.*, 1997).

Work investigating tumour antigens on *Xenopus* tumour cell lines has highlighted the possible importance of a heat shock protein called gp96 (Robert and Cohen, 1998). This protein is expressed on the surface of all known *Xenopus* tumour cell lines and when isolated from the 15/0 tumour cell line has been used successfully to immunise against challenge with 15/0 cells, whereas gp96 isolated from non-tumour cells was unable to elicit the same anti-tumour response (Robert and Cohen, 1998). These results suggest a possible role for heat shock proteins in antigen presentation and/or delivery of danger signals to the immune system. The MHC-deficient phenotype of 15/0 cells is suggestive of NK cell involvement in this anti-tumour activity but the involvement of T cells is not ruled out (Robert and Cohen, 1998).

4.4.2 Identification of Three Monoclonal Antibodies to *Xenopus* NK cells

Given the close functional and developmental relationship of T cells and NK cells, the use of Tx animals has been crucial in identifying the putative NK-like population in *Xenopus* (Horton *et al.*, 1996). As such, the attempts made in this Chapter to raise NK-specific mAb's through hybridoma technology used Tx animals as a source of NK-like cells for the immunisation of mice. Furthermore, it was the ability of three mAb's, namely 1F8, 4D4 and 1G5, to label a greater number of spleen cells in Tx than control animals which facilitated their identification as putative markers of *Xenopus* NK cells.

Having identified the three potentially NK-specific antibodies, mAb 1F8 was used in FACS analysis to determine the levels of putative NK cells in control and Tx liver and gut IEL. As with the results in the spleen, the percentage of positively stained cells was higher in Tx than control animals for both liver and gut cells which eradicated the possibility that 1F8 was binding to an antigen on thymus-derived T cells. The levels of 1F8-positive staining in control spleen and liver, which were around 5% and 10% respectively, are fairly comparable with the percentages of NK cells found in those

organs in mammals. The approximately 25% of control gut IEL which labelled with 1F8, however, is considerably higher than the frequency of NK cells in human and mouse intestine, but is more comparable with the levels of NK cells in the intestine of the chicken, which can constitute 50% of IEL (Göbel *et al.*, 1994).

1F8 was used to immunomagnetically isolate the putative NK cell population from Tx spleen which consequently allowed these cells to be used in cytotoxicity assays using the allogeneic thymus tumour cell line B_3B_7 as a target. The ability of 1F8-positive cells to lyse B_3B_7 tumour cells far more efficiently than 1F8-negative cells confirmed this mAb as a marker of *Xenopus* NK cells. Further confirmation of the ability of 1F8 to label *Xenopus* NK cells has been achieved through FACS analysis of 1F8 on cultured cells from both control and Tx animals and the demonstration that 1F8 is not expressed on T or B cells (Horton *et al.*, manuscript submitted).

FACS analysis of immunomagnetically-purified 1F8-positive cells was used to show that the antigen bound by mAb D12 was present on the majority of 1F8-positive cells. The D12 mAb is of interest because it was originally thought to bind to a 36 kDa receptor on *Xenopus* γδ T cells (Ibrahim *et al.*, 1991). The FACS data shown here indicate that D12 is also capable of binding to *Xenopus* NK cells.

The flow cytometric binding studies of mAb 1F8 on human PBL's revealed an interesting anomaly. Whilst it was unable to co-stain human NK or T cells, it appeared that 1F8 did bind to human B cells. Any non-specific binding of 1F8 antibody by Fc receptors on human B cells was deemed unlikely as the PE and FITC-conjugated control mouse IgG antibodies did not show any such binding. Therefore, it appears that there are two possibilities to explain why a mAb which is specific for *Xenopus* NK cells should bind to human B cells. Firstly it is conceivable that 1F8 could, by chance, cross react in humans with a B cell-specific antigen which is entirely unrelated to the original NK-specific antigen in *Xenopus*. Alternatively, it is possible that 1F8 binds to the same antigen in both *Xenopus* and humans and that the antigen is expressed on different cell types in the two species. Further FACS analysis of lymphoid cells from a variety of other species may shed some light on the antigen specificity of 1F8 but the only definitive answer would come from the molecular characterisation of both the *Xenopus* and human 1F8 antigens.

4.4.3 Investigation of the Antigens bound by the Anti-*Xenopus* NK mAb's

The data collected from Western blotting experiments using *Xenopus* lymphoid lysates probed with mAb 1G5 suggested a convincing ability of this mAb to specifically bind two proteins at 72 and 74 kDa. These proteins were found in both control and, at a higher level, in Tx lymphoid lysates and their expression was greatest in gut IEL, followed by liver and then spleen. These levels of expression appear to correlate well with the percentages of NK cells identified by FACS analysis of these organs as the relative numbers of NK cells are highest in the gut, followed by liver and then spleen. 1G5 also identified a higher molecular weight protein band of around 84 kDa in spleen and liver and this variation may reflect alternative splicing of the encoding mRNA or a difference in glycosylation of this protein when expressed on NK cells in these organs.

1F8, 4D4 and D12 were also used to probe Western blots of *Xenopus* lymphoid lysates and were all able to bind the two proteins at 72 and 74 kDa in Tx gut. Of the three mAb's, D12 reacted most strongly to gut lysates and was also able to identify the 72, 74 and 84 kDa bands in Tx spleen whereas 1F8 and 4D4 staining was weak in Tx gut and undetectable in Tx spleen. The evidence from these Western blots seems to suggest that all four mAb's are capable of binding to the same 72 and 74 kDa antigens expressed in Tx gut whereas only mAb's 1G5 and D12 can detect the 72, 74 and 84 kDa bands, which are expressed at lower levels in spleen and liver. The D12 Western blot results reported here disagree with the previously published 36 kDa size of the D12 antigen which was determined by immunoprecipitation (Ibrahim et al., 1991).

The immunoprecipitations carried out with mAb's 1F8, 4D4 and 1G5 on radiolabelled spleen and gut lymphoid lysates from control *Xenopus* do not appear to correlate with the Western blotting results. While 4D4 and 1G5 appear to precipitate a protein of around 55kDa from spleen and gut, 1F8 precipitates a protein of around 60 kDa from spleen and gut. However, it should be noted that these preliminary immunoprecipitation reactions showed a high level of background and all the mAb's appeared to precipitate proteins non-specifically at around 55 kDa in the gut. As such, it is concluded that the Western blotting results, especially with mAb 1G5, which were repeated on more than ten occasions, currently offer a more reliable identification of the NK surface antigen. Further work is required to obtain more convincing results from immunoprecipitation reactions with these mAb's.

Figure 4.1   Identification of Putative anti-NK mAb's 1F8, 1G5 and 4D4 Through Screening on Control and Thymectomised *Xenopus* Splenocytes

Flow cytometric traces comparing the ability of three mAb's, 1F8, 4D4 and 1G5, to bind control and Tx *Xenopus* splenocytes. Bound mAb's were detected using a FITC-conjugated anti-mouse Ig antibody.
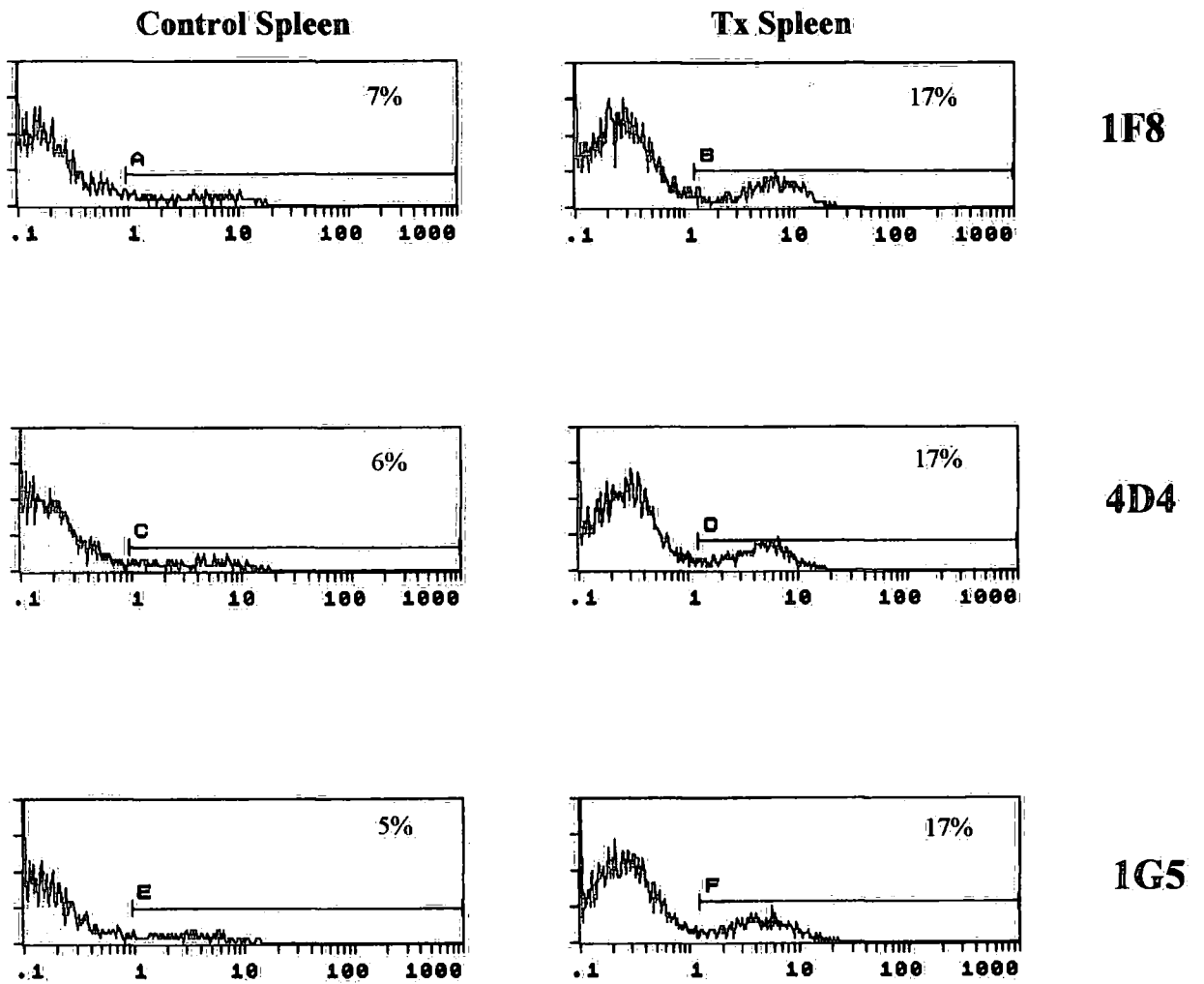
Figure 4.2   Flow Cytometric Testing of mAb 1F8 on Lymphoid Cells from Various Organs of Control and Tx *Xenopus*

Flow cytometric traces are shown which demonstrate the ability of mAb 1F8 to bind spleen, liver and gut lymphoid cells from both control and Tx *Xenopus*. As in the previous figure, the bound mAb was detected using a FITC-conjugated anti-mouse Ig antibody.
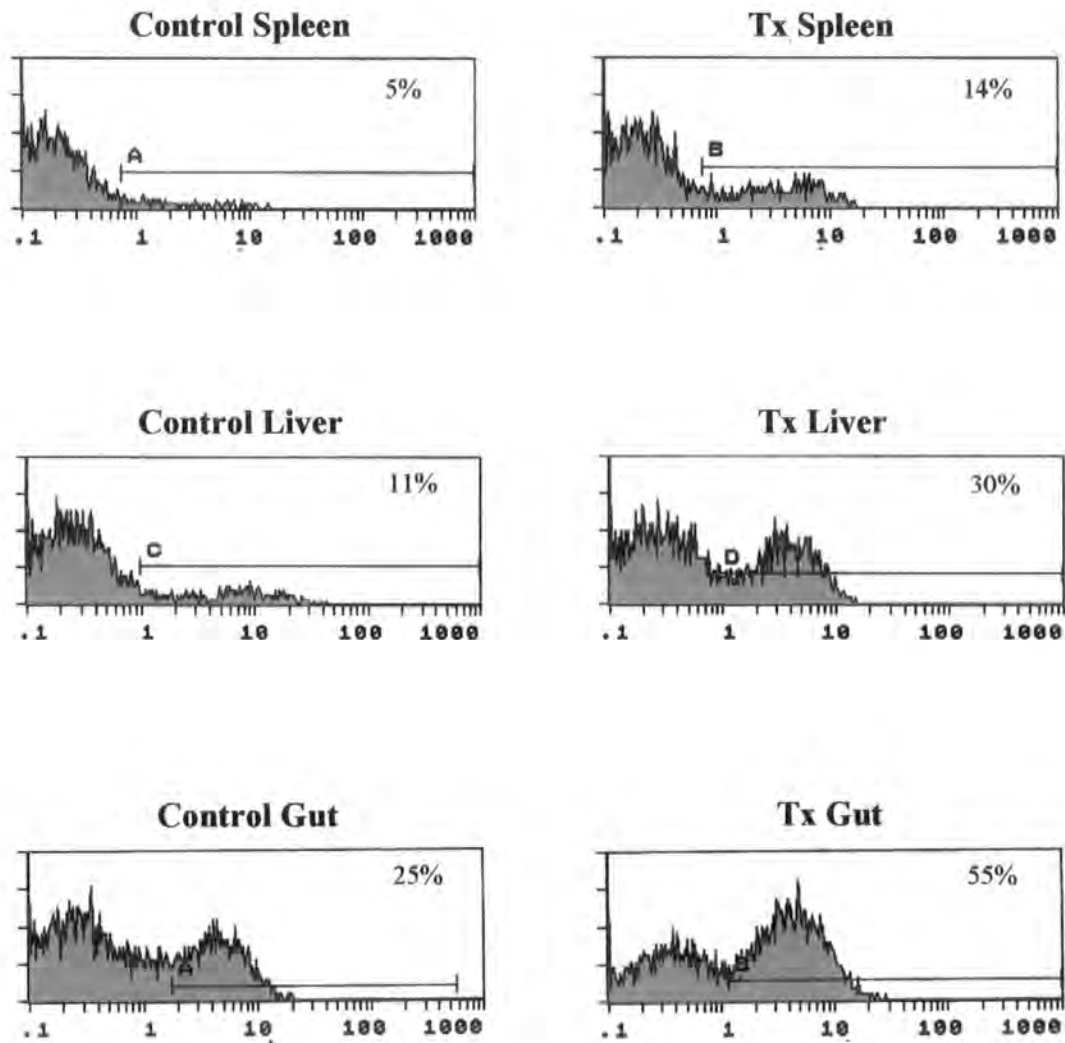
Figure 4.3   Immunomagnetic Separation of Cells from Tx Spleen with mAb 1F8 and Dual Stain Analysis of 1F8-Enriched Cells

The first trace (A) shows the binding of 1F8 to Tx spleen cells prior to immunomagnetic separation. Following separation of the splenocytes into 1F8 non-adherent and 1F8 adherent populations, the cells were re-tested by flow cytometry with mAb 1F8 (B and C). The 1F8-adherent cells were also used in a dual stain with 1F8 in conjunction with the PE-conjugated mAb D12 (D).

