THE ROLE OF FAS SIGNALLING AND THE C-*MYC* ONCOGENE IN T CELL APOPTOSIS AND TRANSFORMATION

by

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SUMMARY

Cancer is a disease characterised by disruption of apoptotic pathways and inappropriate expression of survival or proliferative signals. Despite its role as a major apoptotic pathway in T cells however, the role of Fas in lymphomagenesis is not clear. To investigate the role of Fas in *MYC* induced lymphomagenesis, animals harbouring a c-*MYC* transgene, with expression targeted to the T cell lineage, were placed on a Fas^{lpr} background. Loss of Fas did not alter the incidence, latency or phenotype of thymic lymphomas arising in these mice. In addition, the incidence and latency of lymphomas in Fas^{lpr} mice infected with MuLV was not significantly different from strain controlled mice. Further, the proportion of lymphomas with retroviral insertions at c-*myc* was not increased in Fas^{lpr} mice. These results indicate that Fas does not act to restrict tumourigenesis, at least in the T cell lineage.

Previous studies have reported that MYC induced apoptosis can occur through Fas and p53 signalling pathways. However loss of Fas did not inhibit MYC induced apoptosis in normal or neoplastic T cells, indicating that MYC induced apoptosis can occur by a Fas independent pathway. Furthermore, MYC induced apoptosis could occur in the combined absence of both Fas and p53 apoptotic pathways. Although loss of these two major apoptotic pathways did not prevent MYC induced apoptosis, protection from MYC induced apoptosis was observed with cell contact in some cell lines. This protection was shown to dependent on a PI3 kinase pathway. In addition, in cell lines that retained functional p53, the PI3 kinase/Akt survival signal was shown to be critical for cell survival.

The response to T cell receptor activation in Fas^{lpr} thymocytes was also examined. In addition to lacking the ability to undergo activation induced cell death, a proliferative defect was revealed in Fas^{lpr} thymocytes, compared with control MRL thymocytes. This defect was rescued by co-stimulation of the cell surface marker CD28. The role of Fas:FasL interactions in tumourigenesis may depend on the cell types in which Fas and FasL are expressed. Signalling through Fas or FasL may be important in the transduction of proliferative signals in T cells, and this may explain why disabling the Fas pathway does not appear to influence T cell lymphomagenesis.

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DECLARATION

I hereby declare that the work presented in this thesis is original and was conducted by the author under supervision except where stated.

I certify that no part of this thesis has been submitted previously for the award of a degree to any University but has been reproduced in parts in the following scientific paper:-

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ABBREVIATIONS

AICD	activation induced cell death
ALG	apoptosis linked gene
ALPS	autoimmune lymphoproliferative disorder
AML	acute myeloid leukaemia
Apc	adenomatous polyposis coli
APC	antigen presenting cell
ATM	ataxia telangiectasia mutated
b/HLH/LZ	basic domain/helix-loop-helix/leucine zipper
BMTC	bone marrow T cell
bp	base pair
BSA	bovine serum albumin
°C	degrees Celsius
$CaCl_2$	calcium chloride
CD	cluster designation
cdk	cyclin-dependent kinase
cDNA	complementary DNA
CH_3COONa	sodium acetate
CIP	cdk-interacting protein
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
DcR	decoy receptor
DED	death effector domain
DISC	death inducing signalling complex
$\Delta\Psi_m$	mitochondrial transmembrane potential
DMEM	Dulbecco's Modified Eagle Medium
DN	dominant negative
DNA	deoxyribonucleic acid
DR	death receptor
dsDNA	double stranded DNA

ECL	electrochemical luminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
Eμ	immunoglobulin heavy chain enhancer
ER^{TM}	oestrogen receptor, tamoxifen modified
ERK	extracellular signal regulated kinase
ES cells	embryonic stem cells
ETn	early transposable element
FADD	Fas-associating protein with death domain
FAK	focal adhesion kinase
Fas^{gld}	homozygous for Fas^{gld}
Fas^{lpr}	homozygous for Fas^{lpr}
$Fas^{lpr/-}$	heterozygous for the Fas^{lpr} mutation
FasL	Fas ligand
FITC	fluoroscein isothiocyanate
FLASH	FLICE-associated huge protein
FLICE	FADD-like ICE
FLIP	FLICE inhibitory protein
GADD45	growth arrest DNA damage inducible gene 45
gld	generalised lymphoproliferation
GSK-3	glycogen synthase kinase 3
HA	hyaluronic acid
HCl	hydrochloric acid
HRP	horseradish peroxidase
HSA	heat stable antigen
ICE	interleukin-1β converting enzyme
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IGF	insulin like growth factor
Il	interleukin
ILK	integrin linked kinase
JNK	c-Jun NH_2-terminal kinase
kb	kilobase

KCl	potassium chloride
kD	kilodalton
L	litre
LCR	locus control region
lpr	lymphoproliferation
M	molar
MACH	MORT-1 associated CED 3 homologue
MAPK	mitogen activated protein kinase
MDM2	murine double minute 2
MEF	mouse embryo fibroblast
MEK	MAP kinase/ERK kinase kinase
MHC	major histocompatibility complex
Mom-1	modifier of *Min-1*
μg	microgram
μCi	micro Curie
mg	milligram
$MgCl_2$	magnesium chloride
μM	micromolar
mM	millimolar
MIA	MYC induced apoptosis
MoMuLV	Moloney Murine Leukaemia Virus
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NF-κB	nuclear factor κB
ng	nanogram
NGF	nerve growth factor
NK	natural killer
4-OHT	4-hydroxy-tamoxifen
OPGL	osteoprotegerin ligand
Trp53-/-	homozygous for the *Trp53* null mutation (murine)
Trp53+/-	heterozygous for the *Trp53* null mutation (murine)
PBS	phosphate buffered saline

PBTC	peripheral blood T cell
PCR	polymerase chain reaction
PI3 kinase	phosphotidylinositol 3-kinase
PT	permeability transition
PTEN	phosphatase and tensin homologue deleted from chromosome 10
RAG	recombination activating gene
RANK	receptor activator of NF-κB
RB	retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
SEB	staphylococcal enterotoxin B
SID	Sin3-interacting domain
SV40	simian virus 40
TCR	T cell receptor
THANK	TNF homologue that activates apoptosis
TGF	transforming growth factor
TNF	tumour necrosis factor
TNF-R	TNF receptor
TRAIL	TNR-related apoptosis inducing ligand
TRANCE	TNF-related activation induced cytokine
TRRAP	transformation/transcription domain associated protein
UV	ultraviolet
V	volts
VDAC	voltage dependent ion channels
VSMC	vascular smooth muscle cell
WAF1	wild type p53-activated fragment 1
whn	winged-helix nude

CHAPTER 1

INTRODUCTION

1.1 CANCER AND GENETICS

Cancer is the broad term for a group of diseases that are characterised by inappropriate and uncontrolled cell proliferation. In general, cancers are caused by clonal expansion of a single somatic cell in which deregulation of the mechanisms that control cell survival and proliferation has occurred. It is now widely recognised that disturbance of the cell cycle in tumour cells is triggered by a series of mutational events. There are several factors which may influence the process of transformation. Elements outwith the cell can affect the probability that mutational events will occur, for example environmental or lifestyle factors such as diet, hormones, radiation and carcinogens such as those found in cigarette smoke and asbestos (reviewed by Tominaga, 1999). By products of normal cellular metabolism such as free hydroxyl radicals derived from oxidative respiration and lipid metabolism also constitute a possible threat to DNA integrity (Cadet *et al.*, 1997). In addition, some infectious pathogens have been causally related to cancers; in particular, human papillomaviruses have been detected in virtually all cervical cancers (Walboomers *et al.*, 1999), and Epstein Barr virus has been linked with B cell malignancies (reviewed by Oudejans *et al.*, 1997). Inside the cell, genetic abnormalities may occur as a result of failure to repair mistakes made during DNA replication (reviewed by Hoeijmakers, 2001). In some cases, predisposition to cancer may be a consequence of inherited mutations in genes which may influence cell survival and proliferation (reviewed by Ponder, 1990). Tumourigenesis is a multi-step process however, and further events are required in these susceptible individuals if transformation is to occur.

The genetic lesions which occur during transformation must convey on the cell the ability to override the normal controls on proliferation and survival. A model has recently been proposed by Hanahan and Weinberg describing six essential categories of genetic alteration which dictate progression towards malignancy (Hanahan &

Weinberg, 2000). In order to overcome the anti-cancer defence mechanisms present in all cells and tissues, cells must lose their dependence on normal growth signalling, while becoming insensitive to anti-growth signals. Cells which progress towards malignancy must also acquire genetic alterations which allow them to exceed their normally finite replicative potential. Since the rate of expansion of tumour cells depends not only on proliferation, but also on cell death, tumour cells must also develop the ability to evade signals to die. In addition, survival of a growing tumour requires angiogenesis to provide a sustained blood supply. Finally, to escape constraints on space and nutrients, it is advantageous for tumour cells to acquire the ability to invade and colonise new tissues, a process known as metastasis.

Although several mutagenic events occur during the process of transformation, in almost all cases, deregulation of proliferation and inhibition of death provide the foundation for clonal expansion. The genes whose alteration bring about the development of malignancy are broadly categorised as either oncogenes or tumour suppressor genes. Gain of function of an oncogene may contribute to tumour development by promoting cell growth, proliferation or survival, while loss of function of a tumour suppressor gene is likely to contribute to malignancy by resulting in loss of the ability to suppress proliferation or promote cell death.

1.2 APOPTOSIS

Multicellular organisms must have the ability to tightly control cell numbers, in order to maintain homeostasis. As well as having the capacity to divide, cells must also be able to mediate their own death, by a process known as apoptosis. This kind of programmed cell death, or apoptosis, occurs throughout development, and is essential for maintaining homeostasis in a number of cell systems, particularly the immune system (Osborne, 1996). Too much cell death however, may lead to developmental disorders or degenerative disorders such as Huntingdon's disease (Portera-Cailliau *et al.*, 1995). Failure of apoptosis might result in autoimmune diseases (Strasser *et al.*, 1991; Rieux-Laucat *et al.*, 1995) or cancer (reviewed by Kerr *et al.*, 1994).

The ability of a tumour cell population to expand depends not only on the rate at which those cells proliferate, but also on the rate of cell death. Under normal circumstances, stress signals in a cell, for example DNA damage, hypoxia, absence of survival factors, or abnormal proliferation as a result of oncogenic activation, lead to death of that cell by apoptosis (reviewed by Hoeijmakers, 2001). It is widely acknowledged however, that most, if not all cancers have evolved to evade apoptosis. Inability to induce apoptosis in response to oncogene-associated stress signals early during tumour development may be sufficient to allow progression of malignancy, while intact apoptotic pathways should facilitate clearance of cells undergoing early transformation events. This is undoubtedly an oversimplification of the complex interaction of events that ultimately result in neoplasia. High levels of apoptosis are not uncommon in end-stage malignant tumours (reviewed by Papac, 1998), and the likelihood is that tumours are composed of proliferating and apoptotic cells, the balance of which must shift towards proliferation to allow tumour outgrowth.