A. Total Tx Splenocytes

12%

A

.1   1   10   100   1000

B. 1F8 Non-Adherent Cells

5%

C

.1   1   10   100   1000

C. 1F8 Adherent Cells

83%

E

.1   1   10   100   1000

D. Dual Stain on 1F8 Adherent Cells

0%   85%

D12

10%

1F8
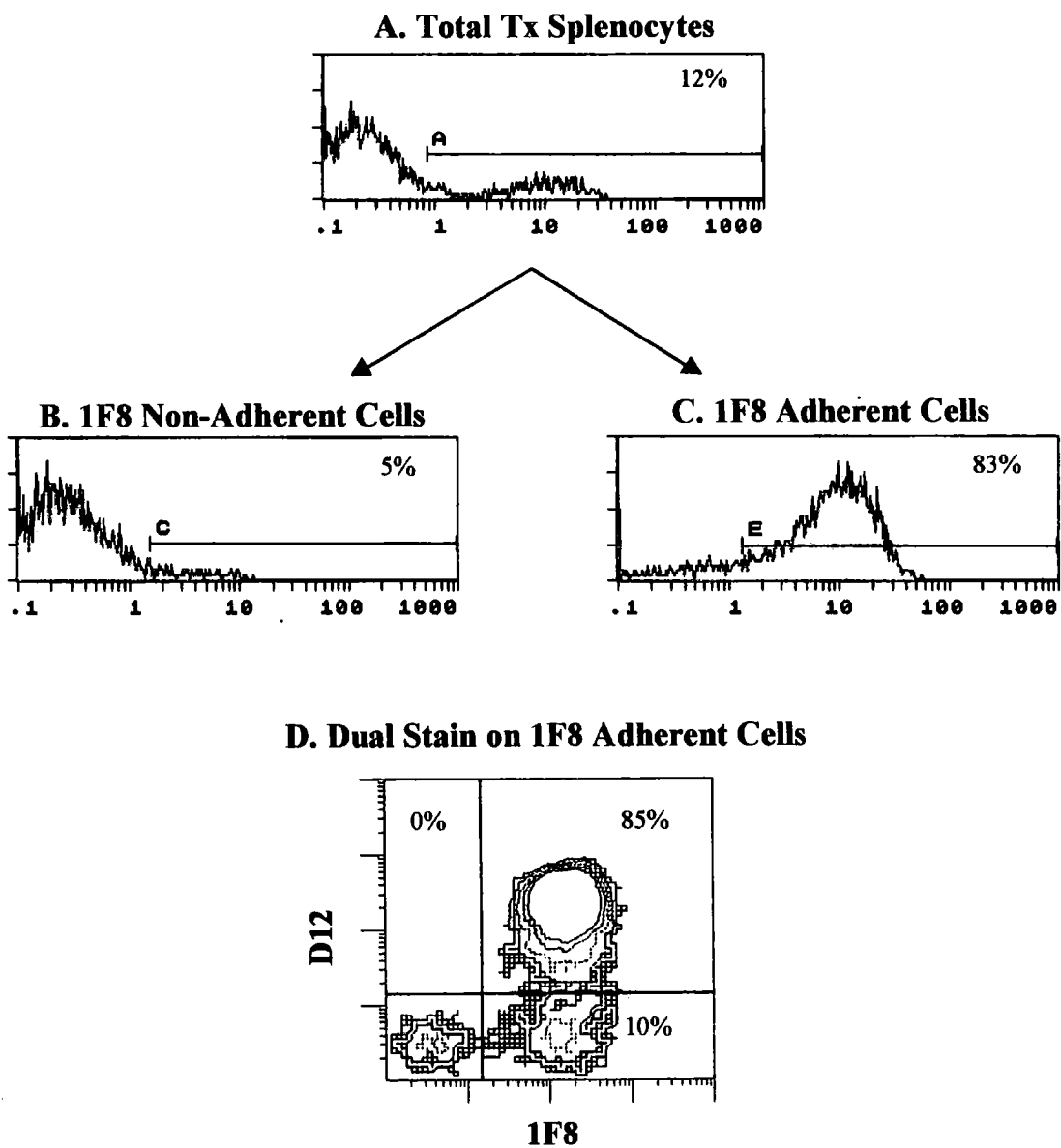
Figure 4.4 Cytotoxicity Assays to Test the Ability of 1F8-Adherent, 1F8-Non-Adherent and Unseparated Cells from Control and Tx Spleen to Kill the Tumour Target B_3B_7

Following immunomagnetic separation of spleen cells with mAb 1F8, the 1F8-adherent, non-adherent and unseparated populations were each tested for their ability to lyse B_3B_7 cells in chromium release assays. The percentage of B_3B_7 cells lysed is shown at different effector:target (E:T) ratios and the data (with mean ± standard errors) from three experiments each on control and Tx spleen are plotted.
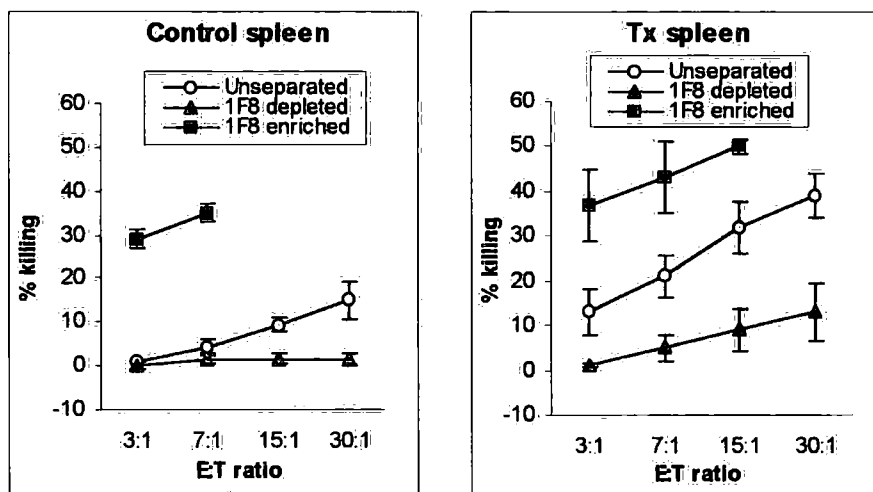
Figure 4.5   Dual Stain FACS Analysis of mAb 1F8 on Human PBL

Human PBL were probed with mAb 1F8 (detected with FITC-conjugated anti-mouse Ig) in conjunction with PE-conjugated mAb's to human CD3 (A), CD56 (B), CD4 (C), CD8 (D), CD16 (E), CD14 (F), CD19 (G), or no PE-mAb (H). Also included was a control sample incubated with mouse IgG-FITC and mouse IgG-PE (I). FITC-labelled cells are detected on the X-axis and PE-labelled cells on the Y-axis.
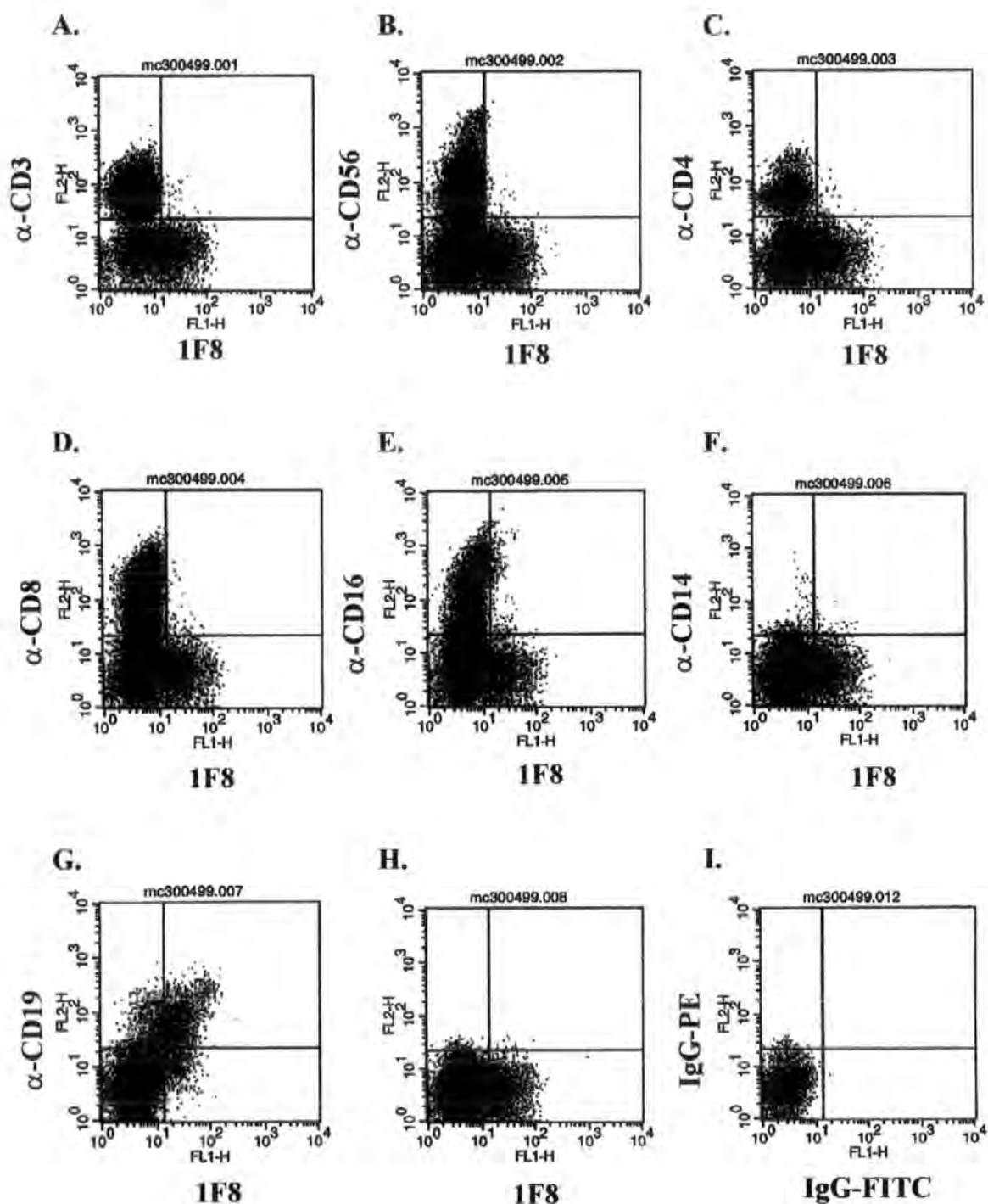
Figure 4.6   A Comparison of Western Blots of Tx Spleen and Gut Lysates Probed with mAb's 1F8, 4D4, 1G5 and D12

Western blots of spleen (S) and gut (G) lysates from Tx *Xenopus* probed with mAb's 1F8, 4D4, 1G5 and D12 and, as a control, with secondary antibody alone (2°). Molecular weight markers are shown for each blot.

Tx Spleen Lysates

Tx Gut Lysates
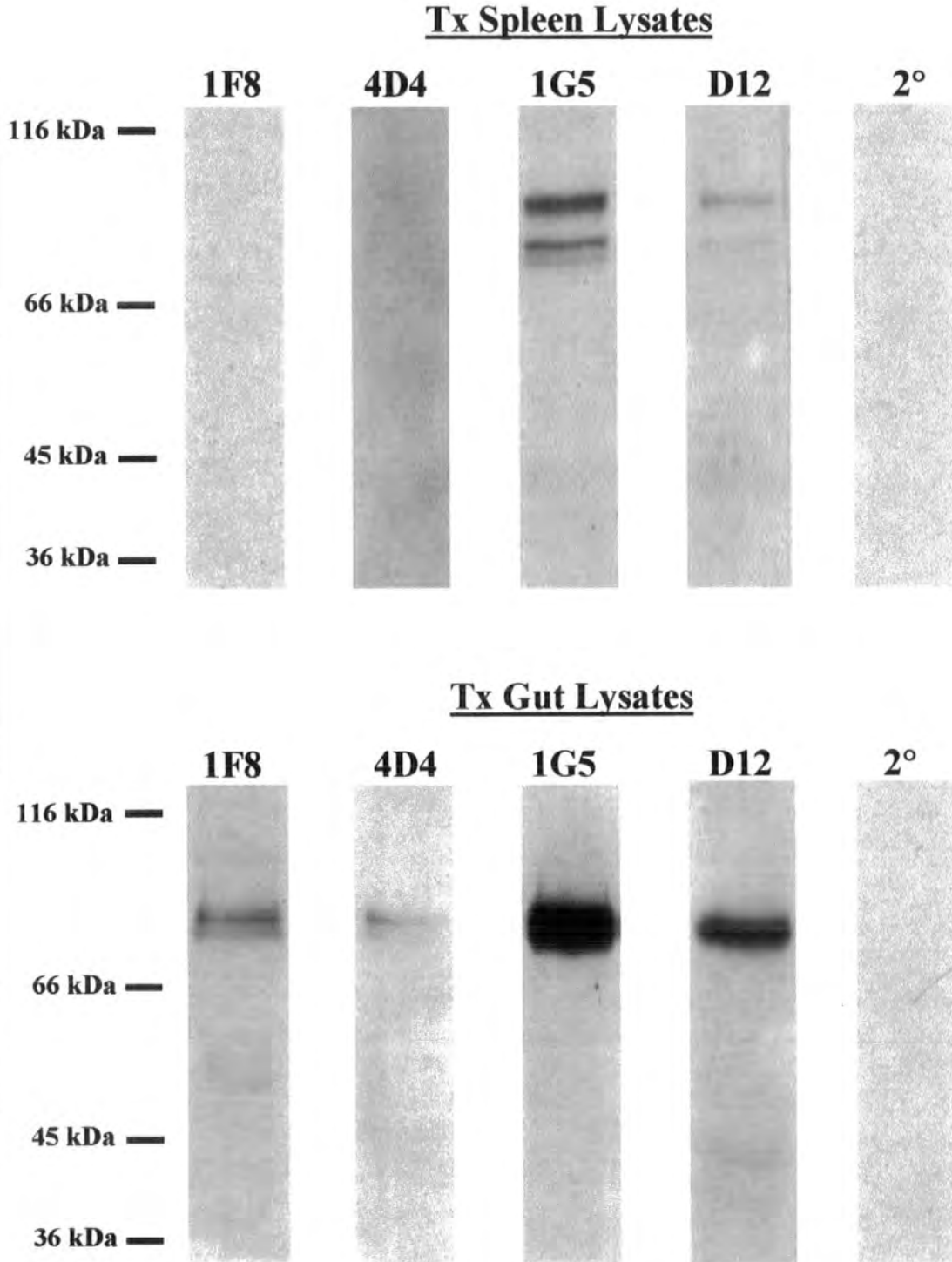
Figure 4.7   Western Blots of *Xenopus* Cell Lysates Probed with mAb 1G5

Western blots of spleen (S) and gut (G) lysates from control *Xenopus* and liver
(L), spleen (S) and gut (G) lysates from thymectomised *Xenopus* probed with
mAb 1G5 and, as a control, with secondary antibody alone (2°). B_3B_7 cell
lysates were probed with mAb's 1G5 and XT-1, which is known to react with a
protein of 120-130 kDa. Molecular weight markers are shown for each blot.
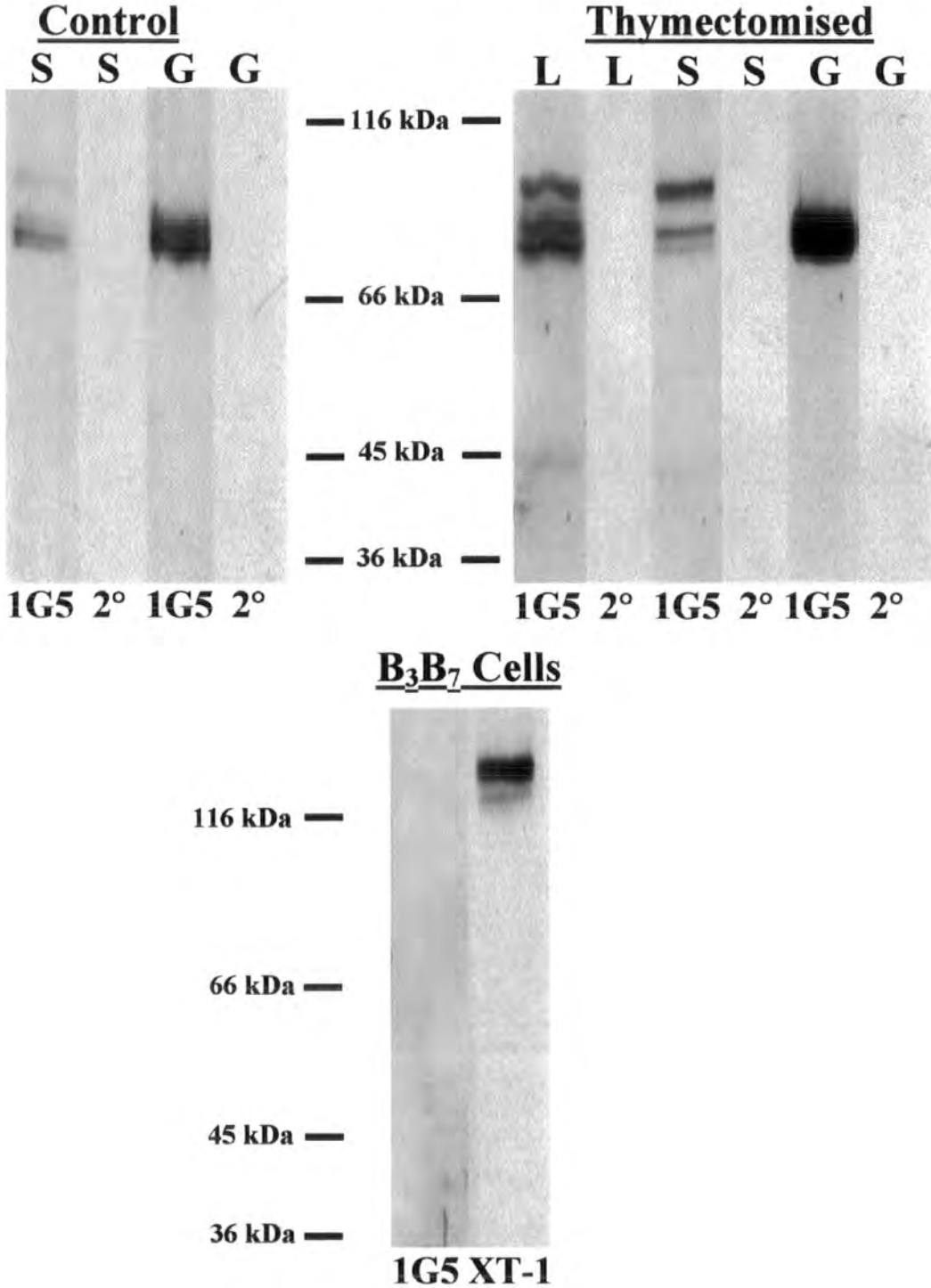
Figure 4.8 Immunoprecipitation Reactions Carried out on Cell Lysates from Control *Xenopus* Spleen and Gut

Autoradiograms showing proteins immunoprecipitated with mAbs 1F8, 4D4 and 1G5 following SDS-PAGE under reducing conditions (arrows indicate specific proteins). Positive control samples immunoprecipitated with mAb 10A9, which identifies IgM heavy (75-80 kDa) and light (25 kDa) chains and mAb TB17, which identifies MHC class I (40-45 kDa) are included for comparison. Also included is a negative control using mAb Gabi, which identifies a molecule called CTX (55kDa) which is not expressed in spleen or gut. Molecular weight markers and their sizes in kilodaltons are shown.

48 hour exposure   2 week exposure
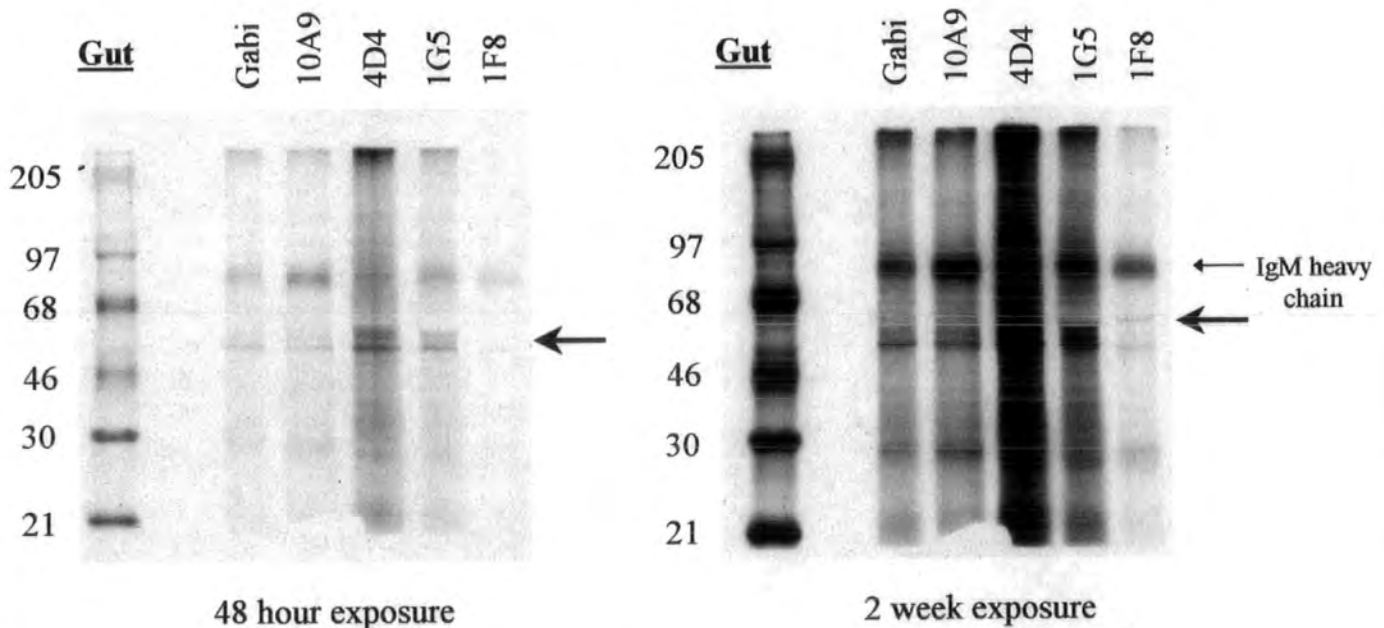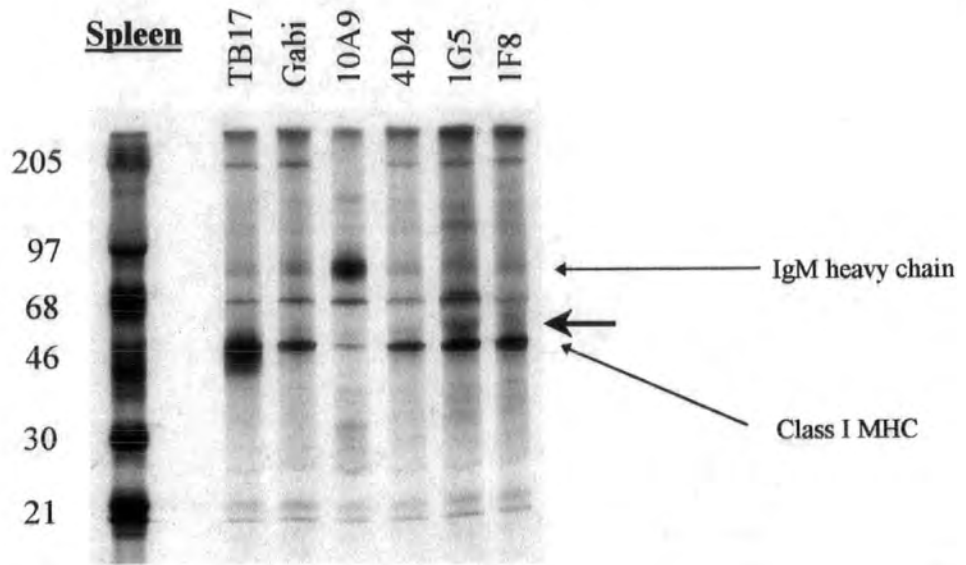
CHAPTER 5

Cloning Strategies for the Isolation of *Xenopus* NK Receptor Genes

Following on from the identification of NK cells in *Xenopus*, as described in Chapter 4, the principal aim of this Chapter was to isolate genes encoding *Xenopus* NK receptor proteins. Two strategies were used, the first being the screening of a Tx *Xenopus* gut cDNA expression library with one of the NK-specific mAb's described in Chapter 4. The second strategy was to design degenerate PCR primers to conserved sequences of mammalian NK receptors and use them to amplify DNA encoding NK receptors from *Xenopus*.

5.1 Introduction

5.1.1 The Benefit of Studying NK Receptor Evolution

The studies of mammalian NK receptors, reviewed in Chapter 1, have revealed a number of receptor families, some of which are not conserved from rodents to humans. This has made difficult the identification of receptors which are of fundamental importance to the function of all NK cells. It would therefore be useful to characterise NK receptors from more distantly related vertebrate species to find out which receptors are conserved through vertebrate evolution. The identification of antibodies, class I and II MHC and T cell receptors in lower vertebrates (reviewed in Horton and Ratcliffe, 1996) has underlined the fundamental importance of these molecules in the immune systems of all vertebrate species. The recent discovery of *bona fide* NK cells in *Xenopus laevis*, as described in Chapter 4, now offers an opportunity to study and characterise amphibian NK cell receptors.

5.1.2 The Use of mAb's to Isolate Specific cDNA's from Bacteriophage Lambda Expression Libraries

The mAb's described in Chapter 4, which identify *Xenopus* NK cells, offer a direct means of isolating cDNA's encoding *Xenopus* NK cell surface proteins. By isolating mRNA from *Xenopus* NK-enriched lymphocytes, reverse transcribing to cDNA and cloning into bacteriophage lambda vectors, it is possible to express a library of *Xenopus* cDNA's as proteins and probe these proteins with the NK-specific mAb's.

Since each cDNA-containing phage can be grown as an individual plaque on a lawn of bacteria, any which express proteins identified by the NK-specific mAb can be isolated and the cDNA sequence derived. One feature of the anti-*Xenopus* NK cell mAb 1G5 is that it reacts strongly with denatured proteins in Western blot experiments (See Figure 4.6), suggesting that it binds to a sequential, rather than a conformational, epitope. Therefore if the *Xenopus* cDNA's are not expressed in their native conformation, which is often the case with eukaryotic proteins expressed in bacterial systems (Sambrook et al., 1989), the polypeptide antigen should still be identified by this mAb. Furthermore, the Western blot reaction with mAb 1G5 was strongest in the gut IEL of Tx animals, suggesting that the level of antigen is elevated in this organ. As such, it was decided to clone a cDNA expression library from the gut IEL of a Tx animal and probe it with the mAb 1G5.

5.1.3 The Isolation of DNA Encoding *Xenopus* NK Receptor Proteins by PCR Amplification

Many proteins have conserved stretches of amino acids which are often inherently linked to their specific function. By identifying conserved motifs in the amino acid sequences of NK receptors in different species, it is possible to design PCR primers to specifically amplify DNA encoding these NK receptors. If the motifs are conserved enough through evolution, specific DNA sequences from highly divergent species, such as *Xenopus* and humans, can be amplified with the same PCR primers.

One of the difficulties of identifying targets for NK-specific PCR amplifications in *Xenopus* is that nearly all of the sequence information for NK receptor proteins has been isolated from mammalian species. So even if an amino acid sequence is conserved from rodent to human, there can be no certainty that it will also be conserved in lower vertebrate species, such as *Xenopus*. However, with more sequence information from non-mammalian species gradually becoming available, opportunities are beginning to arise for the PCR amplification of NK receptor transcripts from lower vertebrates such as *Xenopus*.

5.2 Materials and Methods

5.2.1 General Materials and Methods

All the general materials and methods used in this Chapter are described in Sections 2.2.1. and 3.3.1.

5.2.2 Isolation of mRNA from Tx *Xenopus* Gut IEL

IEL were prepared from the gut of a Tx animal (as described in Section 4.2.3.1) and total RNA was purified from these cells (as described in Section 2.2.2.4). mRNA was isolated from the total RNA using an Oligotex™ mRNA Miniprep Kit (Qiagen) which works by capturing the poly-A tail of mRNA molecules on Oligotex particles, which have poly-T oligonucleotides covalently linked to their surface.

Total RNA was resuspended in 500µl 10mM Tris-HCl (pH 7.5), 0.5M NaCl, 1mM EDTA, 0.1% SDS in an eppendorf tube and 15µl Oligotex particles (10% w/v in 10mM Tris HCl (pH 7.5), 0.5M NaCl, 1mM EDTA, 0.1% SDS) were added before heating for 3 minutes at 60°C to disrupt the secondary structure of the RNA. The tube was incubated at RT for 10 minutes to allow hybridisation of the mRNA to the oligo-dT on the Oligotex particles. The Oligotex particles were pelleted by centrifugation for 2 minutes at 15,000 rpm and the supernatant (containing non-messenger RNA) removed and saved. The pellet was washed by resuspending in 400µl 10mM Tris HCl (pH 7.5), 150mM NaCl, 1mM EDTA, pipetting onto a small spin column (Qiagen) and centrifuging for 1 minute at 15,000 rpm. The flow-through was discarded and the wash step repeated. 20µl elution buffer (5mM Tris HCl pH7.5), pre-heated to 70°C, was applied to the column before centrifugation at 15,000 rpm for 1 minute and collection of the flow through. This elution step was repeated and the eluates combined.

5.2.3 PCR Amplification of *Xenopus* β-actin DNA from purified mRNA

Two cDNA amplification reactions were performed, one using the purified mRNA as a template and the other using the RNA left behind following removal of the mRNA (non-mRNA). The following components were added to two nuclease-free eppendorf tubes:

mRNA Reaction		Non-mRNA Reaction	
5x First strand buffer (Gibco)	4µl	5x First strand buffer	4µl
40 units RNase inhibitor (Promega)	1µl	40 units RNase inhibitor	1µl
10mM dNTP's (Gibco)	2µl	10mM dNTP's	2µl
100µM Oligo dT primer (Gibco)	1µl	100µM Oligo dT primer	1µl
0.1M DTT (Gibco)	2µl	0.1M DTT	2µl

200 units Superscript II Reverse		200 units Superscript II Reverse	
Transcriptase (Gibco)	1µl	Transcriptase	1µl
Nuclease-free water (Promega)	8µl	Nuclease-free water	-
10ng mRNA	1µl	10ng non-mRNA	9µl
TOTAL	20µl	TOTAL	20µl

The tubes were incubated at 37°C for 1 hour. Two PCR reactions were then performed using an aliquot of each cDNA amplification. The sequences of the primers for the amplification of a 541bp fragment of β-actin DNA (Prof C. Secombes; pers. comm.) were as follows:

β-ActFor   5' ATC GTG GGG CGC CCC AGG CAC C 3'
β-ActRev   5' CTC CTT AAT GTC ACG CAC GAT TTC 3'

PCR reactions were performed as follows in eppendorf tubes:

10x PCR buffer (Boehringer Mannheim)	3µl
10mM dNTP's	1µl
100ng cDNA	4µl
100µM β-ActFor (Synthesised by MWG Biotech)	1µl
100µM β-ActRev (Synthesised by MWG Biotech)	1µl
2.5 units Taq polymerase (Boehringer Mannheim)	0.5µl
Water	19.5µl
TOTAL	30µl

The tubes were heated in a Thermal Cycler (Perkin Elmer) as follows:

92°C	3 minutes		(Denaturation step)
92°C	1 minute		(Denaturation step)
50°C	1 minute	x 30	(Annealing step)
72°C	1 minute		(Elongation step)
72°C	7 minutes		(Elongation step)

10µl of each completed PCR reaction was analysed by agarose gel electrophoresis, as described in Section 2.2.1.3.

5.2.4 Construction of the Tx *Xenopus* Gut cDNA Library

The amplification of cDNA from the Tx *Xenopus* gut mRNA was carried out using the ZAP-cDNA® Synthesis Kit (Stratagene) and all components, except for the mRNA, were supplied.

5.2.4.1 Bacterial Strains

The strains of *E. coli* used in the construction and manipulation of the cDNA library were as follows:

XL1-Blue MRF'   Δ(*mcrA*) 183 Δ(*mcrCB-hsdSMR-mrr*) 173 *endA*1 *supE44thi*-1 *recA*1 *gyrA*96 *relA*1 *lac* [F' *proAB lacI*^{q} ZΔM15 Tn*10* (Tet^r)]

SOLR   e14-(McrA-) Δ(*mcrCB-hsdSMR-mrr*) 171 *sbcC recB recJ uvrC umuC::*Tn*5* (Kan^r) *lac gyrA*96 *relA*1 *thi*-1 *endA*1 λ^R [F' *proAB lacI*^{q} ZΔM15] Su-

5.2.4.2 First Strand cDNA Synthesis

The primer used for the amplification of first strand cDNA, called the linker-primer, is described in Appendix 4. The following components were assembled in a nuclease-free eppendorf tube:

400ng *Xenopus* mRNA	37.5μl
10x First strand buffer	5μl
10mM dNTP's	3μl
2.8μg Linker-primer	2μl
40 units RNase inhibitor	1μl

The tube was incubated at RT for 10 minutes to allow the linker-primer to anneal before the addition of 1.5μl Moloney murine leukaemia virus reverse transcriptase (50 units/μl). The first strand synthesis reaction was incubated at 37°C for 1 hour.