The morphological and molecular changes associated with apoptosis have now been extensively characterised. Electron microscopy has shown that apoptotic cells often undergo cytoplasmic shrinkage and plasma membrane blebbing (Kerr *et al.*, 1972). Their chromosomes rapidly condense and aggregate around the nuclear periphery, forming small apoptotic bodies (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). More recently, swelling of the outer mitochondrial membrane and release of cytochrome *c* into the cytoplasm from the mitochondrial intermembrane space has also been reported (Kluck *et al.*, 1997; Vander *et al.*, 1997; Yang *et al.*, 1997a). In addition, internucleosomal cleavage of the DNA occurs, generating a characteristic ladder of DNA fragments which can be used to identify apoptotic cells (Wyllie *et al.*, 1980). Disruption of the plasma membrane causing randomisation of phosphatidyl serine between the inner and outer leaflets of the membrane is another feature of apoptosis which can be used to detect apoptotic cells (Koopman *et al.*, 1994; Fadok & Henson, 1998).

The basic components of the apoptotic machinery can be split into two broad categories, namely sensors and effectors. Sensors are responsible for monitoring the intracellular and extracellular environment for changes which affect the decision of

the cell to live or die. The sensors are then responsible for regulation of the effectors of cell death. Among the sensors of cell death are death receptors, which transmit a signal to the cell to die, when bound by death ligand. Examples of these death ligands are tumour necrosis factor (TNF), and other members of the TNF family, Fas ligand, and TNF related apoptosis inducing ligand (TRAIL). The p53 tumour suppressor protein is also capable of eliciting cell death by upregulating the pro-apoptotic molecule Bax which in turn stimulates the mitochondria to release cytochrome *c* (Miyashita & Reed, 1995; Schuler *et al.*, 2000).

Most effectors of apoptosis have downstream signals in common however, as they share as central executioners of the apoptotic pathway a group of cysteine proteases, collectively known as caspases (reviewed by Kidd, 1998). Ligation of death receptors, and/or cytochrome *c* release, activates a cascade of caspases which finally results in cleavage of cellular DNA and destruction of subcellular structures and organelles (reviewed by Hengartner, 2000). The idea that apoptosis may act as a natural barrier to tumourigenesis was conceived when massive apoptosis was observed in the cells of rapidly growing hormone-dependent tumours, following hormone withdrawal (Kerr *et al.*, 1972). It is thought that apoptosis must be blocked, or at least overcome, before oncogenic activation can lead to malignancy, and that some apoptotic sensors and effectors therefore act, at least in part, as tumour suppressors.

1.3 ONCOGENES

Oncogenes were originally discovered when studies of many tumour retroviruses revealed that specific genes were responsible for malignancy. Initially, these genes were believed to be viral genes, until the first oncogene was cloned. Following cloning of v-*src*, from the Rous sarcoma virus which affects chickens, it was discovered that a very close relative of v-*src* was present in normal avian DNA (Stehelin *et al.*, 1976). Further analysis in other species revealed that relatives of this gene are present in other vertebrates (Spector *et al.*, 1978). These findings led to the conclusion that the retroviral genes which cause cells to be transformed are in fact

derived from normal cellular genes. These 'normal' genes became known as proto-oncogenes; genes which could become oncogenes when deregulated by viral integration. The term proto-oncogene can refer to any gene which becomes oncogenic as a result of viral integration, or somatic mutation of the cellular DNA in uninfected cells. Most oncogenes typically act in a dominant manner, so mutation of one allele is usually sufficient to contribute to tumourigenesis.

Because oncogenes lead to malignancy in the cells in which they are expressed, they are generally derived from mutations which cause either amplification or deregulated expression of a gene encoding a protein that enhances cellular growth, proliferation, or cell survival. The most common proto-oncogenes are therefore those encoding growth factors or their receptors, intracellular signal transducers, or transcription factors. A typical example of a proto-oncogene is the c-*MYC* gene, which encodes a transcription factor involved in regulating cell proliferation (Dang *et al.*, 1999). Mutations resulting in overexpression of this gene have been found in many human and animal cancers (Nesbit *et al.*, 1999).

1.4 THE C-*MYC* ONCOGENE

The c-*MYC* gene belongs to a family of genes, that also includes L-*MYC*, N-*MYC*, B-*myc* and s-*myc* (Nau *et al.*, 1985; Slamon *et al.*, 1986; Ingvarsson *et al.*, 1988; Sugiyama *et al.*, 1989). Of these however, only c-*MYC*, L-*MYC* and N-*MYC* have been associated with malignancy (reviewed by DePinho *et al.*, 1991; Nesbit *et al.*, 1999). The first of these oncogenes to be identified was c-*MYC*. In 1978, a retroviral oncogene, v-*myc* was identified in avian tumours (Duesberg *et al.*, 1977). Subsequent research showed that, in common with many oncogenes identified in retrovirally transformed cells, there was a cellular homologue of this gene, referred to as c-*MYC* (Sheiness *et al.*, 1980; Vennstrom *et al.*, 1982). Since then, the c-*MYC* proto-oncogene has been shown to be deregulated in a wide range of human and animal malignancies (Spencer & Groudine, 1991; Dang *et al.*, 1999). Although identified as an oncogene however, it became clear that c-*MYC* was also vital for normal development, and for regulation of cell proliferation. Mice nullizygous for c-

myc cannot survive past embryonic day 10.5 (Davis *et al.*, 1993). The reason for this may be that c-*myc* plays a pivotal role in cell proliferation. Highly elevated levels of c-*MYC* mRNA have been observed in lymphocytes in response to mitogenic stimulation (Kelly *et al.*, 1983), and anti-c-*MYC* antibody has been shown to block DNA replication in HL-60 cells *in vitro* (Iguchi-Ariga *et al.*, 1987). In addition, inactivation of c-*myc* in fibroblast cell lines was shown to cause apparent constraint of the cell cycle, and accumulation of cells in G1 and G2M phases, in addition to a decreased rate of proliferation (Mateyak *et al.*, 1997). Over the past decade research has predicted a role for c-*MYC* not only as a crucial regulator of proliferation, but also of differentiation, cellular metabolism, and apoptosis (reviewed by Dang *et al.*, 1999).

1.4.1 The c-*MYC* Gene and c-MYC Protein

The c-*MYC* gene is comprised of three exons located on chromosome 8q24 in humans (Neel *et al.*, 1982). The major product of the c-*MYC* gene is c-MYC2, a 64kDa polypeptide, translation of which is initiated at an AUG start codon on exon 2 (Ramsay *et al.*, 1984). A second longer product, c-MYC1 results from initiation of translation at a CUG codon on exon 1, 15 codons upstream of the major start site (Hann *et al.*, 1988). Both products are expressed in all normal cells examined so far, although the synthesis of these two proteins is differentially regulated, with c-MYC2 transcripts accounting for 75-90% of the c-*MYC* mRNA (Hann & Eisenman, 1984). In normal cells, expression of c-MYC2 is responsible for the majority of c-MYC activity in growing cells, however as the cells approach high density, synthesis of c-MYC1 increases to levels equal or greater than those of c-MYC2 (Hann *et al.*, 1992).

The ratio of the two c-MYC proteins has been reported to be disrupted in several tumour cell types, either as a result of rearrangement of the c-*MYC* gene, by chromosomal translocation, or mutation, or by increased synthesis of c-MYC2 in some tumour cells (Hann & Eisenman, 1984; Hann *et al.*, 1988). As yet the reason for this is unclear, although the two forms of the MYC protein are functionally distinct (Hann *et al.*, 1994). It has been reported that overexpression of c-MYC1 but not c-MYC2 can inhibit cell growth (Hann *et al.*, 1994). In tumours, deregulation of

c-*MYC* frequently occurs by separation of the c-*MYC* coding region from its regulatory elements, by translocation or proviral insertion for example. These rearrangements often bring about a loss of exon 1 sequences which are required for transcription of c-MYC1 (Blackwood *et al.*, 1994). It may be that, loss of c-MYC1, or an increase in the ratio of c-MYC2 protein has functional significance for cells undergoing malignant transformation. In normal cells, expression of c-MYC is tightly controlled by the cellular environment (Kelly *et al.*, 1983), but it is possible that the ability of c-*MYC* to encode two functionally distinct proteins at specific stages of cell growth represents a further level of control over c-MYC function.

1.4.2 The c-MYC Protein and its Binding Partner MAX in the Control of Transcription

The c-MYC protein is the main member of the MYC family of bHLH/LZ proteins which regulate proliferation and apoptosis. Evidence so far suggests that c-MYC functions chiefly as a transcription factor to mediate its effects (Dang *et al.*, 1999). In common with the other MYC family members, c-MYC is comprised broadly of three sections, with the crucial regions required for its transcriptional activities located at its terminal domains.

Contained within the first 143 amino acids of the N terminal domain is the transactivation domain required for transcriptional regulation (Kato *et al.*, 1990). The mechanism by which the N terminal domain activates transcription appears to be through an N terminal interacting protein called TRRAP (for transformation/ transcription domain associated protein, McMahon *et al.*, 1998), which is part of a complex containing histone acetyl transferases which have been implicated as transcriptional co-activators (Utley *et al.*, 1998). The N terminal domain may have more than one function however, since a role for the N terminal domain of c-MYC in transcriptional repression has also been described (Lee *et al.*, 1996). Repression of growth arrest genes might be another way in which MYC mediates proliferation and transformation.

The C terminal domain contains a dimerisation domain consisting of a basic domain/helix-loop-helix/leucine zipper (bHLH/LZ) motif. The basic domain is responsible for direct contact with DNA through specific recognition of CACGTG sequences in the target genes (Blackwell *et al.*, 1990; Prendergast & Ziff, 1991). c-MYC is unable to bind DNA alone however, and requires oligomerisation with a binding partner to bind DNA effectively (Kato *et al.*, 1992). An HLH/LZ protein called MAX was identified as a binding partner for c-MYC in humans (Blackwood & Eisenman, 1991), and the murine homologue of MAX, known as myn, was shown to have the same function (Prendergast *et al.*, 1991).

Initially it was expected that MYC-MAX heterodimers bound to target DNA to activate transcription, and that MAX-MAX homodimers repressed the activity of MYC by competing with MYC-MAX heterodimers for DNA binding sites on target genes (Kato *et al.*, 1992). This theory was complicated somewhat by the discovery of a third family of proteins, the MAD proteins, which are also able to bind MAX (Ayer *et al.*, 1993). MAD-MAX heterodimers are also able to repress the transcriptional activity of MYC. This transcriptional silencing by MAD proteins has been attributed to their ability to recruit histone deacetylases through their SID (Sin3-interacting domain) motif (Ayer *et al.*, 1995; Heinzel *et al.*, 1997). Histone deacetylation, which results in remodelling of the chromatin into a closed conformation, has been reported to be the major mechanism by which MAD proteins repress transcription (Sommer *et al.*, 1997). These results have led to formation of a model in which MYC activity is regulated by MAD proteins, due to the competition between MYC and MAD for binding with MAX (Baudino & Cleveland, 2001). The fact that there are at least 4 MAD family members and two other distantly related bHLH/LZ factors, mnt and mga, all of which effectively antagonise MYC function (Baudino & Cleveland, 2001), emphasises the importance of tight regulation of MYC to maintain normal cell function.