5.2.4.3 Second Strand cDNA Synthesis

The following components were added to the first strand reaction tube:

10x Second strand buffer	20μl
10mM dNTP's	6μl
Water	111μl
E.coli RNase H (1.5 units/μl)	2μl
E.coli DNA polymerase I (9 units/μl)	11μl
TOTAL	200μl

The tube was incubated for 2.5 hours at 16°C

5.2.4.4 Blunting of cDNA Termini, Ligation of *Eco*RI Adapters and Phosphorylation of Cohesive Ends

The following components were added to the second strand synthesis reaction to create blunt-ended cDNA termini:

2.5mM dNTP's	23μl
Pfu DNA polymerase (2.5 units/μl)	2μl

The blunting reaction was carried out at 72°C for 30 minutes. Following completion of the reaction, 200μl phenol-chloroform (1:1 v/v) was added and the tube vortexed before centrifugation at 15,000 rpm for 2 minutes. The upper aqueous layer, containing the cDNA, was transferred to a new eppendorf tube and an equal volume of chloroform was added. The tube was again centrifuged at 15,000 rpm for 2 minutes and the upper aqueous layer transferred to a new tube and the following were added:

3M sodium acetate	20μl
100% ethanol	400μl

The tube was incubated at -20°C overnight to allow the cDNA to precipitate and then centrifuged at 15,000 rpm for 60 minutes at 4°C. The supernatant was discarded and the DNA pellet washed by adding 500μl 70% (v/v) ethanol and centrifuging at 15,000 rpm for 2 minutes. The supernatant was removed and the pellet allowed to air dry for 2 minutes at RT. The pellet was resuspended in 9μl of *Eco*RI adapters (See Appendix 4) and the following components added:

10x Ligase buffer	1μl
10mM Adenosine triphosphate (ATP)	1μl

T4 DNA Ligase (4 units/µl)   1µl

The ligation reaction was incubated at 4°C for 2 days before heat inactivating the ligase enzyme by incubation at 70°C for 30 minutes.

Following ligation of the *EcoRI* adapters to the ends of the cDNA molecules, the cohesive ends were phosphorylated by the addition of the following components:

10x Ligase buffer   1µl
10mM Adenosine triphosphate   2µl
Water   6µl
T4 Polynucleotide kinase (10 units/µl)   1µl

The tube was incubated at 37°C for 30 minutes, then at 70°C for 30 minutes to heat inactivate the kinase enzyme.

5.2.4.5 *XhoI* Digestion of cDNA

The cDNA was digested with the restriction endonuclease *XhoI* at the site contained within the linker-primer to give each cDNA molecule an *XhoI* and an *EcoRI* cohesive end. The following components were added to the phosphorylated cDNA from Section 5.2.4.4:

XhoI buffer   28µl
XhoI (40 units/µl)   3µl

The reaction was incubated for 1.5 hours at 37°C before the addition of 5µl 10xSTE buffer (1M NaCl, 200mM Tris HCl (pH 7.5), 100mM EDTA) and 125µl 100% ethanol. The tube was placed at -20°C overnight to precipitate the cDNA, before centrifugation at 15,000rpm for 60 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 14µl 1xSTE buffer and 3.5µl column loading dye (50% v/v glycerol, 10% v/v 10x STE buffer, 40% w/v bromophenol blue) was added.

5.2.4.6 Size Fractionation of cDNA

cDNA was size fractionated by passing it through a Sepharose CL-2B column and eluting progressive fractions from the column. The column was prepared by adding

Sepharose CL-2B resin on top of a cotton plug in the tip of a sterile 1ml plastic pipette (Greiner). The resin was left to pack down and form a column before being washed by the addition of 10ml 1xSTE buffer. As the last of the 1xSTE buffer entered the column, the cDNA was loaded on top and allowed to migrate through the column. When the leading edge of the dye reached the -0.4ml gradation on the pipette, fractions were collected from the column in eppendorf tubes in aliquots of around 100µl (3 drops). 8µl of each fraction was removed and saved for electrophoretic analysis while an equal volume of phenol-chloroform (1:1 v/v) was added to the remainder. Each tube was vortexed and centrifuged at 15,000 rpm for 2 minutes. The upper aqueous layer, containing the cDNA, was transferred to a new eppendorf tube and an equal volume of chloroform was added. The tube was again vortexed and centrifuged at 15,000 rpm for 2 minutes and the upper aqueous layer transferred to a new tube and 2 volumes of 100% ethanol were added. The tube was incubated at -20°C overnight, centrifuged at 15,000 rpm for 60 minutes at 4°C and the DNA pellet washed by the addition of 200µl 80% (v/v) ethanol. Following a further centrifugation at 15,000 rpm for 2 minutes, the supernatant was removed and the pellet resuspended in 3.5µl sterile water and stored at -20°C.

5.2.4.7 Electrophoretic Analysis of cDNA Fractions

A 5% polyacrylamide gel was poured using the Bio-Rad Mini-Gel apparatus as described in Section 2.2.1.3. Each 8µl cDNA fraction was added to 2µl 5x DNA loading buffer (See Section 2.2.1.1) and loaded on the gel before electrophoresis at 100V for 1.5 hours. Gels were removed from the electrophoresis apparatus and stained using a silver stain kit (Bio-Rad). Gels were fixed by incubating in 50ml 40% (v/v) methanol for 30 minutes, followed by 15 minutes in two changes of 50ml 10% (v/v) ethanol. Following fixation, the gels were incubated in 50ml oxidising solution (Bio-Rad), containing potassium dichromate and nitric acid, for 5 minutes before 3 washes in 50ml sterile, distilled water. The gels were then incubated in 50ml silver reagent (Bio-Rad), containing silver nitrate, for 20 minutes before washing once in 50ml sterile, distilled water. 50ml developing solution (Bio-Rad), containing sodium carbonate and paraformaldehyde, was added to the gels until the DNA bands became visible, at which point 5% (v/v) acetic acid was added to stop the reaction.

5.2.4.8 Ligation of cDNA into the Phage Lambda Uni-Zap XR Vector

cDNA was ligated into the Uni-ZAP XR vector (Appendix 5), which had been digested with *EcoRI* and *XhoI*, in a 5µl reaction volume as follows:

cDNA	2.5μl
10x Ligase buffer	0.5μl
10mM ATP	0.5μl
Uni-ZAP XR Vector	1μl
T4 DNA Ligase (4 units/μl)	0.5μl

The ligation reaction was incubated at 4°C for two days.

5.2.4.9 Packaging of the Uni-Zap XR Vector DNA into Lambda Phage Particles and Quantification of Phage Numbers by Titering

A Gigapack III Gold packaging extract (Stratagene) was removed from -80°C storage and placed on dry ice. The extract was then removed from dry ice, allowed to thaw and 4μl of the ligation reaction added immediately. The tube was incubated at RT for 2 hours before the addition of 500μl SM buffer (0.1M NaCl, 8mM $MgSO_4 \cdot 7H_2O$, 50mM Tris HCl (pH 7.5), 0.01% (w/v) gelatin) and 20μl chloroform. The completed packaging reaction was then stored at 4°C.

XL1-blue MRF' *E.coli* cells were cultured overnight at 30°C in 10ml 2xYT medium (See Section 2.2.2.2), supplemented with 10mM $MgSO_4$ and 0.2% (w/v) maltose, before being resuspended at an OD_{600} of 0.5 in 10mM $MgSO_4$. A serial dilution of the packaged phage was performed down to a final dilution of 10^{-6}. 1μl aliquots of the 10^{-4}, 10^{-5} and 10^{-6} dilution's were each added to 200μl XL1-blue cells and incubated at 37°C for 15 minutes. 3 universal tubes, each containing 3ml 2xYT soft agar which had been melted and cooled to 48°C, were supplemented with 2.5mM IPTG and 5mg/ml 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-gal). Following the 15 minute incubation at 37°C, each 200μl aliquot of XL1-blue cells was added to a tube of soft agar which was consequently poured onto a 2xYT agar plate. The plates were incubated for 8 hours at 37°C before counting the respective numbers of white and blue plaque forming units.

5.2.4.10 Amplification of the Uni-Zap XR Library in Phage Lambda

XL1-blue MRF' *E.coli* cells were cultured overnight at 30°C in 10ml 2xYT medium, supplemented with 10mM $MgSO_4$ and 0.2% (w/v) maltose, before being resuspended at an OD_{600} of 0.5 in 10mM $MgSO_4$. 500μl of the phage packaging reaction from

Section 5.2.4.9 was added to 1ml XL1-blue cells and incubated at 37°C for 15 minutes. The XL1-blue cells were then added to 30ml 2xYT soft agar, which had been melted and cooled to 48°C, and poured on to a 21x21cm square 2xYT agar plate and incubated at 37°C for 8 hours. The plate was then overlaid with 50ml SM buffer and incubated overnight at 4°C before transferring the SM buffer, into which the phage had diffused, to a 50ml Falcon tube. The tube was centrifuged at 4,000 rpm for 10 minutes to pellet the cell debris and the supernatant divided into 1ml aliquots, which were supplemented with 7% dimethyl sulphoxide (DMSO) and stored at -80°C. One aliquot was titred, as described in Section 5.2.4.9.

5.2.5 *In vivo* Excision of cDNA-Containing Plasmid's from the Uni-ZAP XR cDNA Library in Phage Lambda

In-vivo excision of the pBluescript phagemid (Stratagene) from the Uni-Zap XR vector was achieved by co-infection with ExAssist helper phage (Stratagene). Separate 10ml cultures of XL1-blue MRF' and SOLR *E.coli* cells in 2xYT medium, supplemented with 10mM Mg SO_4 and 0.2% (w/v) maltose, were grown overnight at 30°C. Each culture was centrifuged at 4,000 rpm for 10 minutes and the cells resuspended at an OD_{600} of 0.5 in 10mM $MgSO_4$ and stored on ice. The following were added to a 50ml Falcon tube:

10^7 plaque forming units (pfu) Uni-ZAP XR lambda phage library
125μl XL1-blue cells (at an OD_{600} of 0.5 in 10mM $MgSO_4$)
10^9 pfu ExAssist helper phage (Stratagene)

The tube was incubated at 37°C for 15 minutes before the addition of 20ml 2xYT medium. The tube was shaken at 37°C for 3 hours and then heated to 70°C for 20 minutes before being centrifuged at 4,000 rpm for 10 minutes. 1μl of the supernatant was added to 200μl SOLR cells in an eppendorf tube and incubated at 37°C for 15 minutes. 100μl was then spread, with a sterile glass spreader, on the surface of a 2xYT agar plate, which had been supplemented with 50μg/ml ampicillin. The plate was incubated overnight at 37°C and the cells harvested by adding 5ml 2xYT medium and scraping the colonies with a sterile glass spreader. The 2xYT medium was then transferred to a 15ml Falcon tube and centrifuged at 4,000 rpm for 10 minutes to pellet the cells. The supernatant was discarded and the cells resuspended in 500μl 2xYT medium. A 50μl aliquot was removed for the isolation of plasmid DNA (As described

in Section 2.2.1.3), while the remainder was supplemented with 500μl 80% (v/v) glycerol and stored at -80°C.

5.2.6 PCR Amplification of *Xenopus* β-actin DNA from the Excised cDNA Library

Following the preparation of excised plasmid DNA (Section 5.2.5), a PCR reaction was performed to amplify *Xenopus* β-actin DNA:

10x PCR buffer (Boehringer Mannheim)	3μl
10mM dNTP's	1μl
100ng Excised plasmid DNA	1μl
100μM β-ActFor	1μl
100μM β-ActRev	1μl
2.5 units Taq polymerase (Boehringer Mannheim)	0.5μl
Water	22.5μl
TOTAL	30μl

The tube was heated in a Thermal Cycler (Perkin Elmer) as described in Section 5.2.3. 10μl of each completed PCR reaction was analysed by agarose gel electrophoresis, as described in Section 2.2.1.3.

5.2.7 Screening the Uni-ZAP XR Lambda Phage Expression Library with mAb's

5.2.7.1 Primary Screening

4×10^5 pfu of the Tx *Xenopus* gut cDNA library in phage lambda were plated out on a 21x21cm 2xYT agar plate as described in Section 5.2.4.10. The plate was incubated at 42°C for 4 hours before 4 sheets of 10x10cm nitrocellulose (Schleicher and Schuell), which had been pre-wetted in 10mM IPTG, were placed side by side on the agar plate. The plate was incubated for a further 4 hours at 37°C before transferring each nitrocellulose filter to a shallow tray containing 50ml TBS (See Section 2.2.1.1). After 5 minutes incubation at RT on a rocker platform, the TBS was poured off and replaced with 50ml blocking solution (5% non fat dried milk (Marvel), 0.05 % Tween 20 in TBS) and the filters incubated with rocking for a further 30 minutes. After the 30 minute incubation, the blocking solution in each tray was replaced with 10ml mAb 1G5 hybridoma supernatant (diluted 1:2 in blocking solution) and the trays incubated for 2 hours. Following incubation, the filters were washed 3 times in 50ml blocking

solution before the addition of 10ml HRP-conjugated anti-mouse IgG antibody (diluted 1:20,000 in blocking solution). The filters were incubated for a further hour at RT on the rocker platform before washing 3 times in TBS. Chemiluminescent detection of bound antibodies was carried out as described in Section 2.2.4.3. Plaques corresponding to positive results on the nitrocellulose filters were isolated by pressing down on the appropriate area of agar with a cut off pipette tip. The plug was transferred to an eppendorf tube containing 500µl SM buffer and left overnight at 4°C to allow diffusion of phage into the buffer. The titre of phage in the buffer was determined using the technique described in Section 5.2.4.9.

5.2.7.2 Secondary Screening

Approximately 100 pfu of each phage carried forward from the primary round of screening was plated on 2xYT as described in Section 5.2.4.9. Following incubation at 42°C for 4 hours, each plate was overlaid with a 5x5cm nitrocellulose filter, which had been pre-wetted with 10mM IPTG. The remainder of the screening process was carried out as in Section 5.2.7.1, except that the filters were incubated in 10ml buffer volumes.

5.2.8 Sequencing and Analysis of cDNA Clones from the Excised cDNA Library

The excised DNA library in *E.coli* SOLR was plated on to a 2xYT agar plate from the 40% glycerol stock at -80°C and the plate incubated at 37°C overnight. Individual colonies were picked with a sterilised nichrome wire, transferred to 5ml 2xYT medium containing 50µg/ml ampicillin and cultured overnight at 37°C. Overnight bacterial cultures were consequently used for the preparation of plasmid DNA, as described in Section 2.2.1.3.

5' to 3' sequencing of cDNA inserts from individual plasmid's was carried out using the M13 reverse primer (Stratagene) on a 373 Stretch™ or 377 Prism™ automated DNA sequencer (Applied Biosystems). Analysis of cDNA sequences was performed using a web site (http://www.ncbi.nlm.nih.gov/BLAST) run by the US National Centre for Biotechnology Information (NCBI). cDNA sequences were entered and compared to either DNA or protein sequence databases using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al.*, 1990). BLASTN searches were used to directly compare the cDNA sequence with the DNA database whereas BLASTX searches compared the 6-frame conceptual translation of the cDNA to the protein database.

5.2.9 Attempts to Isolate DNA Encoding *Xenopus* NK Receptor Proteins by PCR Amplification

5.2.9.1 Bacterial Strain

The strain of *E.coli* used in the cloning of PCR products was as follows:

TOP10   F-, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lacX74 deo*R *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

5.2.9.2 Alignment of NK Receptor Protein Amino Acid Sequences

NK receptor amino acid sequences were obtained from the NCBI Entrez database (http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez) and gapped alignments were performed using the MegAlign program, which is part of the LaserGene software package (DNASTAR Inc.). Conserved motifs within the NK receptor protein NKRP-1 were found by aligning amino sequences from different species (See Figure 5.6). These motifs were consequently used to derive the PCR primers NK-F and NK-R (Figure 5.7).

5.2.9.3 RT-PCR Reactions to Amplify NKRP-1 DNA from *Xenopus* Spleen RNA

A spleen was dissected from a control *Xenopus*, as described in Section 2.2.3.2, and total RNA prepared from the spleen (as in Section 2.2.2.4). In order to remove contaminating genomic DNA, the following components were added to 14μl *Xenopus* spleen RNA in a nuclease-free eppendorf:

5x First strand buffer (Gibco)   4μl
2 units RNase-free DNase I (Promega)   2μl

The tube was incubated at RT for 15 minutes before heating to 65°C for 10 minutes. Two cDNA amplification reactions were then performed on the RNA, one of which was a control reaction conducted in the absence of the reverse transcriptase enzyme:

Test Reaction		Control Reaction	
20ng RNA	10µl	20ng RNA	10µl
5x First strand buffer (Gibco)	2µl	5x First strand buffer	2µl
40 units RNase inhibitor (Promega)	1µl	40 units RNase inhibitor	1µl
10mM dNTP's (Gibco)	2µl	10mM dNTP's	2µl
100µM Oligo dT primer (Gibco)	1µl	100µM Oligo dT primer	1µl
0.1M DTT (Gibco)	2µl	0.1M DTT	2µl
200 units Superscript II Reverse		Nuclease-free water	1µl
Transcriptase (Gibco)	1µl	TOTAL	20µl
Nuclease-free water (Promega)	1µl		
TOTAL	20µl		

The reactions were incubated at 37°C for 1.5 hours and then each was used in a PCR reaction with the NKR-P1 primers NK-F and NK-R (synthesised by MWG Biotech):

cDNA	4µl
10mM dNTP's (Gibco)	1µl
100µM NK-F primer	1µl
100µM NK-R primer	1µl
10x Expand™ buffer (Boehringer Mannheim)	5µl
Expand™ polymerase mixture (Boehringer Mannheim)	0.5µl
Water	37.5µl
TOTAL	50µl

The tubes were heated in a Thermal Cycler (Perkin Elmer) as follows:

95°C	3 minutes		(Denaturation step)
95°C	1 minute		(Denaturation step)
50°C	1 minute	x 30	(Annealing step)
72°C	1 minute		(Elongation step)
72°C	7 minutes		(Elongation step)

10µl of each completed PCR reaction was analysed by agarose gel electrophoresis, as described in Section 2.2.1.3.

5.2.9.4 Cloning and Sequencing of PCR Products

PCR products were purified following agarose electrophoresis by silica fines, as described in Section 2.2.1.3. The PCR products were cloned into the pCR® vector (Invitrogen) using a TOPO™ TA cloning kit (Invitrogen). 4µl PCR product was added to 1µl pCR vector and incubated for 5 minutes at RT. 1µl of the supplied 6x stop solution was added and the tube incubated for a further 10 seconds at RT before being placed on ice. 2µl of the reaction was added to a vial of TOP10 *E.coli* cells (Invitrogen) and incubated on ice for 30 minutes. The cells were subjected to heat shock by incubating at 42°C for 30 seconds and then placing immediately on ice. Following the heat shock, 250µl NE medium (See Section 2.2.2.2) was added to the cells and the tube shaken horizontally for 30 minutes at 37°C. 50µl was then plated onto a 2xYT agar plate, which had been supplemented with 50µg/ml ampicillin, 40µg/ml X-gal and 0.1mM IPTG, and the plate was incubated overnight at 37°C.

White colonies were picked from the 2xYT agar plate with a sterilised nichrome wire, transferred to 5ml 2xYT medium containing 50µg/ml ampicillin and cultured overnight at 37°C. Overnight bacterial cultures were consequently used for the preparation of plasmid DNA, as described in Section 2.2.1.3. Bi-directional sequencing of cDNA inserts from individual plasmid's was carried out using the universal M13 forward (-20) and reverse (-40) primers (Invitrogen) on a 373 Stretch™ or 377 Prism™ automated DNA sequencer (Applied Biosystems). Analysis of cDNA sequences was performed as described in Section 5.2.8.

5.3 Results

5.3.1 RT-PCR Amplification of β-actin DNA from Tx *Xenopus* Gut mRNA

Following preparation of mRNA from the total RNA isolated from Tx *Xenopus* gut IEL, an RT-PCR reaction was performed to amplify β-actin DNA (As described in Section 5.2.3). The same RT-PCR was performed on both the mRNA and the RNA left behind following removal of the mRNA. The result (Figure 5.1) shows amplification of a 541bp DNA fragment, which is the correct size for β-actin DNA (Prof C. Secombes; pers. comm.), in the mRNA lane but not in the non-mRNA lane. This result suggested that intact mRNA had been isolated from the total RNA prepared from gut IEL.

5.3.2 Construction of Tx *Xenopus* Gut cDNA Library in Phage Lambda

5.3.2.1 Size Fractionation of cDNA Molecules

Double stranded cDNA was synthesised from Tx *Xenopus* gut mRNA and size fractionated by passing through a sepharose column, as described in Section 5.2.4. The cDNA fractions collected from the column were analysed by polyacrylamide gel electrophoresis and the DNA visualised by silver staining (See Section 5.2.4.7). The two gels displayed in Figure 5.2 show that the largest cDNA fragments were present in fractions 4, 5 and 6, whereas later fractions (7 to 12) show progressively smaller fragments of cDNA.

5.3.2.2 Cloning of cDNA Fractions into the Phage Lambda Uni-Zap XR Vector

Equal amounts of cDNA fractions 4, 5 and 6 were ligated into the Uni-Zap XR Vector as described in Section 5.2.4.8. Following packaging of the ligation reaction into phage lambda particles, the phage were titred using blue-white selection to determine how many contained cDNA inserts. 3×10^5 white plaques (containing cDNA inserts) and 1×10^4 blue plaques (no inserts) were counted. The library was amplified and stored at -80°C.

5.3.2.3 PCR Amplification of β-actin DNA from the Tx *Xenopus* cDNA Library

In order to verify that *Xenopus* cDNA inserts had been successfully cloned, an aliquot of the phage lambda library was converted in an *in vivo* excision reaction to a plasmid library (As described in Section 5.2.5). The plasmid cDNA library was then used as a template in a PCR reaction to amplify β-actin DNA (See Section 5.2.6). Figure 5.3 shows that the 541bp β-actin DNA was successfully amplified from the cDNA library.

5.3.3 Screening of Tx *Xenopus* Gut cDNA Library in Phage Lambda with anti-*Xenopus* NK mAb 1G5

Proteins expressed by the Tx *Xenopus* gut cDNA library in phage lambda were screened with mAb 1G5, as described in Section 5.2.7. The primary round of screening (Figure 5.4) highlighted three potentially positive plaques, denoted A, B and C, which were subjected to a secondary round of screening with mAb 1G5. The second round of screening (Figure 5.5) showed no positive reactions to cDNA's expressed by plaques A, B or C.

5.3.4 Sequencing of 5 Tx *Xenopus* Gut cDNA's from the Excised Plasmid cDNA Library

Five cDNA inserts, denoted *Xen1* to *Xen5*, from individual excised plasmid's were sequenced in a 5' to 3' direction and the deduced amino acid sequences compared to a protein database using the BLAST algorithm (See Section 5.2.8). One of the five sequences (*Xen4*) resulted from the re-cloning of vector DNA whereas the other four showed significant similarity to known mouse or human proteins (Table 5.1). The most significant result was the cDNA *Xen3* which showed 92% identity to a 208 amino acid sequence found in a human GTP-binding protein (Data not shown). Of the other cDNA's, *Xen1* showed similarity to the murine protein Keap1, a possible suppressor of the transcription factor Nrf2 (Itoh *et al.*, 1999), *Xen2* was similar to a human protein called OS-9, which is over-expressed in osteosarcomas (Kimura *et al.*, 1998) and *Xen5* showed homology to a mouse protein called Bim, a novel member of the Bcl-2 family that promotes apoptosis (O'Connor *et al.*, 1998).

5.3.5 Attempts to Amplify DNA Encoding NK Receptors from *Xenopus* spleen RNA by RT-PCR

5.3.5.1 Alignment of NKR-P1 Amino Acid Sequences

The only NK receptor for which non-mammalian homologous sequences could be found on the NCBI database was NKR-P1. These homologous sequences were both cDNA's and were cloned from the Japanese flounder (*Paralichthys olivaceus*) and the chicken (*Gallus gallus*). When translated to amino acid sequence they showed significant homology to mammalian NKR-P1 amino acid sequences from mouse, rat and human (See Figure 5.6).

5.3.5.2 Design of PCR Primers for the Amplification of NKR-P1 DNA

Two short regions which showed a high level of homology between the 5 aligned amino acid sequences were chosen for the design of PCR primers (See Figure 5.6). The flounder and chicken cDNA regions encoding these sequences were compared and in the case of one region were found to be identical (Figure 5.7). This sequence was thus chosen as the forward PCR primer (NK-F). The flounder and chicken cDNA sequences for the second region were not identical and differences between the two were incorporated into the reverse PCR primer (NK-R) as degenerate bases (Figure

5.7). Based on the flounder and chicken cDNA sequences, the predicted size of the DNA fragment amplified by NK-F and NK-R would be between 150 and 180 bp.

5.3.5.3 RT-PCR with Primers NK-F and NK-R on *Xenopus* Control Spleen RNA

Two RT-PCR reactions were performed on control *Xenopus* spleen RNA with primers NK-F and NK-R (Described in Section 5.2.9.3). One of these was carried out in the absence of reverse transcriptase as a control for amplification from contaminating genomic DNA. This control sample showed no DNA bands when examined by agarose electrophoresis, whereas the sample in which reverse transcriptase was included showed amplification of 2 bands at around 180 and 450 base pairs (Figure 5.8). This suggested that amplification from mRNA rather than genomic DNA had taken place.

5.3.5.4 Sequencing and Analysis of PCR Products

Both the 180 and 450 base pair PCR products were cloned and sequenced, as described in Section 5.2.9.4. The 180 base pair fragment contained the NK-F primer sequence at one end but the NK-R primer sequence was not found. The 450 base pair product contained neither the NK-F or NK-R primer sequences but did contain a poly-T region, probably transcribed from the poly-A tail of an mRNA molecule. Unfortunately, neither sequence showed significant homology to nucleic acid or protein sequences contained within the NCBI database when entered into the BLAST algorithm.

5.4 Discussion

5.4.1 Screening the *Xenopus* cDNA Expression Library with an Anti-NK mAb

The first strategy for isolating a cDNA encoding a *Xenopus* NK cell surface protein was to screen a cDNA expression library cloned from the gut IEL of a Tx animal with the anti-NK mAb 1G5. This strategy of immunological screening has been used successfully in the molecular cloning of MHC class I cDNA from *Xenopus* (Flajnik *et al.*, 1991). A library of 3×10^5 recombinants was constructed from the Tx gut IEL mRNA. PCR with primers specific for β-actin DNA were able to amplify a correctly sized band from the cloned library. In addition, 5 cDNA's from the library were sequenced and 4 were found to be *Xenopus* cDNA's with similarity to 4 different mouse or human sequences. This evidence was taken as an indication that the library contained a diverse selection of *Xenopus* cDNA sequences. Primary screening of the

expression library with mAb 1G5 revealed three putative positive clones which were isolated and subjected to a further round of screening with 1G5. Unfortunately, none of the clones were true positives and a repeated attempt to isolate clones by screening with 1G5 was unsuccessful.

There are several possible reasons for the failure of this strategy to clone a cDNA encoding a *Xenopus* NK cell surface protein. Firstly, the library size of 3×10^5 may have been too small to contain the cDNA encoding the 1G5 antigen. Although the library was enriched for larger cDNA molecules which roughly approximated to the size of the protein identified by Western blotting (See Figure 4.6) by size fractionation, it is possible that rare mRNA transcripts, which can be present at a frequency of 1 per cell (Toole *et al.*, 1984; Wood *et al.*, 1984), were not represented. In this respect it is worth noting that the successful cloning of *Xenopus* MHC class I cDNA (Flajnik *et al.*, 1991) resulted from the screening of a library of 6×10^6 recombinants. The factor which limited the size of this library was the number of IEL which could be obtained from a Tx animal and hence the amount of mRNA which could be used for cDNA library construction. The ZAP-cDNA® Synthesis Kit (Stratagene) is designed for cDNA amplification from 5μg of mRNA, whereas the amount of total RNA obtainable from the gut IEL was around 20μg (Data not shown), of which 1-5% (0.2 - 1μg) would be expected to be mRNA. Although RNA from several organs or even several animals could have been combined in order to obtain enough mRNA, it was thought that this would have introduced an increased number of mRNA transcripts from non-NK cells and reduced the frequency of NK-specific transcripts in the library.

A second explanation for the failure of this method is that the correct cDNA clone was present in the library but was not expressed in a form which could be recognised by mAb 1G5. Although, 1G5 reacted very strongly to Western blots of denatured Tx gut protein, it is possible that when the cDNA-encoded antigen was expressed in bacteria it no longer contained the epitope to which 1G5 reacted. The initial amplification of cDNA from the 3' end of mRNA molecules with an oligo-dT primer tends to favour the production of C-terminal polypeptides which 1G5 may fail to recognise if its epitope is found in the N-terminus of the protein. Alternatively, 1G5 may react with a carbohydrate moiety which would not be present when the protein is expressed in *E.coli*, as glycosylation of proteins does not occur in bacteria. However, the high level of specificity of mAb 1G5 for NK cells in *Xenopus*, suggests that it is not simply binding to a carbohydrate antigen. With respect to the problem of eukaryotic protein expression in bacteria, it is desirable to screen bacterial expression libraries with polyclonal antisera which react to several epitopes on the target antigen, thus

increasing the chances of identifying a positive clone (Sambrook *et al.*, 1989). Polyclonal antisera were used in the cloning of *Xenopus* MHC class I by this strategy (Flajnik et al., 1991) but unfortunately a polyclonal antiserum raised against the 1G5 antigen was not available for this study.

Given the reasons for the failure of this strategy, any future attempts to clone cDNA's for *Xenopus* NK cell surface proteins by immunological screening of cDNA expression libraries will require certain improvements. Firstly, the frequency of the desired clone in the cDNA library can be increased by employing immunomagnetic separation (as described in Section 4.2.3.3) to obtain highly enriched populations of NK cells prior to the preparation of mRNA. In order to obtain sufficient mRNA for the construction of a representative library it may be necessary to combine NK-enriched cells from several organs or even from several animals. Secondly, the cDNA could be initially amplified with a combination of oligo-dT and random primers so as not to favour too heavily the production of C-terminal polypeptides. Thirdly, and perhaps most importantly, screening the library with polyclonal antisera raised against the purified *Xenopus* NK antigen would increase the chances of identifying a positive clone despite some of the problems of bacterial expression described above. As an alternative to this third improvement, the *Xenopus* cDNA library could be expressed in a eukaryotic host, such as a yeast, insect or mammalian cell, to overcome some of the difficulties of bacterial protein expression. The eukaryotic expression library could then be screened by flow cytometry using the original anti-*Xenopus* NK mAb's.

5.4.2 Attempts to Isolate NKR-P1 DNA from *Xenopus* by RT-PCR

The isolation of DNA encoding a *Xenopus* NKR-P1 homologue by RT-PCR relies upon the identification of conserved sequences in NKR-P1 proteins from several species. As mentioned earlier, NKR-P1 was the only NK receptor for which sequence homology was found in non-mammalian organisms, namely the Japanese flounder and the chicken. Although these sequences were not highly homologous, they did contain short amino acid motifs which appeared to be quite highly conserved and these were chosen for the design of the PCR primers NK-F and NK-R. The ability of these primers to amplify two PCR products, one of which was at the expected 180 base pair size, from control *Xenopus* spleen RNA initially suggested that the strategy may have been successful. However, sequencing and analysis of these products revealed no significant homology to any known DNA or protein sequences. The NK-F primer was found at one end of the 180 base pair sequence but the NK-R primer sequence could not be found, suggesting that an NKR-P1 homologue had not been amplified. The

larger 450 base pair PCR product contained a poly-T region but neither the NK-F or NK-R primers could be found within this PCR product.

The failure of the PCR strategy to isolate a *Xenopus* NK receptor homologue may reflect the use of the chicken and flounder cDNA sequences to design PCR primers. Although the conceptual translations of these sequences were found to show homology to regions of mammalian NKR-P1, there has been no verification that these cDNA's actually encode NKR-P1 homologues. However, the design of PCR primers based on the mammalian sequences would have required the use of highly degenerate primer sequences which would have decreased the chance of amplifying a specific product.