Transcriptional activation by c-MYC is central to its function. The understanding of exactly how this is achieved has been the subject of a great deal of research. A number of factors have now been reported to interact with c-MYC (reviewed by Sakamuro & Prendergast, 1999). Some of these may facilitate direct interaction of c-

MYC with the transcriptional machinery, for example the TATA-binding protein (Hateboer *et al.*, 1993; Maheswaran *et al.*, 1994; McEwan *et al.*, 1996). Whether or not each factor which binds c-MYC can regulate its transcriptional activity is not yet determined, since the mechanisms by which MYC can regulate transcription are still not clearly defined. One of these factors, TRRAP, is a likely candidate as a mediator of the transactivational properties of MYC. Binding to TRRAP has been reported to allow c-MYC to regulate transcription, at least in part, by regulation of histone acetylation (Cole & McMahon, 1999). The ability of MYC-MAX heterodimers to bind chromatin-bound target sites, in addition to free DNA, supports the hypothesis that MYC-MAX complexes can promote transcription through site-specific histone acetylation and contact with the basal transcriptional machinery (Wechsler *et al.*, 1994). A number of c-MYC target genes have now been identified (Grandori & Eisenman, 1997), and analysis of these has been vital to gaining understanding of the mechanism by which c-MYC induces both malignant transformation and apoptosis.

1.4.3 c-MYC and Cell Cycle Regulation

The potency of c-*MYC* as an oncogene relies upon the fact that c-MYC is a powerful inducer of proliferation, through its ability to regulate the cell cycle. For many years studies have linked functional MYC expression to cell cycle progression. Ectopic expression of c-MYC has been reported to drive G0 phase quiescent cells back into cycle (Eilers *et al.*, 1991), and over expression of c-MYC in cycling cells has been shown to shorten G1 phase and reduce growth requirements (Karn *et al.*, 1989). Expression of c-*MYC* appears to correlate closely with proliferation. In quiescent cells, c-*myc* expression is absent, but is rapidly induced following addition of growth factors (Dean *et al.*, 1986). Subsequent removal of growth factors results in immediate down-regulation of c-*myc* expression (Dean *et al.*, 1986). Inactivation of c-*myc* in rat fibroblast lines confirmed the importance of myc function in driving the cell cycle, since these cells exhibit significantly impaired growth (Mateyak *et al.*, 1997). The profound influence that c-MYC has on the cell cycle has led to intensive studies into the mechanisms by which c-MYC interacts with the cell cycle machinery.

Regulation of the cell cycle by c-MYC has been attributed to its ability to transcribe a number of genes involved in cell cycle progression, for example the cyclins and the cyclin dependent kinases (cdks, Jansen-Durr *et al.*, 1993; Daksis *et al.*, 1994; Barrett *et al.*, 1995; Rudolph *et al.*, 1996; Perez-Roger *et al.*, 1997). Quiescent cells differ from proliferating cells in that they do not transcribe those genes. The correlation between expression of *MYC* and cell cycle progression led to the hypothesis that c-MYC was able to initiate transcription of several of these genes (reviewed by Obaya *et al.*, 1999). The relationship between c-MYC and the cell cycle is complicated however. Control of the cell cycle by c-MYC has been limited mainly to progression from G0 through to G1/S phase. Overexpression of c-MYC shortens G1 phase (Karn *et al.*, 1989), and a requirement for c-myc to drive the G0-G1/S transition has been reported in hepatocytes (Skouteris & Schroder, 1996). Although cyclin D1 plays a major role in progression through G1 (Baldin *et al.*, 1993), and follows c-MYC expression, there are conflicting reports in the literature of the association between c-MYC and cyclin D1 expression, and more research is required to identify the role played by c-MYC in regulation of cyclin D1 and cyclin D1-cdk4 complexes (reviewed by Obaya *et al.*, 1999). More conclusive evidence has come from studies of c-MYC regulation of the cyclin E-cdk2 complex, and cyclin A. Rapid induction of cyclin E-cdk2 activity, in response to activation of c-Myc has been reported (Steiner *et al.*, 1995), and inhibition of cdk2 has been shown to block all downstream responses to Myc in the cell cycle, including transcription of cyclin A (Rudolph *et al.*, 1996). The evidence suggests that c-Myc activates cyclin E-cdk2 complex activity indirectly, particularly since expression of dominant negative c-*myc* induces arrest of the cell cycle, while the cells maintain high levels of cyclin E and cdk2 (Berns *et al.*, 1997). One candidate for direct transcriptional activation by c-MYC is the cdc25A gene, which encodes a phosphatase that removes two inhibitory phosphate groups from cdk2 (Galaktionov *et al.*, 1996; Pusch *et al.*, 1997). c-MYC also decreases the levels of the $p27^{Kip1}$ cdk inhibitor (Perez-Roger *et al.*, 1997), and growth arrest of cells due to ectopic expression of $p27^{Kip1}$ is repressed by overexpression of c-Myc (Steiner *et al.*, 1995; Vlach *et al.*, 1996). These results suggest that c-Myc may also transcribe some protein involved in regulating the metabolism or degradation of $p27^{Kip1}$, or inhibiting its binding to cdk2 complexes. Recently activation of Myc was reported to induce expression of cyclin D2, through

TRRAP recruitment and histone acetylation at the cyclin D2 promoter (Bouchard *et al.*, 1999; Bouchard *et al.*, 2001). Sequestration and subsequent destruction of p27 at cyclin D2/cdk4 complexes has been implicated as another mechanism by which c-Myc can regulate cellular proliferation (Bouchard *et al.*, 1999). There is now a growing body of evidence that c-MYC regulation of the cell cycle can occur at multiple levels.

1.4.4 c-MYC and Apoptosis

The central role of c-MYC in the proliferation of normal cells has been clearly demonstrated in a number of studies (reviewed by Dang *et al.*, 1999; Obaya *et al.*, 1999). Over the past decade it has become apparent that c-MYC can also activate apoptosis. Constitutive expression of c-*myc* resulted in significant acceleration of apoptosis in Il-3 deprived murine 32D.3 myeloid cells which are Il-3 dependent (Askew *et al.*, 1991). Overexpression of c-MYC in serum deprived Rat-1 fibroblasts was also shown to induce dramatic apoptosis (Evan *et al.*, 1992). In both these studies apoptosis occurred in cells in all phases of the cell cycle, while c-MYC could still be seen to be stimulating cell cycle progression in other cells in the population which were not immediately apoptotic (Askew *et al.*, 1991; Evan *et al.*, 1992). c-MYC was also reported to induce apoptosis in response to amino acid deprivation (Evan *et al.*, 1992), indicating that induction of apoptosis might be a result of conflicting growth and arrest signals. The machinery used by c-MYC to induce apoptosis has been the subject of controversy. Since there appears to be overlap in the regions of c-MYC required for induction of apoptosis, cell cycle progression and transformation, and as the transactivation and DNA binding domains are required for apoptosis, it has been suggested that c-MYC may affect the transcription of certain genes involved in apoptosis (Stone *et al.*, 1987; Evan *et al.*, 1992).

Some investigators have suggested a requirement for wild-type p53 in c-Myc induced apoptosis (Hermeking & Eick, 1994; Wagner *et al.*, 1994), and elevated expression of p53 has been observed following ectopic c-MYC expression (Yu *et al.*, 1997). Studies from other groups however, have reported p53 independent c-MYC induced apoptosis (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Blyth *et al.*, 2000). The Fas

pathway has also been implicated in c-MYC induced apoptosis (Hueber *et al.*, 1997), but a separate study indicated that the apoptosis mediated by c-MYC was independent of the Fas signalling pathway (Yeh *et al.*, 1998). The relationship between c-*MYC* and Fas will be discussed in more detail later in this chapter.

More recent research has suggested that the mechanism by which c-MYC induces apoptosis is more complex than originally supposed. c-MYC may be required for efficient response to a number of apoptotic stimuli, including hypoxia, glucose withdrawal, heat shock, DNA damage and cytotoxic therapy (Graeber *et al.*, 1996; Jiang *et al.*, 1996; Li *et al.*, 1996; Rupnow *et al.*, 1998; Shim *et al.*, 1998) although apoptosis may still occur in response to these stimuli in the absence of elevated c-MYC. Juin *et al.* (1999) have proposed that rather than having the ability to directly mediate cell death as a result of all these triggers, c-MYC can sensitise cells to a wide range of apoptotic stimuli by causing release of cytochrome *c* into the cytoplasm, the consequence of which will be dependent on other cellular signals. This hypothesis is supported by the results showing that c-Myc induced apoptosis is inhibited by ectopic expression of Bcl-2 (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993), since Bcl-2 has been reported to block apoptosis by inhibiting release of cytochrome *c* from the mitochondria (Kluck *et al.*, 1997; Yang *et al.*, 1997a). The co-operation between c-MYC and Bcl-2 in tumourigenesis (Strasser *et al.*, 1990a), adds weight to the theory that loss of apoptotic function may be important for the full oncogenic potency of c-*MYC*.

1.4.5 The c-*MYC* Gene and Tumourigenesis

The c-*MYC* gene has been inextricably linked with proliferation and cell cycle control in all normal cells, and it is loss of this high level of control that explains why deregulated c-*MYC* expression has been detected in approximately one third of human cancers in many different cell types (Spencer & Groudine, 1991; Nesbit *et al.*, 1999). Amplification or overexpression of the human c-*MYC* gene has been detected in tumours of various origin, for example lung, colon, breast, cervical and ovarian carcinomas (Little *et al.*, 1983; Escot *et al.*, 1986; Erisman *et al.*, 1988; Munzel *et al.*, 1991; Pinion *et al.*, 1991; Augenlicht *et al.*, 1997; Wang *et al.*, 1999b). Deregulation

of c-*MYC* appears to be a fundamental event in the development of lymphoid neoplasia. Nearly all cases of Burkitt's lymphoma for example, involve the translocation of a c-*MYC* allele to the regulatory elements of one of the three immunoglobulin chain genes on chromosomes 2, 14 or 22, which causes deregulation of c-*MYC* expression (Croce, 1993). Further, proviral integration at c-*myc* has been observed in approximately 45% of murine leukaemia virus induced T cell lymphomas (Selten *et al.*, 1984), and transgenic animals that have deregulated c-*myc* expression develop tumours at an increased incidence (reviewed by Morgenbesser & DePinho, 1994; Pelengaris *et al.*, 2000). These findings highlight the importance of the contribution made by c-*MYC* in the development and progression of cancer, particularly of lymphoid origin.

1.4.6 c-*MYC* Transgenic Mouse Models

The ability to target gene expression to specific tissues and cell lineages in transgenic mice, as well as the ability to regulate that expression, has made transgenic mice a valuable resource for studying oncogenic function, and the importance of different oncogenes in the process of tumourigenesis (reviewed by Macleod & Jacks, 1999). Expression of a putative oncogene can be activated in tissues of interest, at the appropriate time, and the resulting changes in phenotype used to understand the actions of that gene (reviewed by Macleod & Jacks, 1999). Oncogenic mouse models are important not only for advancing the understanding of oncogenic activity *in vivo*, but may also result in the generation of cell lines expressing particular oncogenes, which assist *in vitro* studies. In addition, collaborating tumourigenic events can be identified in transgenic mice already harbouring a constitutively activated oncogene, and synergy between different oncogenes can be examined by interbreeding of oncogenic transgenic mice (reviewed by Macleod & Jacks, 1999).

The c-*MYC* oncogene is among the best studied transgenic models of malignancy, and c-MYC overexpression has been targeted to particular tissues using transgenic constructs with different regulatory elements. CD2-*MYC*ER^{TM} mice for example express regulatable *MYC* in T cells (Blyth *et al.*, 2000). In this model, a transgenic construct of c-*MYC*, fused to the tamoxifen inducible oestrogen receptor, is placed

under the control of a CD2 locus control region promoter which targets expression to the T cell lineage (Lang *et al.*, 1988). Induction of the transgenic construct by tamoxifen led to an increased incidence and reduced latency of lymphoma development (Blyth *et al.*, 2000). Eμ-*MYC* mice express the c-*MYC* oncogene under the control of an immmunoglobulin heavy chain enhancer, which targets expression to B cells (Langdon *et al.*, 1986). Tetracycline inducible *MYC* transgenic mice also have targeted expression to B cells, through an Eμ-*MYC* linked tetracycline activating protein (Felsher & Bishop, 1999). These mice developed haematopoietic tumours at a frequency of 100% when the transgene was active, however when the transgene was inactivated, tumour regression was observed in 90% of the animals (Felsher & Bishop, 1999). The potency of *MYC* as a major driving force behind haemopoietic malignancies has been demonstrated in these mice (Langdon *et al.*, 1986; Felsher & Bishop, 1999; Blyth *et al.*, 2000). One advantage of inducible models, is the ability not only to regulate transgene expression *in vivo*, but also in explanted tumour cells *in vitro*.

Because cancer is a multi-stage process requiring a series of genetic events which may affect cell growth, proliferation, survival or death, it is important to understand which genetic lesions occur together during tumourigenesis, and in which order. There may be some genetic alterations which are functionally equivalent, and others which synergise strongly, and crossing of transgenic mouse models bearing different lesions associated with tumour progression allows improved understanding of these events. A number of strong collaborations between genetic lesions in tumourigenesis have been observed using this approach. In *MYC* transgenic mouse models a number of collaborating oncogenes have been reported. In Eμ-*MYC* mice, acceleration has been observed following introduction of the *ras* oncogene (Langdon *et al.*, 1989), the Bcl-2 oncogene (Vaux *et al.*, 1988), and the *pim*-1 oncogene (Verbeek *et al.*, 1991). *MYC* transgenic models have also been crossed with mice lacking tumour suppressor genes, and in particular, there has been great interest in *MYC* induced tumourigenesis on a *Trp53* deficient background. Because of the ability of MYC to induce apoptosis as well as proliferation, the potential collaboration between deregulated *MYC* and loss of p53, which might mediate apoptosis, has been studied by a number of groups. Many studies have shown that overexpression of *MYC* and loss of p53 co-operate in

tumourigenesis (Blyth *et al.*, 1995; Elson *et al.*, 1995; Hsu *et al.*, 1995). CD2-*MYC* mice null for *Trp53* for example, develop thymic lymphoma at an increased incidence and decreased latency, compared to *Trp53* heterozygous littermates (Blyth *et al.*, 1995), while in Eμ-*MYC* animals, overexpression of c-*MYC* could collaborate with a heterozygous *Trp53* mutation in B cell lymphomagenesis (Hsu *et al.*, 1995). Investigation of the levels of apoptosis in tumours arising in these models however, indicated that loss of p53, or loss of heterozygosity in *Trp53* +/- tumours, did not inhibit the ability of MYC to induced apoptosis (Blyth *et al.*, 2000; Hsu *et al.*, 1995). These results suggested that in these tumours MYC did not require p53 to induce apoptosis, and rather, loss of *Trp53* could collaborate with overexpression of *MYC*, because of enhanced cell cycle progression and proliferation in the absence of p53.

1.5 TUMOUR SUPPRESSOR GENES

As the name suggests, a tumour suppressor gene is one whose normal function prevents tumour development (reviewed by Knudson, 1993). In contrast to oncogenes, tumour suppressor genes encode proteins that limit the replicative potential of a cell, or induce cell death. Mutations in these genes that result in loss of function, contribute to the tumourigenic process. Function of the normal gene is generally dominant however, so unlike oncogenes, loss of function of both alleles of a tumour suppressor gene is usually required for tumourigenesis. This may occur as a result of two somatic mutations, or in individuals who have an inherited mutation in a single allele of a tumour suppressor gene, one somatic mutational event in the remaining wild-type allele. Individuals who harbour a germline mutation in a tumour suppressor gene carry an increased risk of cancer, due to the high probability of loss of function of the wild-type allele in at least one cell.

A number of familial cancer syndromes caused by germline mutations in tumour suppressor genes have been described in humans (reviewed by Tomlinson, 1997). For example, 90% of humans carrying a germline mutation in the retinoblastoma susceptibility gene, *RB-1*, acquire a somatic mutation in the wild-type allele and develop retinoblastoma, while 15% also develop osteosarcomas (Hooper, 1998).

Mutations at *RB-1* have also been observed in a variety of spontaneously occurring human carcinomas (Knudson, 1993). An inherited predisposition to cancer is also observed in individuals affected by Li Fraumeni syndrome. These individuals develop diverse mesenchymal and epithelial neoplasms at multiple sites and this is due to a germline mutation in the p53 tumour suppressor gene (Srivastava *et al.*, 1990). Mutations in the p53 gene have been detected in over half of all human tumours, and inactivation of p53 is considered to be an important step in tumourigenesis (Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994; Hollstein *et al.*, 1994; Hollstein *et al.*, 1996; Hainaut & Hollstein, 2000). This realisation has stimulated a great deal of research over the past decade, on the functions of p53 in human cancer.

1.5.1 The p53 Tumour Suppressor Gene and Protein

One of the first tumour suppressor genes to be identified was the p53 gene. Initially this was believed to be an oncogene due to the high levels of expression of the p53 protein, (so called because of its apparent molecular weight of 53 kD), in transformed mouse cells, but not in normal mouse tissues (DeLeo *et al.*, 1979). At the same time, studies into the mechanism by which cells were transformed by the simian virus 40 (SV40) -encoded large T antigen, revealed that a cellular protein of 53 kD bound to this viral protein, and was thought to mediate its transforming potential (Lane & Crawford, 1979; Linzer & Levine, 1979). Further work revealed that the highly expressed p53 found in many tumours (Crawford *et al.*, 1981; Rotter *et al.*, 1983) was actually mutated, and wild-type p53 could in fact inhibit transformation (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). The definitive experiments demonstrating the role of p53 as a tumour suppressor gene were carried out in transgenic mice null for *Trp53*. Mutating the *Trp53* gene in the germline of mice was found to predispose animals to cancer (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Purdie *et al.*, 1994). Studies of human tumours showed that many harbour p53 gene mutations (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Hollstein *et al.*, 1994; Hollstein *et al.*, 1996). These observations implicated the loss of p53 as a major factor in tumourigenesis.

The p53 protein is normally unstable and rapidly degraded, and is stabilised and thus activated only as the main cellular response to stress or damage (Hall *et al.*, 1996;

Levine, 1997). Stressful stimuli, which include hypoxia, DNA damage, such as that caused by ionising radiation, changes in metabolism, heat shock and certain cytokines result in activation of the p53 protein (Kuerbitz *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993; Graeber *et al.*, 1996; Linke *et al.*, 1996; Meek, 1999). Activated p53 is then responsible for initiating a series of events leading to either cell cycle arrest to allow repair to take place, or apoptosis, if the damage is excessive. Damaged cells pose a considerable threat to the organism, since they are more likely to harbour mutations which will eventually lead to malignancy. The p53 protein senses mutations and prevents the damaged cells from multiplying, thereby providing a critical brake on tumourigenesis. This perhaps explains why damage to the p53 gene itself predisposes to tumour development, and why p53 mutations have been detected in 50-55% of human tumours (Hollstein *et al.*, 1991; Hollstein *et al.*, 1994; Hollstein *et al.*, 1996). Malfunction of the p53 system may result in genomic instability, which then permits accumulation of the multiple mutations required for tumour development (Lengauer *et al.*, 1998).

1.5.2 p53 Knockout Mouse Models

Donehower *et al.* (1992), developed the first reported *Trp53* knockout mice, by substituting a neo cassette in place of parts of intron 4 and exon 5 of the *Trp53* gene. Analysis of these mice, and the other strains of *Trp53* null mice, generated broadly the same conclusions. Firstly, and perhaps surprisingly given p53's role in regulation of the cell cycle, these mice were viable and appeared developmentally normal, indicating that p53 is not required for embryonic development (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Purdie *et al.*, 1994). These mice did however inherit a strong predisposition to cancer (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Purdie *et al.*, 1994). The mice of Donehower *et al.*, for example, developed tumours rapidly and at high frequency on a genetic background of 75% C57Bl6 and 25% 129/Sv. Seventy four percent of these *Trp53* null mice developed tumours by 6 months of age, and one hundred percent by 10 months of age. The average latency of tumours was 4.5 months.