1% agarose gel showing the amplification of ß-actin DNA from Tx Xenopus gut mRNA in an RT-PCR reaction. As a control reaction, the same RT-PCR was performed on RNA left behind after the removal of mRNA. Lane 1 contains λPstI DNA markers.

541 base pair ß-actin DNA

Primers
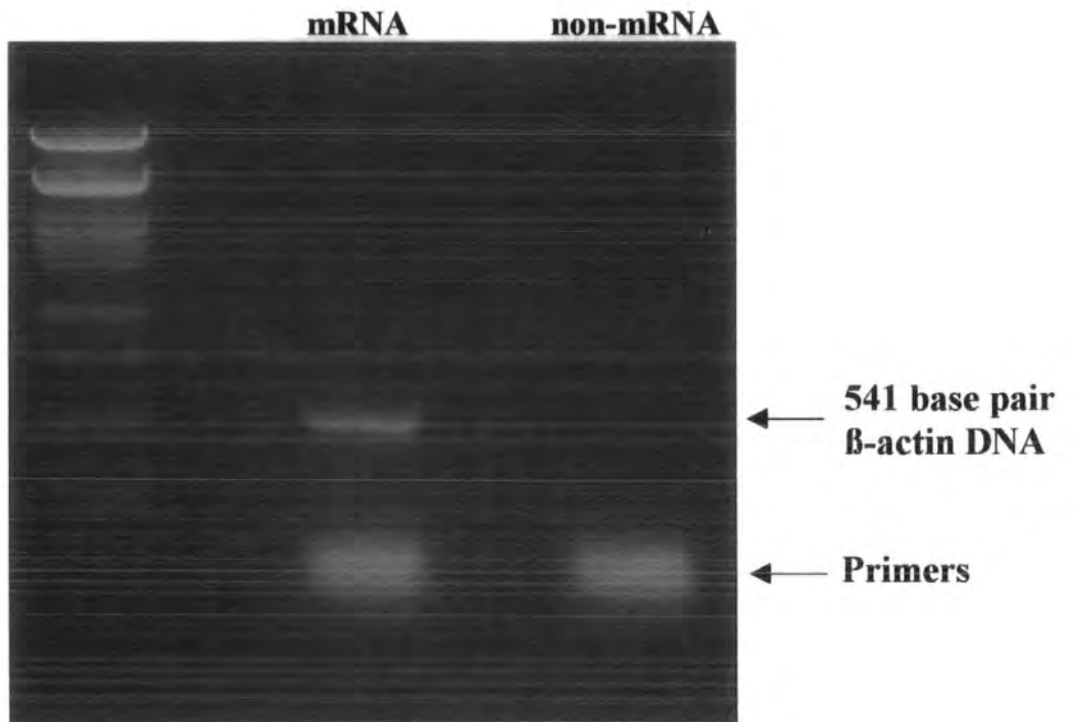
Figure 5.2   Size Fractionation of Tx Xenopus Gut cDNA

2 silver-stained polyacrylamide gels showing aliquots of *Xenopus* gut cDNA following size fractionation on a sepharose column. Progressively-eluted fractions from the column are indicated (1 to 12) as are the DNA size markers λHindIII and λPstI.
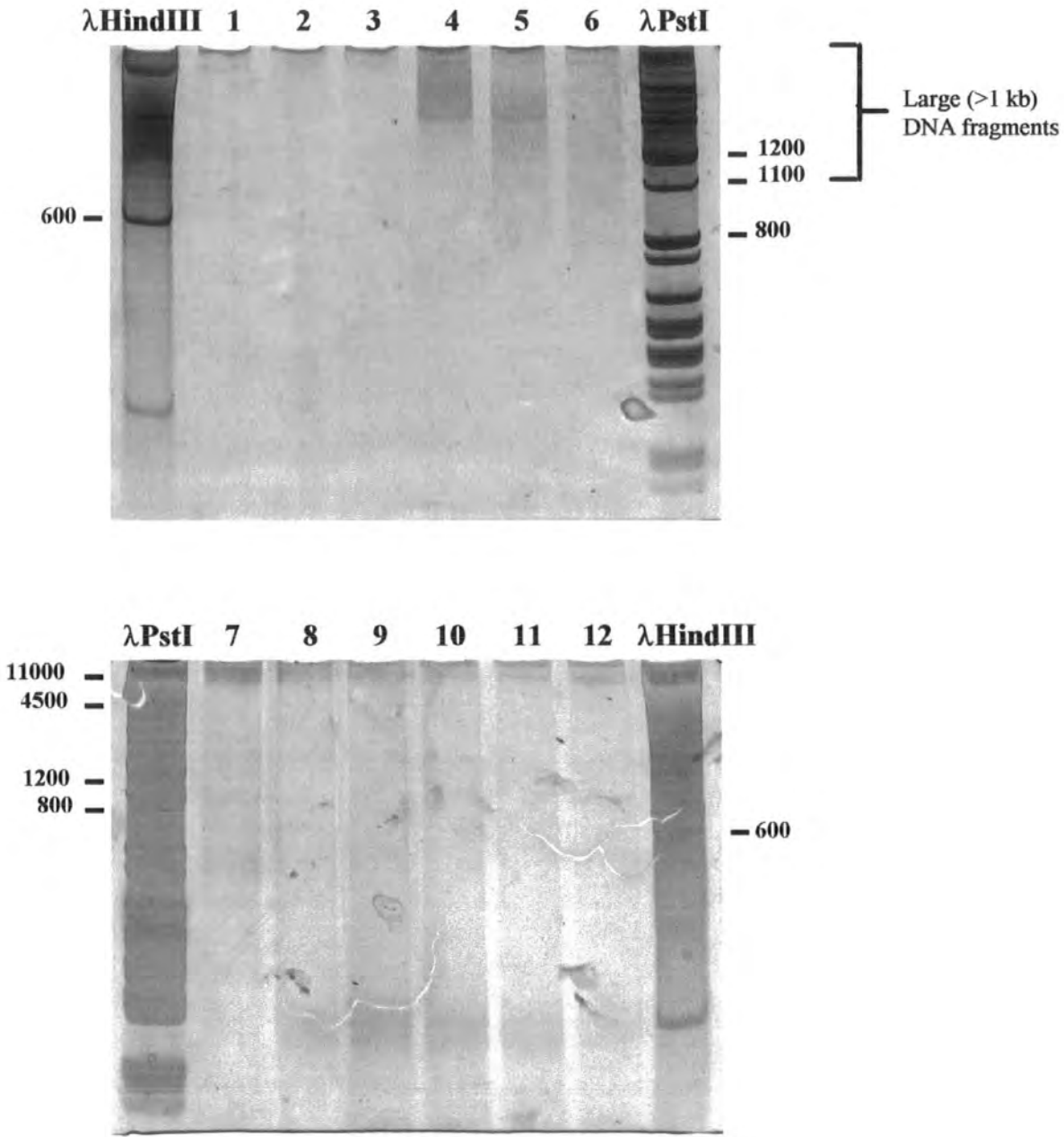
Large (greater than 1 kilobase) DNA fragments are indicated.

Figure 5.3 Amplification of ß-actin DNA by PCR from the *Xenopus* Gut cDNA Library

Having excised the cDNA library as plasmids from the bacteriophage lambda vector, the plasmid library was used as a template in a PCR reaction to amplify ß-actin DNA. Lane 1 contains λPstI DNA size markers.

541 base pair
ß-actin DNA

Primers
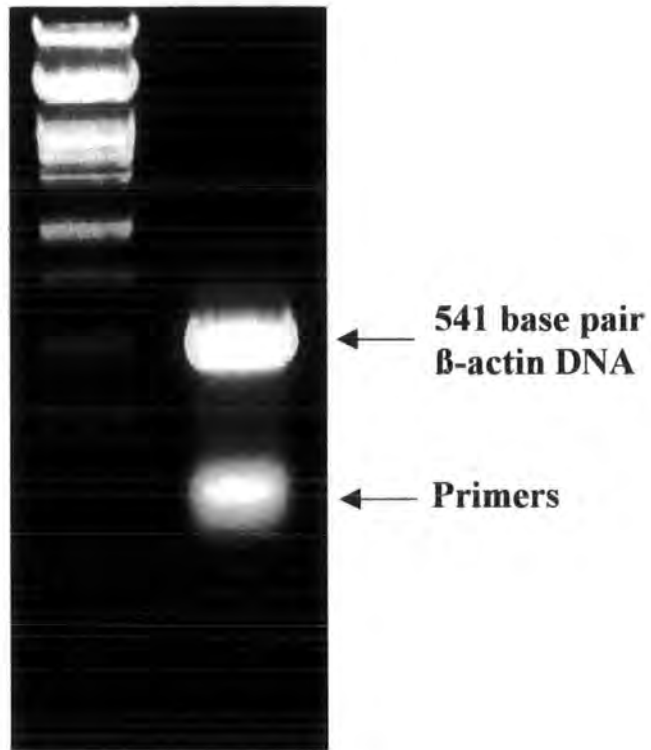
Figure 5.4   Primary Screening of *Xenopus* Gut cDNA Expression Library with anti-NK mAb 1G5

The Tx *Xenopus* gut cDNA library in the lambda ZAP vector was plated out on a bacterial lawn and the cDNA-encoded proteins were expressed, transferred to nitrocellulose filters and consequently probed with mAb 1G5. Four filters, each of which has corresponds to around 1×10^5 cDNA-containing lambda plaques, are shown with arrows highlighting putative positives. The circles indicate marks made on the nitrocellulose for orientation.
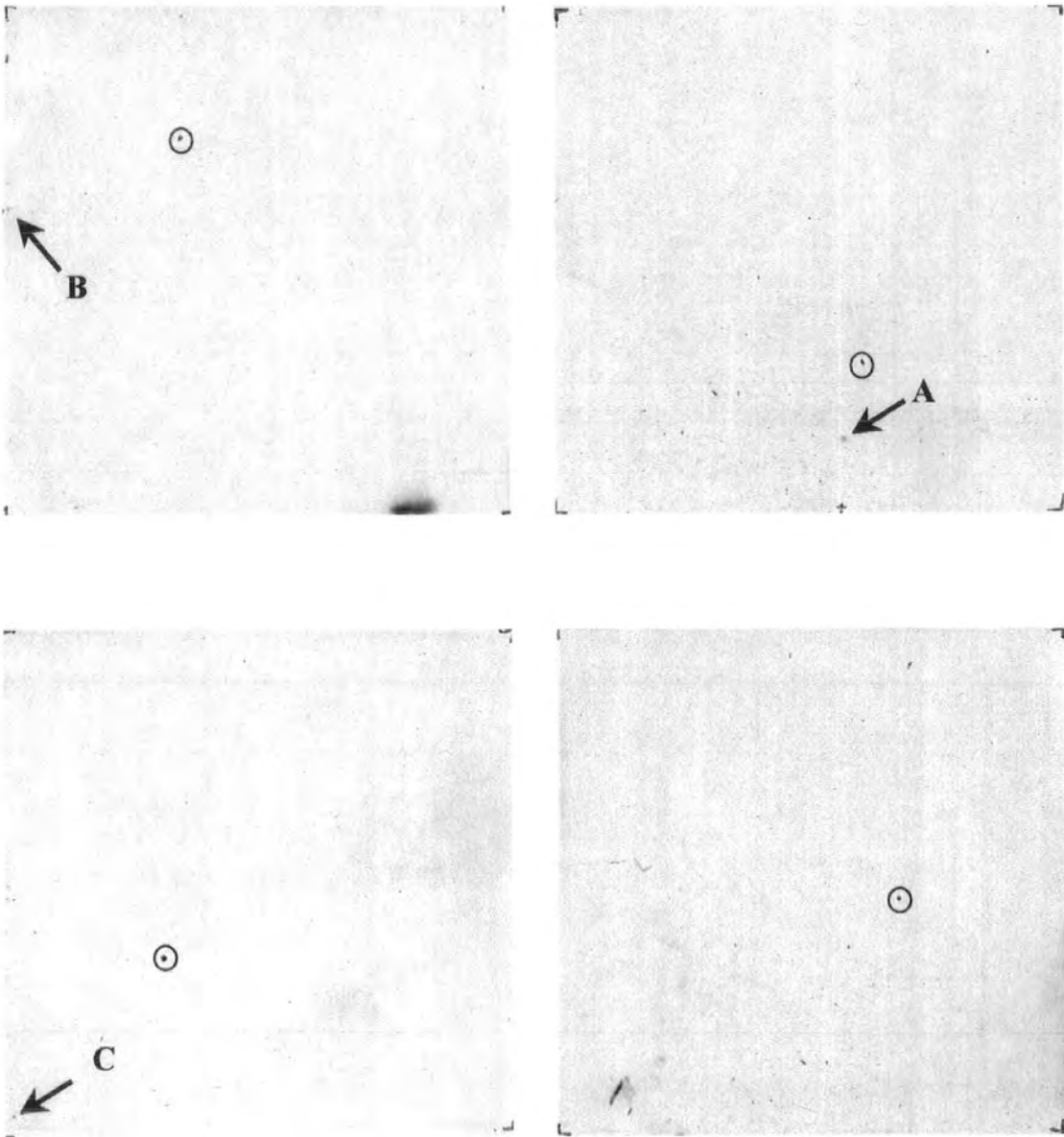
Figure 5.5   Secondary Screening of Xenopus Gut cDNA Expression Library with anti-NK mAb 1G5

Bacteriophage isolated from the primary screen with 1G5 (designated A,B and C in Figure 5.4) were individually plated out and re-screened with 1G5. The circles indicate marks made on the nitrocellulose for orientation.
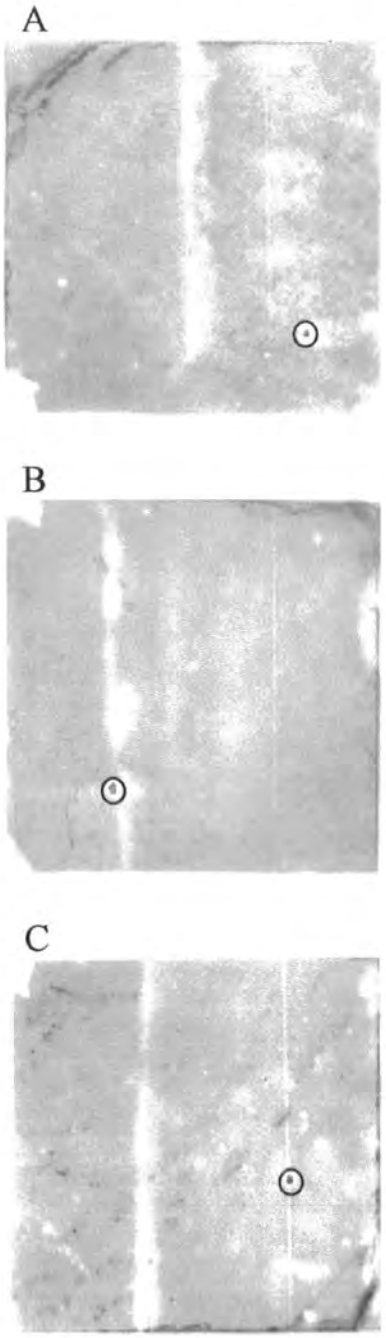
Table 5.1 Sequencing Results for 5 Random cDNA's from the Tx *Xenopus* Gut Library

cDNA's from 5 randomly isolated plasmids were sequenced from the 5' end and the sequences entered into BLAST searches to allow comparison with DNA and protein sequence databases. BLASTN searches were used to directly compare the cDNA sequence with the DNA sequence database whereas BLASTX searches compared the 6-frame conceptual translation of the cDNA to the protein database. The degree of similarity is indicated by the score and E-values, with a high score signifying similarity found over a long stretch of amino acids or bases and a low E-value indicating a low probability of a match being found by chance.

cDNA	BLAST search	Similarity Found	Accession Number	Score	E-value
*Xen*1	BLASTX	Mouse Keap1 protein	AB020063	79	2×10^{-15}
*Xen*2	BLASTX	Human OS-9 precursor protein	AB002806	62	3×10^{-9}
*Xen*3	BLASTX	Human GTP-binding protein	AF120334	405	1×10^{-113}
*Xen*4	BLASTN	Bluescript vector DNA	X52324	1223	0
*Xen*5	BLASTX	Mouse BimEL protein	AF032459	66	1×10^{-13}

Figure 5.6 Sequence Alignment of NKR-P1 Proteins from Various Vertebrate Species

Aligned amino acid sequences are shown for NKR-P1 receptor proteins from mouse, rat and human. Also included are NKR-P1 homologous sequences translated from cDNA's cloned from the Japanese Flounder (*Paralichthys olivaceus*) and the chicken (*Gallus gallus*). The one-letter amino acid code is used and consensus sequences (found in at least two species) are contained in shaded boxes. The sequences used for the design of forward and reverse PCR primers are highlighted.