This relatively long latency period gives credence to the hypothesis that cancer represents a multi-step process. It is apparent that several other genetic events are required before the onset of neoplasia in these mice. Further evidence for this comes from analysis of the tumour types in *Trp53* null mice, since tumours arose in various different cell types. More than 70% of *Trp53* null mice developed lymphomas, the majority of which were thymic lymphomas. Among the other tumours, haemangiosarcomas, undifferentiated sarcomas, osteosarcomas, testicular tumours, and a small number of carcinomas were detected. The results also confirmed the importance of the *Trp53* tumour suppressor gene in multiple tissues in the mouse. Interestingly, the spectrum of tumours was altered in *Trp53* heterozygotes. These mice perhaps expectedly, developed tumours with an increased latency compared with their *Trp53* null counterparts (Donehower *et al.*, 1992; Harvey *et al.*, 1993; Jacks *et al.*, 1994; Purdie *et al.*, 1994), however tumours were predominantly sarcomas, and lymphomas were detected at the much lower frequency of 25%. This is likely to be a reflection on the increased latency of these tumours. The susceptibility of *Trp53* null mice to thymic lymphomas in particular, has been attributed to the fact that thymocytes must undergo DNA rearrangements of the TCR loci during maturation. Given the importance of p53 in ensuring that only cells with 'normal' DNA rearrangements survive, it follows that aberrant DNA events during thymocyte development will persist in *Trp53* null mice, and may lead to cancer. In *Trp53* heterozygous mice however, loss of heterozygosity must occur at the *Trp53* locus before p53 function is lost. The rapid growth of the T cell compartment, and the maturation of most T cells may have taken place before loss of heterozygosity occurs, making the T cell compartment less susceptible to tumours induced by *Trp53* loss in these mice. Recent studies however, have shown that p53 deficient mice which were unable to carry out V(D)J recombination of the TCR loci due to RAG (recombination-activating gene) deficiency still developed lymphomas at high frequency (Nacht & Jacks, 1998; Liao *et al.*, 1998). These results suggest that development of lymphoma in p53 deficient mice is not dependent on aberrant DNA rearrangement during recombination events. The study of *Trp53* null and heterozygous mice alone, or in crosses with other transgenic strains has contributed greatly to the knowledge of the function of p53 as a tumour suppressor (reviewed by Attardi & Jacks, 1999).

1.5.3 Activation and Control of p53

The p53 protein is a transcription factor, and functions in part by enhancing transcription and expression of a number of cellular proteins involved in cell cycle control, DNA repair and apoptosis (Levine, 1997). It is this function of p53 that also enables it to regulate its own activity. In normal cells, the levels of p53 are subject to a negative feedback loop, and the amount of active p53 depends not on the rate at which it is synthesised, but on the rate at which it is degraded. Degradation of p53 occurs by a process known as ubiquitin-mediated proteolysis (Huibregtse *et al.*, 1991). Through this process, the p53 protein becomes labelled with several copies of ubiquitin, a small peptide that acts as a recognition signal and enables the protein degradation machinery to recognise the protein to be degraded (Hershko & Ciechanover, 1998). Through its role as a transcription factor, p53 is able to control its own ubiquitination, by enhancing transcription of MDM2 (Barak *et al.*, 1993; Wu *et al.*, 1993), the enzyme chiefly responsible for labelling p53 with ubiquitin (Momand *et al.*, 2000). When p53 levels are high, MDM2 is expressed, binds to p53 and stimulates ubiquitination which is followed by degradation. The levels of p53 then fall, transcription of MDM2 is reduced, and p53 levels are allowed to rise again. This negative feedback system may explain why very high levels of p53 have been found in so many tumours (Hollstein *et al.*, 1991; Hollstein *et al.*, 1994; Hollstein *et al.*, 1996). Mutations at p53 may result in expression of a product that can no longer control transcription and therefore has no method for autoregulation.

Activation of p53 occurs when p53 is stabilised, however the method of activation and stabilisation of p53 may depend on the trigger. Activation of p53 in response to oncogenic stress, for example, is controlled by a protein called $p14^{Arf}$ (human)/ $p19^{Arf}$ (mouse, Pomerantz *et al.*, 1998). Oncogenes such as *MYC* (Zindy *et al.*, 1998) and E1A (de Stanchina *et al.*, 1998) have been shown to regulate p53 activity by stimulating Arf. Then Arf, itself a tumour suppressor protein, binds to MDM2 and sequesters it into the nucleolus, where it is prevented from binding to, and stimulating degradation of p53 (Weber *et al.*, 1999). The p53 pathway invoked by DNA damage is activated in a different way. DNA damage is recognised by 'checkpoint' proteins that cause the cell cycle to be delayed until the damage is

repaired (reviewed by Carr, 2000). Here activation of p53 is dependent on two protein kinases, ATM, which is stimulated by double stranded DNA breaks, and Chk2 which is stimulated by ATM (reviewed by Meek, 1999). These kinases stabilise p53 by phosphorylating p53 protein at sites close to the MDM2 binding domain, thus blocking MDM2-p53 interactions (reviewed by Meek, 1999). Other forms of DNA damage may stabilise p53 in a similar way, but through different kinase pathways which are less well understood (reviewed by Meek, 1999; Vogelstein *et al.*, 2000). Following stabilisation, p53 is free to suppress cell growth, either by cell cycle arrest, or by apoptosis.

1.5.4 Mechanisms of p53 Action

Since the identification of p53 as a tumour suppressor gene, a number of physiological functions have been attributed to activated p53. Activation of p53 has been shown to affect cell cycle arrest, senescence, apoptosis, differentiation, and blood vessel formation (angiogenesis), and this is partly due to p53's role as a transcription factor (reviewed by Levine, 1997; Steele *et al.*, 1998; Dang *et al.*, 1999; Vogelstein *et al.*, 2000). Several target genes that are directly controlled by p53 have been implicated in mediating these biological effects (El-Deiry, 1998).

In nearly all mammalian cells, activation of p53 results in cell cycle arrest. This is due mainly to a block in the G1 phase of the cell cycle, which is brought about by inhibition of various cyclin-dependent kinases (CDKs) by $p21^{WAF1/CIP1}$, a transcriptional target of p53 (El-Deiry *et al.*, 1993). CDKs and the cyclins with which they function, are responsible for ensuring progression of the cell cycle from the G1 resting phase into the replicative S phase, and from G2 phase into mitosis. It is at these transitions, particularly at G1, that p53 activation of p21 has been reported to cause arrest of the cell cycle (Harper *et al.*, 1993; Deng *et al.*, 1995; Waldman *et al.*, 1995). Other cell cycle control genes which are regulated by p53, and may contribute to G2 arrest have also been reported (Hermeking *et al.*, 1997; Utrera *et al.*, 1998; Zhan *et al.*, 1998).

In addition to its ability to inhibit cell cycle progression, an important role of p53 as a tumour suppressor, is to induce apoptosis in cells in which damage cannot be repaired (Symonds *et al.*, 1994). Radiation induced DNA damage causes apoptosis in normal thymocytes for example, but not in p53 null thymocytes (Clarke *et al.*, 1993; Lowe *et al.*, 1993). There are many potential mechanisms by which p53 mediates apoptosis (Bates & Vousden, 1999). One of the transcriptional targets of p53 for example is Bax, a pro-apoptotic member of the Bcl-2 family, that heterodimerises with Bcl-2 (Oltvai *et al.*, 1993), and prevents Bcl-2 from blocking release of cytochrome *c* from the mitochondria (Rosse *et al.*, 1998). p53 has also been shown to induce transcription independent apoptosis in a number of systems, since p53 mediated apoptosis can occur in the absence of *de novo* protein synthesis (Caelles *et al.*, 1994). It may be that p53 can induce transcription independent apoptosis by forming a complex with one of the cellular proteins with which it is reported to interact (reviewed by Levine, 1997). As yet however, there is no firm evidence of this.

Death receptors and their ligands have also been proposed as effectors of p53 mediated apoptosis. Expression of the Fas receptor for example has been reported to be dependent on p53 regulation (Owen-Schaub *et al.*, 1995). The relationship between p53 and the Fas pathway will be discussed in more detail later. Other putative death receptors appear to play a role in p53 induced apoptosis. Expression of one of the TRAIL receptors, named KILLER or DR5, appears to be increased following exposure to DNA damaging agents in *Trp53* wild-type mice, indicating that KILLER may be involved in p53 dependent apoptosis (Wu *et al.*, 1997). Further, inhibition of transcription in cells undergoing p53 dependent apoptosis blocked induction of KILLER, suggesting that KILLER is a transcriptional target of p53. There may be many other genes involved in the apoptotic function of p53. Indeed the effects of Fas, p21, Bax and MDM2 on p53 mediated apoptosis have been tested *in vivo*, and the results showed that no single target of p53 is required for apoptosis (Reinke & Lozano, 1997). There may be functional redundancy between the targets of p53. Research in this field is ongoing, not least because it is this feature of p53 which may be of most therapeutic value.

There are other activities associated with p53 which may prevent tumour formation. As mentioned earlier, p53 loss may result in genomic instability, due to the persistence of cells with damaged DNA. p53 may also have a more direct role in maintaining genomic stability, by regulating the genes that are involved in DNA repair, for example GADD45 (Kastan *et al.*, 1992, reviewed by Wahl *et al.*, 1997). There is also a reported role for p53 in inducing cellular senescence, and dominant mutant p53 has been shown to rescue human cells from senescence (Bond *et al.*, 1995). Finally, p53 has been reported to stimulate expression of genes that prevent angiogenesis, the growth of new blood vessels (El-Deiry, 1998). If a tumour is to grow to a size where the host organism is endangered, then a new blood supply must be encouraged to grow around, and into the tumour. Loss of p53 may therefore enable this to occur, thus highlighting the importance of p53 as a tumour suppressor with various means of action. Clinical research has sought to understand the importance of p53 as a prognostic indicator. In general, p53 mutations are associated with more aggressive, higher grade tumours, and can be correlated with reduced patient survival (Wallace-Brodeur & Lowe, 1999). These observations to some extent confirm p53 as a major tumour suppressor.

1.6 THE FAS/FASL PATHWAY

Among the main effectors of apoptosis are the death ligands and receptors. Fas and its ligand, FasL are important mediators of cell death, particularly in the immune system, and have been the subject of a great deal of research over the past few years. The question of whether or not this system has any role in prevention of tumourigenesis remains the subject of much controversy.

1.6.1 A Novel Death Receptor

Fas (CD95/Apo-1) is a 45kD cell surface protein belonging to the tumour necrosis factor (TNF) / nerve growth factor (NGF) receptor superfamily. Activation of Fas by crosslinking either with the natural ligand or the anti-Fas agonistic antibody leads to apoptotic cell death with characteristic morphological changes and DNA

fragmentation (Trauth *et al.*, 1989; Itoh *et al.*, 1991).

The Fas antigen was identified in 1989 as a cell death signalling receptor (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). Two groups working independently had isolated mouse derived antibodies cytolytic for various human cell lines. These antibodies recognised novel cell surface proteins named Fas and Apo-1 (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). The identity of these two receptors was subsequently established when their respective cDNAs were cloned (Itoh *et al.*, 1991; Oehm *et al.*, 1992).

The isolation and characterisation of human Fas cDNA from KT-3 lymphoma cells revealed a 325 amino acid protein consisting of a membrane spanning region in the middle of the molecule and a signal sequence at the cytoplasmic NH_2-terminus (Itoh *et al.*, 1991). The structure indicated that Fas is a type I membrane protein belonging to the TNF/NGF receptor superfamily (Itoh *et al.*, 1991; Oehm *et al.*, 1992; Watanabe-Fukunaga *et al.*, 1992b). The members of this family all have relatively well conserved extracellular regions consisting of between two and six cysteine rich domains. In contrast, there is little similarity in the cytoplasmic region between various members except for the intracellular death domain shared by Fas and TNF-R1 (Itoh *et al.*, 1991; Oehm *et al.*, 1992). The family includes Fas, two TNF-Rs (TNF-R1 and TNF-R2), the NGF receptor, CD40, CD27, CD80, the lymphotoxin β receptor and the TRAIL receptors (Nagata & Golstein, 1995). This list is not complete however; the family is still growing and will be discussed later.

The human Fas gene spans 12kb on the long arm of chromosome 10 (Inazawa *et al.*, 1992), while in the mouse, the gene is located on chromosome 19 and spans more than 70kb (Watanabe-Fukunaga *et al.*, 1992b). The receptor is ubiquitously expressed in various tissues of both lymphoid and non-lymphoid origin in mice, with abundant expression in thymus, liver, heart, lung, kidney and ovary (Watanabe-Fukunaga *et al.*, 1992b). High expression of Fas on murine hepatocytes was highlighted by the lethality of the anti-Fas antibody in mice, due to severe liver damage (Ogasawara *et al.*, 1993). Expression of Fas is also high in mature activated lymphocytes (Trauth *et al.*, 1989) and in virally transformed lymphocytes (Falk *et al.*,

1992; Debatin *et al.*, 1994). Expression of Fas in human thymocytes is weak (Yonehara *et al.*, 1994) compared to the high levels of expression observed in almost all mouse thymocyte populations (Ogasawara *et al.*, 1995).

The apoptotic function of Fas was assessed in transformed murine lymphocytes transfected with human Fas. The constitutive expression of human Fas in these transfectants conferred sensitivity to anti-Fas antibody induced apoptosis (Itoh *et al.*, 1991; Oehm *et al.*, 1992). As induction of cell death appeared to be the major outcome of receptor binding, Fas was described as a cell surface mediator of apoptosis. It was therefore important to understand how Fas was activated in cells, and under what physiological circumstances, since any cell expressing Fas could be susceptible to Fas-mediated apoptosis.

The structure of Fas led to the belief that it was a receptor for an unidentified cytokine. The finding that the CTL hybridoma, PC60-d10S required the presence of Fas on target cells to induce cytotoxicity implied that these cells expressed a ligand that could induce death via Fas (Rouvier *et al.*, 1993). In 1994, Suda and Nagata constructed a soluble fusion protein consisting of the extracellular region of Fas and the Fc region of human IgG1, which they used to isolate and characterise the Fas ligand (Suda & Nagata, 1994). They identified and purified a 40kDa membrane glycoprotein which showed strong cytotoxic activity against Fas-expressing cells, indicating that FasL is a death factor (Suda & Nagata, 1994). Expression of recombinant FasL on the cell-surface was sufficient to induce apoptosis in Fas-bearing cells within a few hours (Suda *et al.*, 1993).

The structure of FasL indicates a type II membrane protein with a hydrophobic membrane-spanning domain in the middle of the molecule, but no cytoplasmic NH_2-terminus signal sequence (Suda *et al.*, 1993). The FasL gene is located on chromosome 1 in both human and mouse (Lynch *et al.*, 1994; Takahashi *et al.*, 1994b), and shows similar organisation to other members of the TNF family. FasL is predominantly expressed in activated T-cells and natural killer (NK) cells, although abundant expression is also found in the tissues of the 'immune privilege' sites such as the eye and the testes (Suda *et al.*, 1993).

1.6.2 Fas^{lpr} and Fas^{gld} Mutations

Loss of function mutations in the Fas system have been important in determining the role of the Fas pathway *in vivo*. Two naturally occurring mutations exist in the mouse, in which expression of either the Fas receptor or its ligand is disturbed. The Fas^{lpr} (lymphoproliferation) mutation is an autosomal recessive mutation in the Fas gene (Watanabe-Fukunaga *et al.*, 1992a), caused by insertion of an early transposable element (ETn) into intron 2 (Lynch *et al.*, 1994). This causes premature termination of the Fas mRNA transcript and aberrant splicing. In fact small mRNAs encoding exons 1 and 2 have been found in the thymus and liver of Fas^{lpr} mice. Inhibition of expression is not complete however, as full length Fas mRNA has also been detected at low levels in these tissues, indicating that Fas^{lpr} is a leaky mutation (Adachi *et al.*, 1993). The Fas^{gld} (for generalised lymphoproliferative disease) mutation is an autosomal recessive mutation and is located near the C-terminus of the FasL coding region on mouse chromosome 1 (Takahashi *et al.*, 1994a). The Fas^{gld} mutation is a T to C point mutation which results in an amino acid change from phenylalanine to leucine, and abolishes the ability of FasL to bind to Fas (Takahashi *et al.*, 1994a).

Although Fas^{lpr} and Fas^{gld} mutations are non-allelic mutations, they show a similar phenotype (Cohen & Eisenberg, 1991). MRL mice homozygous for either mutation develop lymphadenopathy and splenomegaly and produce large quantities of IgG and IgM antibodies including anti-DNA antibody and rheumatoid factor autoantibody. At around five months of age, the mice die of nephritis or arteritis (Cohen & Eisenberg, 1991). Other strains of mice carrying Fas^{lpr} and Fas^{gld} mutations develop lymphadenopathy and splenomegaly, but not nephritis or arteritis (Izui *et al.*, 1984). The full manifestation of the disease requires background genes which are only present in the MRL strain (Wang *et al.*, 1997), suggesting that Fas^{lpr} and Fas^{gld} mutations induce autoimmune disease which is enhanced in these animals by an underlying genetic susceptibility.

Another Fas^{lpr} mutation, known as $Fas^{lpr\text{-}cg}$, has been described (Watanabe-Fukunaga *et al.*, 1992a). The $Fas^{lpr\text{-}cg}$ mice express full length Fas mRNA as abundantly as wild type mice do. The mRNA carries a T to A point mutation in the middle of the

Fas cytoplasmic region however, resulting in the replacement of isoleucine with asparagine (Watanabe-Fukunaga *et al.*, 1992a). This mutation occurs in the Fas death domain, and abolishes the ability of Fas to transduce the apoptotic signal into cells (Watanabe-Fukunaga *et al.*, 1992a). Unlike the original Fas^{lpr} mutant, a heterozygous $Fas^{lpr\text{-}cg}$ mutation results in a weak *lpr* phenotype in mice that are also heterozygous for Fas^{gld}, hence the name $Fas^{lpr\text{-}cg}$ (for *lpr* complementing *gld*, Matsuzawa *et al.*, 1990).

Fas null mice have also been generated, and these show more accelerated and pronounced lymphadenopathy and splenomegaly than Fas^{lpr} mice, as well as lymphocytic infiltration in the liver and lungs (Adachi *et al.*, 1996). The general phenotype of lymphoproliferative disease and autoimmunity is observed in these mice however. The human equivalent of Fas^{lpr} disease has now been described. Patients with ALPS (autoimmune lymphoproliferative syndrome) show phenotypes similar to those of Fas^{lpr} mice, and carry a heterozygous mutation in the Fas gene (Rieux-Laucat *et al.*, 1995).

The lymphocytes that accumulate in the lymph nodes and spleen of Fas^{lpr} and Fas^{gld} mice express the T-cell marker Thy-1 and the B cell marker B220. They express a rearranged T-cell receptor but not a rearranged IgG gene (Morse *et al.*, 1982). The conclusion that it is T-cells that accumulate in Fas^{lpr} and Fas^{gld} mice was confirmed by the observation that neonatal thymectomy prevents the accumulation of such lymphocytes (Steinberg *et al.*, 1980). Unlike normal mature T-cells however, the lymphocytes that accumulate in Fas^{lpr} and Fas^{gld} mice do not express the CD4 and CD8 cell surface antigens, however the gene loci encoding them are demethylated, indicating that these genes have been expressed in the past (Landolfi *et al.*, 1993). In addition, when mature single positive T-cells from young Fas^{lpr} mice were transplanted into wild type mice, these T-cells became double negative T-cells (Laouar & Ezine, 1994). The evidence suggests that the double negative T-cells that accumulate in Fas^{lpr} and Fas^{gld} mice are derived from mature single positive cells by suppressing the expression of the CD4 or CD8 antigen. In parallel with accumulation of abnormal double negative T cells, the proportion of phenotypically normal T cells bearing CD4 or CD8 progressively declines, until these cells may

constitute less than 10% of the total population in the lymph nodes (Cohen & Eisenberg, 1991). As a result of the huge increase in total lymphocyte numbers in the mice however, overall numbers of single positive T cells in these animals are increased (Cohen & Eisenberg, 1991).

The accumulation of T-cells in these mice is a result of a breakdown in the apoptotic process that occurs during the normal maturation of T-cells. The Fas/FasL system has been implicated in this and other apoptotic processes employed by the immune system (Lynch *et al.*, 1995; Nagata, 1997).

1.6.3 Homeostatic Regulation of the Immune Response

T lymphocytes, the cells responsible for removing malignant and virally transformed cells, die at various stages during their development. Less than 5% of thymocytes survive during their development. Most immature T-cells are eliminated by positive or negative selection as a result of incorrect rearrangement of the T-cell receptor, failure to be recognised by the MHC complex, or self-reactivity. In the peripheral immune system, mature T-cells recognising self-antigens are also deleted. It is also necessary that in the periphery, elimination of lymphocytes occurs after they have been activated by antigen, to ensure that there is no accumulation of activated lymphocytes.

Thymic clonal deletion was originally considered to be apparently normal in Fas^{lpr} and Fas^{gld} mice (Singer & Abbas, 1994), even though normal thymocytes abundantly express Fas and are sensitive to Fas-mediated apoptosis. These results indicated that a Fas-mediated mechanism is unlikely to be involved in the thymic deletion process, although redundant mechanisms may be activated where the Fas system is disrupted. More recent research in neonatal thymocytes has highlighted a role for Fas in negative selection however (Kishimoto & Sprent, 1997; Kishimoto *et al.*, 1998). TCR activation induced apoptosis of semi-mature HSA^{hi} CD4 single positive thymocytes was revealed to be severely impaired in cells from Fas^{lpr} mice, compared with normal cells, implicating Fas in negative selection (Kishimoto & Sprent, 1997). Fas dependency however was shown to be relevant only with high doses of anti-TCR

antibody (Kishimoto & Sprent, 1997). At low level stimulation, anti-TCR mediated apoptosis was not significantly reduced in Fas^{lpr} thymocytes. This phenotype was not observed however in mature HSA^{lo} $CD4^{+}CD8^{-}$ thymocytes or in CD4 single positive peripheral T cells, in which AICD was Fas dependent. Further evidence for a role for Fas in negative selection was generated subsequently. Injection of the superantigen, staphylococcal enterotoxin B (SEB), induces thymic clonal deletion of normal cells at various concentrations, however in Fas^{lpr} thymocytes, deletion was only induced at low to moderate doses of antigen, but not at higher doses (Kishimoto *et al.*, 1998). These results suggested for the first time, that thymic clonal deletion might be Fas dependent at higher antigen levels. In contrast, most evidence suggests that peripheral clonal deletion and antigen activation induced death are impaired in Fas^{lpr} and Fas^{gld} mice (Singer & Abbas, 1994).