Key: Forward primer [   ]   Reverse primer [   ]

```
1    - - - - - - - - - - - - - - - - - - - - - - - -              FLOUNDER
1    - - - - - - - - - - - - - - - - - - - P P A - - -            CHICKEN
1    M D S T T L V Y A D L N L A R I Q E P K H - D S P P S L S P   MOUSE
1    M D - T A R V Y L S L K P S K T A A G A Q C V S P P S L P P   RAT
1    M D Q Q A - I Y A E L N L P - T D S G P E S S S P S S L P R   HUMAN

1    - - - - - - - - - - - - - C S - - - - - - - - - -            FLOUNDER
4    - - - - A P H W A R L T K P G G C S A S T P C S M E P A G P   CHICKEN
30   D T C R C P R W H R L A L K F G C A G L I L L V L V V I G L   MOUSE
30   D A C R C P R S H R L A L K L S C A G L I L L V L A L V G M   RAT
29   D V C Q G S P W H Q F A L K L S C A G I I L L V L V V T G L   HUMAN

3    - - - - - - - - - - - - - - - - - - - - - - -                FLOUNDER
30   P P P P E P R V E V M S L S P - - - - - - - - - - - - - -     CHICKEN
60   C V L V - L S V Q K S S V Q K I C A D V Q E N R T H T T D C   MOUSE
60   S I L V R V L V Q K P S V E P - C R V L I Q E N L S K T G S   RAT
59   S V S V T S L I Q K S S I E K C S V D I Q Q S R N K T T E R   HUMAN

3    - - - - - E C P A G W F Q V A D Q C F Q I S T D R Q D W L T S FLOUNDER
45   A A C L L C P Q F W R L G D R C Y E L S T E K G N W T Q A     CHICKEN
89   S V N L E C P Q D W L S H R D K C F R V F Q V S N T W E E G   MOUSE
89   P A K L K C P K D W L S H R D K C F H V S Q T S I T W K E S   RAT
89   P G L L N C P I Y W Q Q L R E K C L L F S H T V N P W N N S   HUMAN

29   A N I C A L G G R H L A I L N T M A Q H E A V E K E S R R I   FLOUNDER
75   K M K C E N L Q S Q L A V L R K K A E E D H L Q Q M A G A E   CHICKEN
119  Q A D C G R K G A T L L L I Q D Q E E L R F L L D S I K E K   MOUSE
119  L A D C G G K G A T L L L V Q D Q E E L R F L R N L T K R I   RAT
119  L A D C S T K E S S L L L I R D K D E L I H T Q N L I R D K   HUMAN

59   G G F Y T H Y W I G L T D V E K E G E W K W V D N S T L E N   FLOUNDER
105  - - - P V - - W I G L E - - V S T N Q W K W V D N S S Y N S   CHICKEN
149  - - - Y N S F W I G L R F T L P D M N W K W I N G T T F N S   MOUSE
149  - - - S S S F W I G L S Y T L S D E N W K W I N G S T L N S   RAT
149  - - - A I L F W I G L N F S L S E K N W K W I N G S F L N S   HUMAN

89   P S W N I L K S E P D N N L S D G P K G E D C A V V D S R S   FLOUNDER
128  T E S D N L S - - - - - - V M - - - - E N R C G T F K N T K   CHICKEN
176  - - - D V L K - - - - - - I T G D T E N G S C A S I S G D K   MOUSE
176  - - - D V L S - - - - - - I T G D T E K D S C A S V S Q D K   RAT
176  - - - N D L E - - - - - - I R G D A K E N S C I S I S Q T S   HUMAN

119  Q S W Y D V P C S Y K Y P R I C Q M A A T P L H               FLOUNDER
148  V - - E G D V C S G E H Q W V C Q K E P L R L H - - - - - -   CHICKEN
197  V - - T S E S C S T D N R W I C Q K E L N H - - E T P S N D   MOUSE
197  V - - L S E S C D S D N I W V C Q K E L K C - - E C M C N D   RAT
197  V - - Y S E Y C S T E I R W I C Q K E L T P V R N K V Y P D   HUMAN

142                                                              FLOUNDER
170  P                                                           CHICKEN
223  S                                                           MOUSE
223  S                                                           RAT
225  S                                                           HUMAN
```

Figure 5.7 Design of Degenerate Primers to Amplify NKR-P1 DNA

The derivation of the NKR-P1 primers from chicken and flounder cDNA sequences which have homology to mammalian NKR-P1 receptors. The amino acid and DNA sequences for each organism are aligned and the primers designed from these sequences are shown. Brackets indicate bases excluded from the primer sequences. Note that the NK-reverse primer is anti-parallel to the chicken/flounder DNA sequence.

Flounder/Chicken (amino acid)

W   K   W   V   D   N   S

Flounder/Chicken (DNA)

5' TGG AAA TGG GTG GAC AAC TCA 3'
                                       C

NK-F (forward primer) (20-mer)

5' TGG AAA TGG GTG GAC AAC TC 3'

Flounder/Chicken (amino acid)

R/W   I/V   C   Q   M/K A/E

Flounder/Chicken (DNA)

5' CGG ATC TGT CAG ATA GAT 3'
    T   G   C   AG CA

NK-R (reverse primer) (16-mer)

3' CC TAG ACA GTC TAT CT 5'
          C   G   TC G

Figure 5.8   RT-PCR Reaction to Amplify NKR-P1 DNA from control *Xenopus* spleen RNA

1% agarose gel showing 2 RT-PCR reactions performed on *Xenopus* spleen RNA, using degenerate primers designed to amplify NKR-P1 DNA. The reverse transcriptase enzyme was omitted from the reaction shown in lane A to act as a control for the amplification of products from contaminating genomic DNA. Lane B contains the RT-PCR reaction in which reverse transcriptase was included and the possible products of specific amplification with the NKR-P1 primers are indicated. The DNA marker in the left hand lane is λPstI.
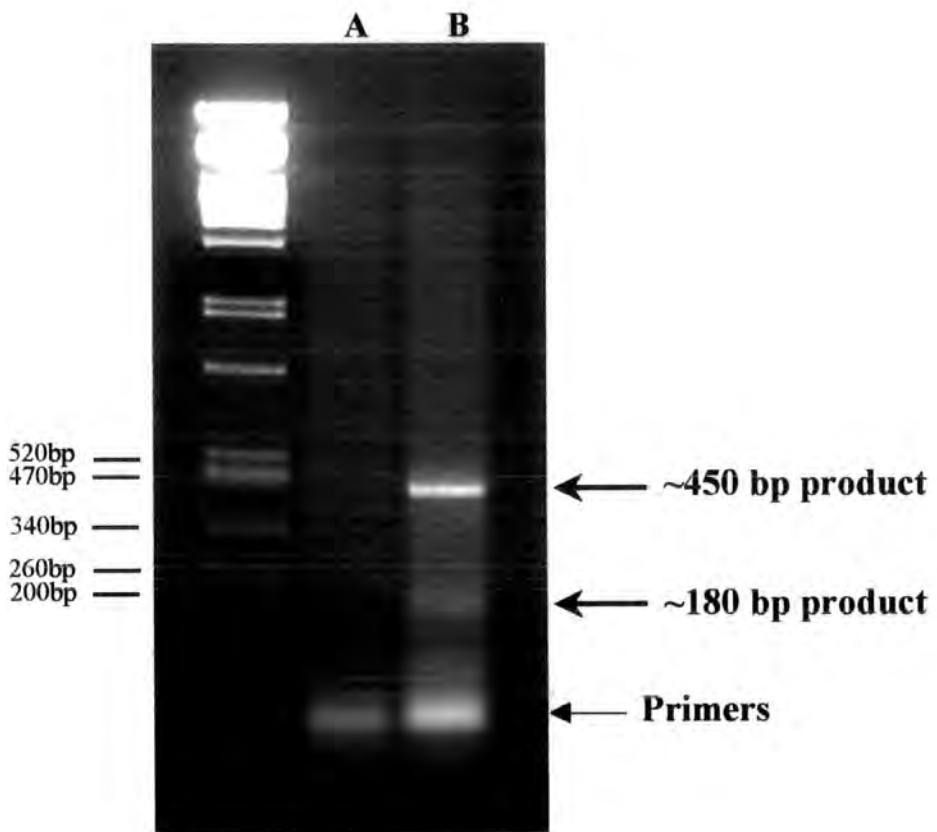
CHAPTER 6

Conclusions and Future Work

The aim of the work described in this Thesis was to raise antibodies to *Xenopus* NK cells with a view to the molecular characterisation of NK cell surface antigens. The main benefits of studying NK cell surface antigens in a comparative model such as *Xenopus* are twofold. Firstly, the identification of NK receptor proteins which are highly conserved through vertebrate evolution underlines their fundamental importance in the function of all NK cells. Secondly, the study of NK cell surface antigens in a lower vertebrate such as *Xenopus* may provide evidence for or against the idea that NK cells are the evolutionary forerunners of CTL as proposed recently (Janeway, 1989).

Before any attempts to raise anti-*Xenopus* NK cell antibodies by phage display were made, the technique was developed in Chapter 2 for the isolation of recombinant antibodies to novel cell surface antigens expressed by the *Xenopus* tumour cell line B_3B_7. This use of the phage display technique differed from previously published examples of phage display in several ways. Firstly, the majority of published examples have used large naïve or '*in-vitro* engineered' libraries, containing as many as 10^{10} different recombinant antibody fragments (reviewed in Sheets *et al.*, 1998). Secondly, many papers have concentrated on the optimisation of phage display for the isolation of antibodies to known, characterised antigens (for example see Nissim *et al.*, 1994). In addition, large amounts of purified antigen, typically 10-100µg, have been used for phage panning in conjunction with a sensitive screening method such as enzyme-linked immunosorbent assay (ELISA) for the detection of bound phage (Krebber *et al.*, 1997; Sheets *et al.*, 1998). In contrast, the protocol in Chapter 2 used panning of a relatively small, immune phage library (10^5 clones) on whole, live cells to enrich for phage which bound to unknown antigens on the cell surface. In this case the levels of surface antigen expressed by the cells would have been significantly lower than the amount of purified antigen used in previously published reports (for example see Krebber *et al.*, 1997). Additionally, flow cytometry, rather than ELISA, was used to screen phage for novel cell binding properties and this has only been described in a minority of publications (Siegel and Silberstein, 1994; De Kruif et al., 1995; Palmer et al., 1997).

The ability of this cell based panning and screening strategy to isolate a phage antibody, XLB-5, which bound the target cell B_3B_7 was demonstrated by several lines

of evidence, including FACS data, RFLP analysis of scFv DNA sequences and the monitoring of phage elution titres during panning. The confirmation of scFv/cpIII fusion protein expression by this phage antibody, using Western blotting, indicated that specific binding was occurring through surface scFv. Phage antibody XLB-5 was found to label approximately two-thirds of T cells, B cells and red blood cells which meant that its target antigen was common to many *Xenopus* cell types. However, as no such pattern of binding has been reported for other anti-*Xenopus* mAb's it appears that XLB-5 is specific for a novel antigenic determinant.

Following on from the optimisation of phage display through panning on the B_3B_7 tumour cell line, attempts were made in Chapter 3 to isolate phage display antibodies specific for *Xenopus* NK cells. Since the α-B_3B_7 antibody XLB-5 cross-reacted with several *Xenopus* cell types, an area which required improvement in order to raise an NK-specific phage antibody was the specificity of the phage selected by panning. Unfortunately, the relatively small size (5×10^5) of the phage library and the lack of a purified population of *Xenopus* NK cells for panning meant that an NK-specific mAb was not isolated. However, the phage antibody XL-6 which was isolated through panning did show specificity for *Xenopus* lymphocytes, which constituted an improvement on the level of specificity achieved in Chapter 2. Another major development was the production of soluble XL-6 scFv from bacteria, which showed the same specificity for *Xenopus* lymphocytes as the phage-displayed XL-6 scFv.

Flow cytometric testing of soluble XL-6 scFv on diverse lymphoid populations from *Xenopus* suggested that it may bind to an amphibian equivalent of the leukocyte common antigen CD45. Particularly interesting was the ability of XL-6 to identify a small population of thymocytes which were thought to represent mature T cells. This finding may be indicative of binding to an isoform of CD45 known as CD45RA which, in mammals, identifies a small subset of thymocytes which are located in the medulla and are thought to be committed to entering the periphery as mature T cells (Deans *et al.*, 1989; Gillitzer and Pilarski, 1990). Although anti-*Xenopus* class II antibodies preferentially stain medullary thymocytes (Flajnik *et al.*, 1990), there are few markers which differentiate thymocytes in *Xenopus* and it would therefore be of interest to investigate the antigen bound by XL-6. Firstly, in order to determine whether the XL-6 positive thymocytes are mature in phenotype, it would be of interest to co-stain thymic lymphocytes with XL-6 and an anti-CTX mAb, which identifies an immature population of *Xenopus* thymocytes (Chrétien *et al.*, 1996), and examine the cells by flow cytometry. If, as predicted, XL-6 identifies a mature thymic population, the two antibodies should not co-stain the same thymocytes. Secondly, despite the failure of

Western blotting with soluble or phage-displayed XL-6 to identify any specific proteins, it may be possible to use soluble XL-6 to immunoprecipitate the target antigen using techniques described previously (Sanna *et al.*, 1995; Sawyer *et al.*, 1997). Determination of the molecular weight of the antigen by this method would help resolve whether or not XL-6 is binding to an amphibian equivalent of the CD45 receptor.

In Chapter 4, the use of hybridoma technology to isolate 3 *Xenopus* NK-specific mAb's was described. FACS analysis of the mAb's 1F8, 4D4 and 1G5 demonstrated binding to a small proportion (5-10%) of spleen lymphocytes in control *Xenopus* and a higher proportion (15-20%) of spleen lymphocytes in Tx animals. Following immunomagnetic separation, 1F8-positive spleen cells from control and Tx animals were shown to be effective killers of the thymus tumour cell line B_3B_7. Further evidence from immunohistochemistry and more detailed FACS analysis has confirmed that 1F8 identifies a population of non-T, non-B lymphocytes which are similar in distribution, morphology and function to mammalian NK cells (Horton *et al.*, manuscript submitted).

One finding of interest resulted from the flow cytometric testing of 1F8 on human PBL, which suggested that this mAb has a specificity for human B cells. This perhaps indicates that the antigen bound by mAb 1F8 is a conserved molecule of importance to the immune systems of many species. As such, it would be interesting to use FACS analysis to test mAb's 1F8, 4D4 and 1G5 on lymphoid populations from other lower vertebrate and mammalian species. Another possibility, if the binding of 1F8 to human B cells is confirmed, would be to test this mAb on a human B cell line, such as an Epstein Barr virus transformed cell line. If 1F8 is shown to bind by flow cytometry, such a cell line could provide a useful source of mRNA and protein for the molecular characterisation of the human 1F8 antigen.

Having established that *Xenopus* NK cells were identified by the mAb's 1F8, 4D4 and 1G5, the surface antigens bound by these mAb's were investigated in Chapter 4 by Western blotting and immunoprecipitation. The results achieved through Western blotting demonstrated that all three mAb's could identify a doublet of protein bands at 72 and 74 kDa, whereas immunoprecipitations indicated that the three mAb's bound to proteins of between 55 and 60 kDa. The significance of these results is not yet known although the Western blotting results were repeated on more than ten occasions, whereas the immunoprecipitations identified a number of non-specific protein bands and were not repeated. Further immunoprecipitation experiments on enriched NK cell

populations from *Xenopus* may help to clarify the true molecular weights of these antigens.