Under normal circumstances and during positive selection, when primary T-cells are activated by engagement of the T-cell receptor/CD3 complex either by immobilised anti-CD3 or by antigen presenting cells, they undergo proliferation and produce cytokines. It has been demonstrated that stimulating T-cells induces Fas ligand and upregulates Fas expression, and interfering with the Fas/FasL interaction inhibits apoptosis (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). In naïve T cells, protective mechanisms must be in place to prevent Fas induced apoptosis. Stimulation of previously activated T-cells however, induces apoptosis in those cells, a process known as activation induced cell death (Smith *et al.*, 1989). This process can be induced in a single cell, indicating that it may be a cell autonomous process. AICD must be mediated at least in part, by the Fas/FasL system, with stimulation of T-cells leading to upregulation of Fas and FasL surface expression (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). FasL would then bind to its receptor on the same cell or on a neighbouring cell, thus triggering the intracellular death-signalling cascade. Susceptibility to AICD has been shown to correlate with cell surface FasL expression, with FasL dependent AICD largely restricted to the Th1 T-cell subset (Oberg *et al.*, 1997). Mature T-cells from Fas^{lpr} or Fas^{gld} mice do not die after activation, but instead accumulate in the lymph nodes and spleen (Russell *et al.*, 1993; Gillette-Ferguson & Sidman, 1994). In addition, T-cells activated in the presence of Fas neutralising antibodies do not die

(Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). These results suggest the involvement of Fas in activation induced cell suicide, and consequently in down-regulation of the immune reaction.

Fas:FasL interactions are also reported to be involved in peripheral clonal deletion of T cells. The cells eliminated during peripheral clonal deletion are those which recognise self antigens expressed by interacting antigen presenting cells (Kabelitz *et al.*, 1993). One study showed that when the superantigen, SEB, was injected into wild-type mice, mature T cells expressing a SEB reactive TCR chain proliferated, and then died by apoptosis. In Fas^{lpr} mice however, apoptosis of these cells was not observed, implicating Fas signalling in the clonal deletion of autoreactive T cells (Nishimura *et al.*, 1995). It is thought that the self peptide-MHC complex interacts with the TCR on autoreactive T cells, causing activation and expression of Fas and FasL, which then induce cell suicide. In addition to autoreactive T cells, Fas deficient mice also accumulate B cells and therefore have elevated levels of autoantibodies (Giese & Davidson, 1994). As with T-cells, Fas may be involved in the deletion of activated B cells in the periphery. Transgenic expression of Fas in the T-cells of MRL-Fas^{lpr} mice has been shown to block lymphoproliferation but not autoimmune disease in these mice (Fukuyama *et al.*, 1998) suggesting that Fas signalling is also required for the killing of activated B cells. Activation of B cells sensitises them to Fas mediated apoptosis (Daniel & Krammer, 1994), although the precise mechanism and role of Fas in the elimination of B cells is not yet understood.

1.6.4 Fas and T Cell Proliferation

One function of Fas which complicates any study of its role in tumour suppression is its ability to transduce activation signals and stimulate cell proliferation under certain circumstances (Alderson *et al.*, 1993). Previously activated mature lymphocytes are killed by Fas ligation on repeated antigen activation by AICD (Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). There is a growing bank of evidence however, which suggests that Fas ligation may stimulate activation and proliferation of T-cells. One of the first pieces of evidence for a role for Fas in proliferation of T-cells came from studies using mAbs against human Fas on purified human T-cells. One of these

antibodies when immobilised in culture wells along with CD3 (representing antigen activation) stimulated proliferation of human T lymphocytes (Alderson *et al.*, 1993). This effect was largely interleukin 2 independent and due to a direct effect on the cells. Many other studies since then, focusing on the apoptotic function of Fas have generated results suggesting involvement of the Fas pathway in T-cell proliferation. Several independent groups have reported that a dominant negative form of FADD, a Fas signalling molecule, can not only inhibit Fas mediated apoptosis, but can also impair mitogen induced and activation induced proliferation of T-cells, suggesting a link between apoptosis and proliferation in T cells (Newton *et al.*, 1998; Zhang *et al.*, 1998; Zornig *et al.*, 1998; Strasser & Newton, 1999). The inhibition of proliferation is not due to impaired Il-2 (Newton *et al.*, 1998; Zhang *et al.*, 1998; Zornig *et al.*, 1998), but has been proposed to be p53 dependent as DN-FADD shows no inhibitory effect on proliferation of *Trp53*-/- cells (Zornig *et al.*, 1998). It is possible that Fas signalling or FADD plays some role in inhibiting p53 mediated growth arrest in normal cells, and thus allows proliferation to occur.

Another route by which proliferation may be induced is by reverse signalling through Fas ligand. FasL has been shown to positively regulate the proliferative capacity of a subset of T-cells which express it. Plate bound anti-FasL antibody is able to upregulate proliferation of FasL bearing cells undergoing sub-optimal anti-CD3 stimulation, with the response limited to $CD8^+$ T cells (Suzuki & Fink, 1998). Conversely, in $CD4^+$ T cells, FasL is reported to transduce signals leading to cell cycle arrest and cell death (Desbarats *et al.*, 1998). In $CD4^+$ T-cells, FasL engagement prevented Il-2 secretion and proliferation, and blocking Fas:FasL interactions brought about an increase in proliferation. In $CD8^+$ T-cells however, FasL engagement did not block cell cycle progression or proliferation (Desbarats *et al.*, 1998). More recently Fas engagement in the presence of anti-CD3 antibody has been shown to induce apoptosis in naïve $CD4^+$ T cells but to co-stimulate proliferation of memory $CD4^+$ T cells (Desbarats *et al.*, 1999). In addition, CD28 co-stimulation altered the response of naïve T cells allowing them to be co-stimulated by FasL engagement (Desbarats *et al.*, 1998).

CD28 is an inducible T cell surface antigen that has been reported to provide a co-

signal in addition to ligation of the T cell receptor/CD3 complex, when bound by its natural ligands, B7-1 or B7-2, or by antibody (Rudd, 1996). CD28 co-stimulation with CD3 crosslinking has been reported to augment deletion of double positive thymocytes (Amsen & Kruisbeek, 1996; Punt *et al.*, 1997), and also to enhance proliferation of $CD3^+$ thymocytes during activation (Turka *et al.*, 1990), an effect which is dependent on presence of the cytokine, Il-2. Enhancement of CD3 stimulated T cell survival and proliferation by CD28 co-stimulation was found to be independent of Fas expression (Noel *et al.*, 1996). This finding suggests that CD28 co-stimulation allows CD3 to signal through an alternative Fas independent pathway to promote activation induced proliferation. In addition, α4 and α5 integrins have been reported to co-stimulate CD3 dependent proliferation of fetal thymocytes (Halvorson *et al.*, 1998). The outcome of CD3 crosslinking will therefore depend very much on the population of stimulated T cells; their expression of surface markers and their stage of development, as will the requirement for Fas or FasL. The ability of Fas:FasL interactions to employ different signalling pathways in individual cell types indicates an important role for the Fas pathway in homeostatic regulation of the immune response.

1.6.5 The Role of Fas in T Cell Mediated Cytotoxicity

Positive and negative selection and apoptosis of previously activated T-cells occurs by default. In contrast, there are situations in the immune system where cells actively induce the death of other cells. An example of this is the induction of apoptosis in malignant or virally transformed cells by cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells. This function of Fas makes it particularly relevant in cancer, and specifically leukaemia research.

The mechanisms involved in the lysis of foreign, or antigen expressing target cells by CTLs were under debate for some time since the known perforin-granzyme-based 'hole-punching' mechanism did not account for all the situations in which CTL killing occurred (Lowin *et al.*, 1994; Ebnet *et al.*, 1995). The demonstration that FasL expressing activated T cells were able to lyse Fas positive cells suggested that the Fas pathway was an alternative mechanism for CTL mediated cytotoxicity

(Rouvier *et al.*, 1993). Evidence for this hypothesis was generated from studies of perforin knock-out mice (Kagi *et al.*, 1994a). Although most CTL mediated cytotoxicity was abolished in these mice, the activity that remained appeared to be FasL dependent (Kagi *et al.*, 1994b). Furthermore, CTLs from these mice appeared to exhibit no cytotoxic activity towards cells lacking Fas (Lowin *et al.*, 1994), implicating the perforin-granzyme and Fas/FasL pathways as the major mechanisms by which CTL killing occurs. Recently however, CTLs from mice deficient in both the perforin and Fas pathways have shown some residual, albeit delayed, cytotoxicity (Braun *et al.*, 1996) suggesting a role for yet another death factor, possibly TRAIL, in CTL killing. The Fas/FasL pathway has also been implicated in NK cell cytolysis of target cells (Arase *et al.*, 1995). Since some cancer cells, particularly in lymphoid tumours, express Fas, an obvious application of this system is the killing of tumour cells. It remains to be seen whether FasL has any therapeutic value for patients with Fas positive tumours. Systemic activation of Fas would cause widespread tissue damage, and an effective method of targeting FasL to the tumour directly has yet to be devised. The possible benefits have been demonstrated in mice however in which allogenic fibroblasts engineered to express surface FasL have been implanted with tumour cells and abolished the tumourigenicity of these cells (Drozdzik *et al.*, 1998).

1.6.6 Immune Privilege

There are some organs that are sites of immune privilege, such as the eye and testis (reviewed by Streilein, 1993). Although most organs can tolerate the non-specific damage caused by inflammatory responses associated with immune reactions, there are sites which cannot, and must have a mechanism by which they protect themselves. It was originally thought that inflammatory cells were prevented from entering these organs, however evidence now points to a situation in which activated cells can enter these organs but are immediately killed. This has been proposed to occur via the Fas pathway, with FasL expressed on the cells in these organs inducing apoptosis in the Fas-bearing invading inflammatory cells (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995).

FasL has been found to be constitutively expressed in the iris, corneal epithelium and

endothelium, and ciliary cells of the eye and in the Sertoli cells of the testis (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995). Evidence for the theory of immune privilege in these sites has been generated in wild type and Fas^{gld} mice. The eyes of wild-type mice were infected with virus, and as expected, very few inflammatory cells were detected associated with the retina. In contrast, when the eyes of Fas^{gld} mice were infected, massive infiltration was detected (Griffith *et al.*, 1995). Perhaps more interesting to researchers in the cancer field, was the observation that allogenic transplantation of these tissues from wild-type mice is tolerated, but allogenic transplant tissues from Fas^{gld} mice are rejected (Bellgrau *et al.*, 1995). A similar situation has been observed in some tumour cells which have become resistant to Fas mediated apoptosis and instead constitutively express FasL on their surface (Hahne *et al.*, 1996; Strand *et al.*, 1996). These cells can then induce apoptosis in Fas-bearing CTL and NK cells and evade destruction themselves. The relevance of this mechanism in cancer will be discussed in more detail later.