Immunoprecipitation with the anti-*Xenopus* NK mAb's could also offer a means of isolating sufficient antigen for amino acid sequencing. The sequences obtained could be used to design degenerate PCR primers with which to amplify the encoding cDNA sequence from *Xenopus*. An alternative route to gaining cDNA sequence information was attempted in Chapter 5 by immunoscreening a lambda expression library with mAb 1G5. The library was constructed from the gut of a Tx animal which, through Western blotting experiments, was known to express a high level of the 1G5 antigen. Unfortunately the initial attempts at screening the library with 1G5 failed to identify any positive clones. As discussed in Chapter 5, improvements in the expression of *Xenopus* cDNA's and perhaps the use of polyclonal antibodies raised against the 1G5 antigen could permit cloning of the appropriate cDNA.

Chapter 5 also described an attempt to clone a *Xenopus* homologue of the mammalian NK cell surface receptor NKR-P1 by RT-PCR. Although an NKR-P1 homologue was not identified, attempts to amplify homologues of other mammalian NK receptor cDNA's with degenerate PCR primers may be worthwhile. In particular, the CD94 molecule is thought to be invariant (Lazetic *et al.*, 1996) and thus may be a good target for PCR amplification. Another possible target is the signal transducing molecule DAP-12 which appears to be closely linked to NK cell activation (Lanier *et al.*, 1998c).

To summarise, the work described in this Thesis demonstrates firstly that phage display technology is capable of raising phage antibodies to novel, unknown epitopes on whole, live cells. These phage antibodies and the soluble scFv molecules derived from them were able to identify distinct lymphoid populations in *Xenopus* using single and dual stain FACS analysis. Secondly, hybridoma technology was used to isolate three monoclonal antibodies which were shown to be specific for *Xenopus* NK cells. These antibodies offer a means of identifying and characterising *Xenopus* NK cell surface antigens which could eventually shed light on NK cell evolution and also the evolutionary relationship between NK and T cells.

Appendix 1

PCR Primers for the Amplification and Combination of Mouse VL and VH Antibody Regions

VL Primers

The VL forward and reverse primer sets are made by mixing the volumes (in μl) of each oligonucleotide indicated under 'Mix' to give the concentrations shown. The variable regions are indicated in capital letters (degenerate bases are denoted as follows: R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T) and the sequence encoding the 4 amino acid tag detected by the α-FLAG mAb is underlined. All oligonucleotide sequences are taken from Krebber *et al.*, 1997.

Primer VL Forward		Mix	Conc. (μM)
LB1	gccatggcggactacaaaGAYATCCAGCTGACTCAGCC	1	1.6
LB2	gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC	2	3.1
LB3	gccatggcggactacaaaGAYATTGTGMTMACTCAGTC	5	7.8
LB4	gccatggcggactacaaaGAYATTGTGYTRACACAGTC	3.5	5.4
LB5	gccatggcggactacaaaGAYATTGTRATGACMCAGTC	4	6.2
LB6	gccatggcggactacaaaGAYATTMAGATRAMCCAGTC	7	10.9
LB7	gccatggcggactacaaaGAYATTCAGATGAYDCAGTC	6	9.3
LB8	gccatggcggactacaaaGAYATYCAGATGACACAGAC	1.5	2.3
LB9	gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC	2	3.1
LB10	gccatggcggactacaaaGAYATTGWGCTSACCCAATC	3.5	5.4
LB11	gccatggcggactacaaaGAYATTSTRATGACCCARTC	8	12.4
LB12	gccatggcggactacaaaGAYRTTKTGATGACCCARAC	8	12.4
LB13	gccatggcggactacaaaGAYATTGTGATGACBCAGKC	6	9.3
LB14	gccatggcggactacaaaGAYATTGTGATAACYCAGGA	2	3.1
LB15	gccatggcggactacaaaGAYATTGTGATGACCCAGWT	2	3.1
LB16	gccatggcggactacaaaGAYATTGTGATGACACAACC	1	1.6
LB17	gccatggcggactacaaaGAYATTTTGCTGACTCAGTC	1	1.6
LBλ	gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC	1	1.6

Primer VL Reverse		Mix	Conc. (μM)
LF1	ggagccgccgccgcc(agaaccaccaccacc)₂ACGTTTGATTTCCAGCTTGG	1	23.5
LF2	ggagccgccgccgcc(agaaccaccaccacc)₂ACGTTTTATTTCCAGCTTGG	1	23.5
LF4	ggagccgccgccgcc(agaaccaccaccacc)₂ACGTTTTATTTCCAACTTTG	1	23.5
LF5	ggagccgccgccgcc(agaaccaccaccacc)₂ACGTTTCAGCTCCAGCTTGG	1	23.5
LFλ	ggagccgccgccgcc(agaaccaccaccacc)₂ACCTAGGACAGTCAGTTTGG	0.25	5.9

VH Primers

The VH forward and reverse primer sets are made by mixing the volumes (in μl) of each oligonucleotide indicated under 'Mix' to give the concentrations shown. The variable regions are indicated in capital letters (degenerate bases are denoted as follows: R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T) and the SfiI restriction sites within the VH reverse primers are underlined and in bold. All oligonucleotide sequences are taken from Krebber *et al.*, 1997.

Primer VH Forward

Primer VH Forward		Mix	Conc. (μM)
HB1	ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTC	4	7.6
HB2	ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC	4	7.6
HB3	ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSASTC	3	5.7
HB4	ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC	4	7.6
HB5	ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC	7	13.2
HB6	ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCAGTC	2	3.8
HB7	ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCAGTC	1	1.9
HB8	ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGAATC	2	3.8
HB9	ggcggcggcggctccggtggtggtggatccGAVGTGAWGYTGGTGGAGTC	5	9.5
HB10	ggcggcggcggctccggtggtggtggatccGAGGTGCAGSKGGTGGAGTC	2	3.8
HB11	ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGAGTC	2	3.8
HB12	ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC	2	3.8
HB13	ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGAGTC	1	1.9
HB14	ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGAGTC	2	3.8
HB15	ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGAGTC	2	3.8
HB16	ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG	5	9.5
HB17	ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC	3.5	6.6
HB18	ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAAGTGTC	0.7	1.3
HB19	ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGAGTC	0.7	1.3

Primer VH Reverse

Primer VH Reverse		Mix	Conc. (μM)
HF1	ggaattc**ggcccccgaggc**CGAGGAAACGGTGACCGTGGT	1	25
HF2	ggaattc**ggcccccgaggc**CGAGGAGACTGTGAGAGTGGT	1	25
HF3	ggaattc**ggcccccgaggc**CGCAGAGACAGTGACCAGAGT	1	25
HF4	ggaattc**ggcccccgaggc**CGAGGAGACGGTGACTGAGGT	1	25

Joining Primers

The primers used to combine VH and VL regions by SOE-PCR are shown below, with the SfiI site shown underlined in bold type and the sequence encoding the 4 amino acid FLAG tag is underlined (Krebber *et al.*, 1997)

ScBack	ttactcgc**ggccc**agcc**ggcc**atggcggactacaaaG	(100μM)
ScFor	ggaattcggcccccgag	(100μM)

Appendix 2

Phagemid Vectors for the Production of Phage-Displayed and Soluble scFv Molecules

pAK100

The pAK100 vector used for the cloning and expression of scFv molecules on the surface of bacteriophage M13 is shown, with the position of the scFv DNA insertion indicated (diagram taken from Krebber *et al.*, 1997). The following features of the plasmid are denoted:

ColE1: *E.coli* origin of replication

t$_{HP}$: terminator sequence

pelB: leader sequence

myc: tag recognised by α-myc mAb

gIII$_{250-406}$: truncated M13 gene III

f1 ori: phage origin of replication

LacI: *LacI* repressor gene

Lac p/o: *Lac* promoter/operator

tet: tetracycline resistance cassette

*: amber codon

t$_{lpp}$: downstream terminator sequence
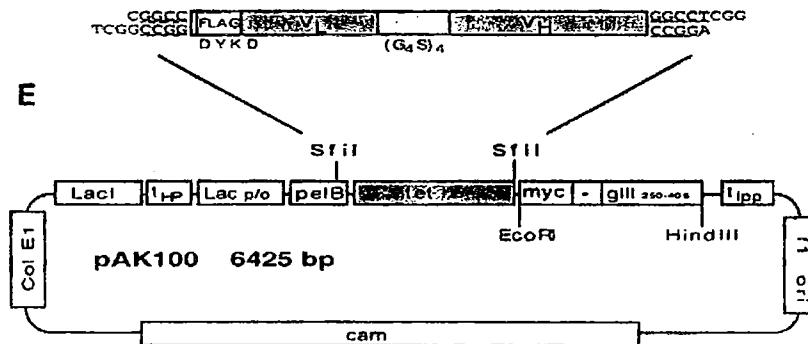
pAK400

The pAK400 vector used for the expression of soluble scFv molecules has the following adaptations from pAK100 (diagram from Krebber *et al.*, 1997):
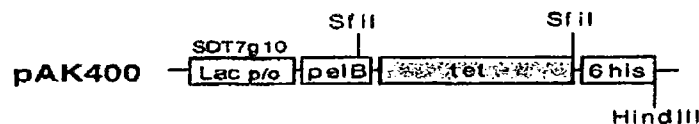
SDT7g10: altered Shine Dalgarno sequence for increased expression

6 his: poly-histidine tag for purification of scFv molecules

Appendix 3

Monoclonal Antibodies to markers on *Xenopus* Cells

The following anti-*Xenopus* mAb's were used during the course of these studies. Asterisks indicate purified mAb's which have been conjugated to phycoerythrin.

Antibody	Isotype	Dilution Used	Antigen (MW when reduced)	Cell Specificity	Reference
2B1 *	IgG1	1:100 (FACS)	CD5 (71-82kDa)	Pan T cell marker	Jürgens et al., 1995
D4-3 *	IgG1	1:200 (FACS)	Putative αβ TCR (40kDa & 60kDa)	Pan T cell marker	Ibrahim et al., 1991
F17 *	IgM	1:200 (FACS)	CD8 (30kDa)	T cell subset	Ibrahim et al., 1991
D12 *	IgG1	1:200 (FACS)	Putative γδ TCR (36 kDa)	Putative γδ T cells	Ibrahim et al., 1991
XT-1	IgG2	1:2 (Western)	XTLA-1 (120kDa)	T cell subset	Nagata, 1986
8E4	IgG	1:30 (MACS)	IgM (25kDa & 75kDa)	Pan B cell marker	Langeberg et al., 1987
D8 *	IgG3	1:100 (FACS)	IgM (25kDa & 75kDa)	Pan B cell marker	Jürgens et al., 1995
10A9	IgG	1:5 (IP)	IgM (25kDa & 75kDa)	Pan B cell marker	Du Pasquier & Hsu, 1983
X71	IgG	1:5 (IP)	CTX (55kDa)	Immature thymocyte	Chrétien et al., 1996
TB17	IgG	1:5 (IP)	Class I MHC (40-45kDa)	All Xenopus cells	Flajnik et al., 1991
XLH-1	IgM	1:4 (FACS)	Not characterised	Thrombocyte	Horton (unpublished)

Abbreviations used in this table:

FACS: Fluorescence Activated Cell Sorting
MACS: Magnetically Activated Cell Sorting
IP: Immunoprecipitation

Appendix 4

Oligonucleotides Used in cDNA Library Construction

Linker-primer

The following oligonucleotide, supplied by Stratagene, was used to synthesise first strand cDNA from mRNA templates. The 'GAGA' sequence is designed to protect the XhoI restriction site which is used for the directional cloning of cDNA's into the Uni-ZAP XR vector. The poly dT sequence anneals to the poly-A tail of mRNA molecules and initiates DNA synthesis.

5' GAGAGAGAGAGAGAGAGAGAACTAGT<u>CTCGAG</u>TTTTTTTTTTTTTTTTTTT 3'
      'GAGA' sequence   XhoI   poly dT

EcoRI Adapters

The *EcoRI* adapters, consisting of the 9- and 13-mer oligonucleotides shown below, are ligated onto the blunt ends of the cDNA molecules to give them *EcoRI* cohesive ends (underlined). These oligonucleotides were also supplied by Stratagene.

5' <u>AATT</u>CGGCACGAG 3'
3' GCCGTGCTC 5'

Appendix 5

The Uni-ZAP XR cDNA Expression Vector

The Uni-ZAP XR Vector (Stratagene) is supplied double digested with *XhoI* and *EcoRI* for directional cloning of cDNA inserts of up to 10 kilobases. cDNA inserts are cloned into the β-galactosidase gene, allowing blue-white colour selection of recombinants. Inserts which are in frame with the *lacZ* sequence can be expressed as fusion proteins and detected by antibody screening.

Lambda ZAP® II vector
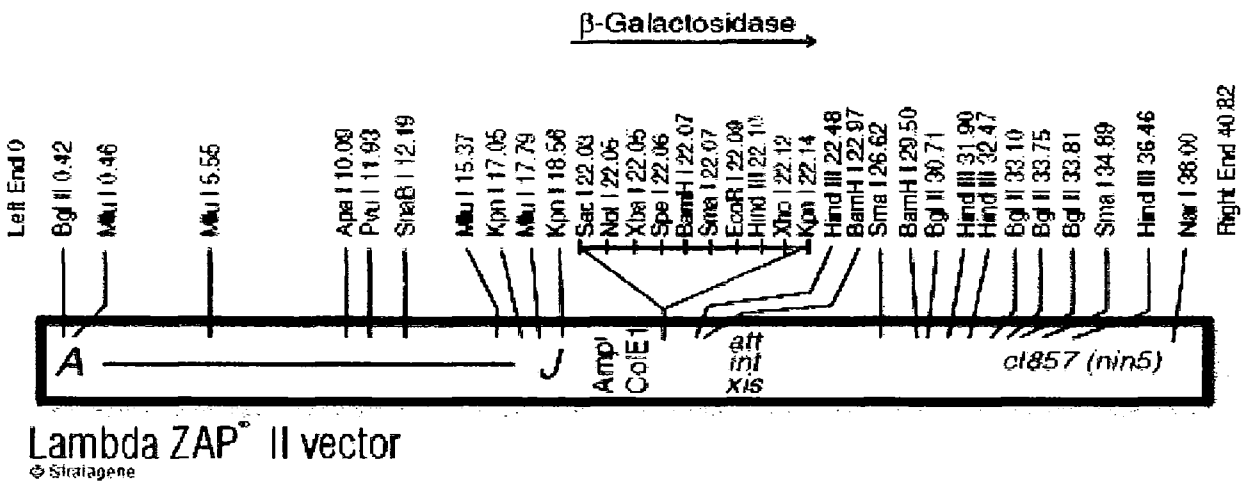
In-vivo excision of the pBluescript phagemid (Stratagene) from the Uni-Zap XR vector can be achieved in SOLR *E. coli* (Stratagene) following co-infection with ExAssist helper phage (Stratagene).
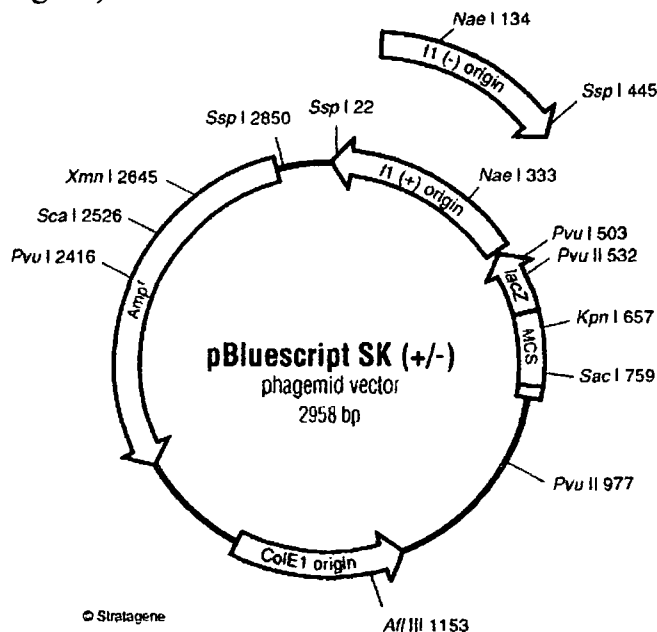
All information and diagrams taken from Stratagene internet site (http://www.stratagene.com)

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