1.6.7 Signalling from Fas

The stimulation of many receptors by ligand binding leads to dimerisation of the receptor and activation of intrinsic catalytic domains. The intracellular region of Fas does not contain any known catalytic domains however, and dimerisation with a divalent anti-Fas monoclonal antibody is insufficient to mediate an apoptotic signal (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). A region of homology of about 80 amino acids, shared by the cytoplasmic domains of Fas and TNF-R1 suggested a common role for these domains in signalling, as so called 'death domains'. Mutational analyses in Fas and TNF-R1 have confirmed that these regions are responsible for transduction of the cytotoxic signal from these receptors (Huang *et al.*, 1996). In addition, tumour cells which express a truncated Fas molecule lacking the intracellular death domain have been shown to be resistant to apoptosis (Cascino *et al.*, 1996). In order for TNF-R1 to be activated, it must be trimerised (Banner *et al.*, 1993). The similarity between Fas and TNF-R1 led to the generation of a model whereby Fas was activated via trimerisation by a FasL homotrimer (Schneider *et al.*, 1997a).

The description of the trimerisation of Fas and the tendency of death domains to self aggregate led to the suggestion that transduction of the apoptotic signal was mediated by the trimerised death domains. Since Fas mediated apoptosis can occur in the presence of transcriptional inhibitors (Itoh *et al.*, 1991) all the components required for this signal are already present in the cell. The first component involved in Fas signal transduction was identified using the yeast two-hybrid system with the intracellular region of Fas. A molecule containing a death domain at its C-terminus was identified using this system, and called FADD (for Fas-Associating protein with Death Domain), or MORT-1 (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995). Fas activation results in recruitment of FADD/MORT-1 which binds to Fas by interactions between the death domains (Kischkel *et al.*, 1995). In addition to the death domain at its C-terminus, FADD/MORT-1 carries a region at the N-terminus which is responsible for transduction of the downstream signal. This region has been called the death effector domain (DED) (Chinnaiyan *et al.*, 1995). N-terminal truncation of FADD/MORT-1 gives rise to a dominant negative derivative of FADD which can inhibit apoptosis initiated by Fas and other TNF-R family members which appear to share FADD as a common signal transducer (Chinnaiyan *et al.*, 1996).

To investigate the role of FADD *in vivo*, FADD deficient mice were generated (Yeh *et al.*, 1998; Zhang *et al.*, 1998), however unlike Fas null mice, FADD -/- embryos die *in utero* of cardiac failure and abdominal haemorrhage (Yeh *et al.*, 1998) at between day 9 and day 11.5 of gestation, indicating that FADD is essential for embryonic development (Yeh *et al.*, 1998; Zhang *et al.*, 1998). FADD -/- chimeras were generated using FADD-/- ES cells on a background lacking the recombinant activating gene RAG-1. Since RAG-1 -/- mice are incapable of producing any B or T-cells, all the lymphocytes in these mice are derived from FADD-/- ES cells. Fas induced apoptosis was completely blocked in these cells (Zhang *et al.*, 1998), and in FADD deficient embryonic fibroblasts (Yeh *et al.*, 1998). These results suggest that FADD is essential for Fas mediated apoptosis, however other putative Fas signalling pathways which are independent of FADD have been studied and will be discussed later, and other receptors may recruit FADD in signalling pathways.

In order to identify the signalling molecule downstream of FADD, the N-terminal

DED domain was used as bait in the yeast two-hybrid system (Boldin *et al.*, 1996). At the same time, another group sequenced a novel component of the Fas signalling complex (Muzio *et al.*, 1996), or DISC (for death inducing signalling complex). This 55kDa molecule showed homology to both FADD and the ICE/CED 3 family of cysteine proteases, or caspases, and was termed FLICE (for FADD-like ICE), or MACH (for MORT-1 associated CED 3 homologue), although it is now more commonly known as caspase 8, a member of the caspase 3 subfamily (Alnemri *et al.*, 1996). Upon recruitment to FADD/MORT-1, the C-terminal protease domain of caspase 8 is proteolytically self-activated by a two step mechanism which may only occur at the DISC. Initial cleavage results in a 43kDa and a 12kDa fragment. The FADD bound 43kDa fragment is subsequently cleaved and an 18kDa proteolytically active fragment is released (Medema *et al.*, 1997). This protein is then able to cleave specific substrates as the first step in the proteolytic cascade leading to cell death (see Figure 1.1A).

Overexpression of caspase 8 was found to induce apoptosis, which was blocked by some caspase family inhibitors (Muzio *et al.*, 1996). Some general caspase inhibitors which were able to block Fas induced apoptosis however, were ineffective as inhibitors of caspase 8 activation (Medema *et al.*, 1997). These results indicate that other downstream caspases are also required for Fas mediated apoptosis, and suggest that caspase 8 is active early in the signalling cascade. The role of caspase 8 in Fas apoptotic signalling was tested in a number of cell lines and in each, its recruitment to FADD was an essential step in death signalling (Medema *et al.*, 1997; Juo *et al.*, 1998). Caspase inhibitors have also been shown to block Fas mediated apoptosis in T cells, confirming a role for caspase involvement in this pathway (Moreno *et al.*, 1996). These results are similar to those found in FADD deficient cells, and suggest that caspase 8, like FADD is an essential component of the Fas signalling system.

Caspase 8 appears to be targeted by viruses seeking to avoid the host's apoptotic response. Thome *et al.* (1997) described a family of viral inhibitors of apoptosis which they called v-FLIPs (for viral FLICE Inhibitory Proteins) which are present in several gamma-herpesviruses and in the proliferative human molluxipoxvirus. v-FLIPs interact with FADD and inhibit recruitment and activation of caspase 8 thus

protecting infected cells against Fas induced apoptosis (Thome *et al.*, 1997). Cellular homologues of these inhibitors have also been identified (Tschopp *et al.*, 1998). The importance of caspase 8 in cells was confirmed in studies of caspase 8 knockout mice (Varfolomeev *et al.*, 1998). Disruption of the caspase 8 gene results in death at day 12.5 to 13.5 of gestation due to hyperaemia, cardiac abnormalities and erythrocytosis in the liver. Loss of this gene appears to result in congested accumulation of erythrocytes and dramatic depletion of the haemopoietic precursor pool (Varfolomeev *et al.*, 1998). This latter defect may be secondary to other abnormalities yet to be clarified in these mice. Predictably, in embryonic fibroblasts derived from these mice, cell death from Fas, the TNF receptors and DR3 was completely blocked, confirming previous results (Varfolomeev *et al.*, 1998).

Until recently, the DISC was believed to be made up of Fas, FADD and caspase 8 alone, however a novel protein has recently been identified, which is reported to be a component of the DISC. FLASH (for FLICE-associated huge protein) was identified by using the yeast two-hybrid system with caspase 8 DED domains as bait (Imai *et al.*, 1999). FLASH is reported to bind activated Fas as well as FADD and caspase 8, and is also able to self-associate. Transient expression of FLASH activates caspase 8, and overexpression of a truncated form of FLASH blocks caspase 8 activation and Fas induced apoptosis (Imai *et al.*, 1999). Further studies of FLASH are necessary to confirm its presence in the DISC, and to assess its importance in signalling.

1.6.8 Two Fas Signalling Pathways

Recently the importance of mitochondria as regulators of apoptosis was recognised (Shimizu *et al.*, 1996; Kroemer *et al.*, 1997). Early after induction of apoptosis (Shimizu *et al.*, 1996), including induction by Fas, a drop in mitochondrial transmembrane potential ($\Delta\Psi_m$) can be detected which may be due in part to the simultaneous opening of permeability transition (PT) pores (Zamzami *et al.*, 1996). Inactivation and release of cytochrome *c* from the mitochondria, into the cytoplasm, occurs in parallel with these events (Krippner *et al.*, 1996; Adachi *et al.*, 1997), resulting in activation of caspase 9 which is activated when complexed with Apaf-1, and in turn activates caspase 3. All apoptogenic activities of mitochondria can be

blocked by overexpression of Bcl-2 or Bcl-x_L which act upstream of Apaf-1. Bcl-2 and Bcl-x_L are members of the Bcl family. Members of this family have been shown to block cell death and protect cells from a wide variety of apoptotic cues (Yang & Korsmeyer, 1996). Bcl-2 family members have recently been reported to regulate the release of cytochrome *c* (Kluck *et al.*, 1997; Yang *et al.*, 1997a) by binding voltage dependent ion channels (VDACs) in the mitochondrial membrane (Shimizu *et al.*, 1999).

The ability of Bcl-2 family members to inhibit Fas mediated apoptosis has been the subject of controversial discussion (reviewed by Peter & Krammer, 1998). Bcl-2 and Bcl-xL have been reported to inhibit Fas-mediated apoptosis *in vitro* and *in vivo* (Itoh *et al.*, 1993; Boise *et al.*, 1995: Rodriguez *et al.*, 1996; Schneider *et al.*, 1997b). Fas mediated apoptosis was reported to be inhibited in murine cell lines overexpressing Bcl-2 (Itoh *et al.*, 1993) and completely blocked in mitochondrial DNA depleted HeLa cells which overexpress Bcl-2 (Asoh & Ohta, 1997). Recent work by Krammer and colleagues however, has resulted in the identification of two distinct cell types each using one of two different Fas signalling pathways: mitochondria independent and mitochondria dependent Fas mediated apoptosis, (Scaffidi *et al.*, 1998, see also Figure 1.1). Characterisation of a number of cell types has predicted that T cells are type I cells while B cells and hepatocytes are type II (Scaffidi *et al.*, 1998). In both type I and type II cells, Bcl-2 or Bcl-xL blocked all mitochondrial apoptogenic activity. In type I cells however, blocking mitochondrial apoptotic function by overexpression of Bcl-2 had no effect on activation of caspase 3 or 8, or on the sensitivity of these cells to Fas induced apoptosis. The DISC activated large amounts of caspase 8 within seconds and caspase 3 within 30 minutes in these cells (Scaffidi *et al.*, 1998). In type II cells apoptosis was dependent on activation of mitochondria, with overexpression of Bcl-2 resulting in complete blockage of caspase 8 and caspase 3 activation, and apoptosis (Scaffidi *et al.*, 1998). In type II cells DISC formation was strongly reduced and activation of caspase 8 and 3 was delayed for about one hour following loss of $\Delta\Psi_m$ (Scaffidi *et al.*, 1998). The mechanism for mitochondrial activation in type II cells is not yet clear. Since type II cells show only moderate DISC formation however, mitochondria may function as an amplifier of the Fas signal in these cells, activating caspase 8 and 3 via caspase 9.

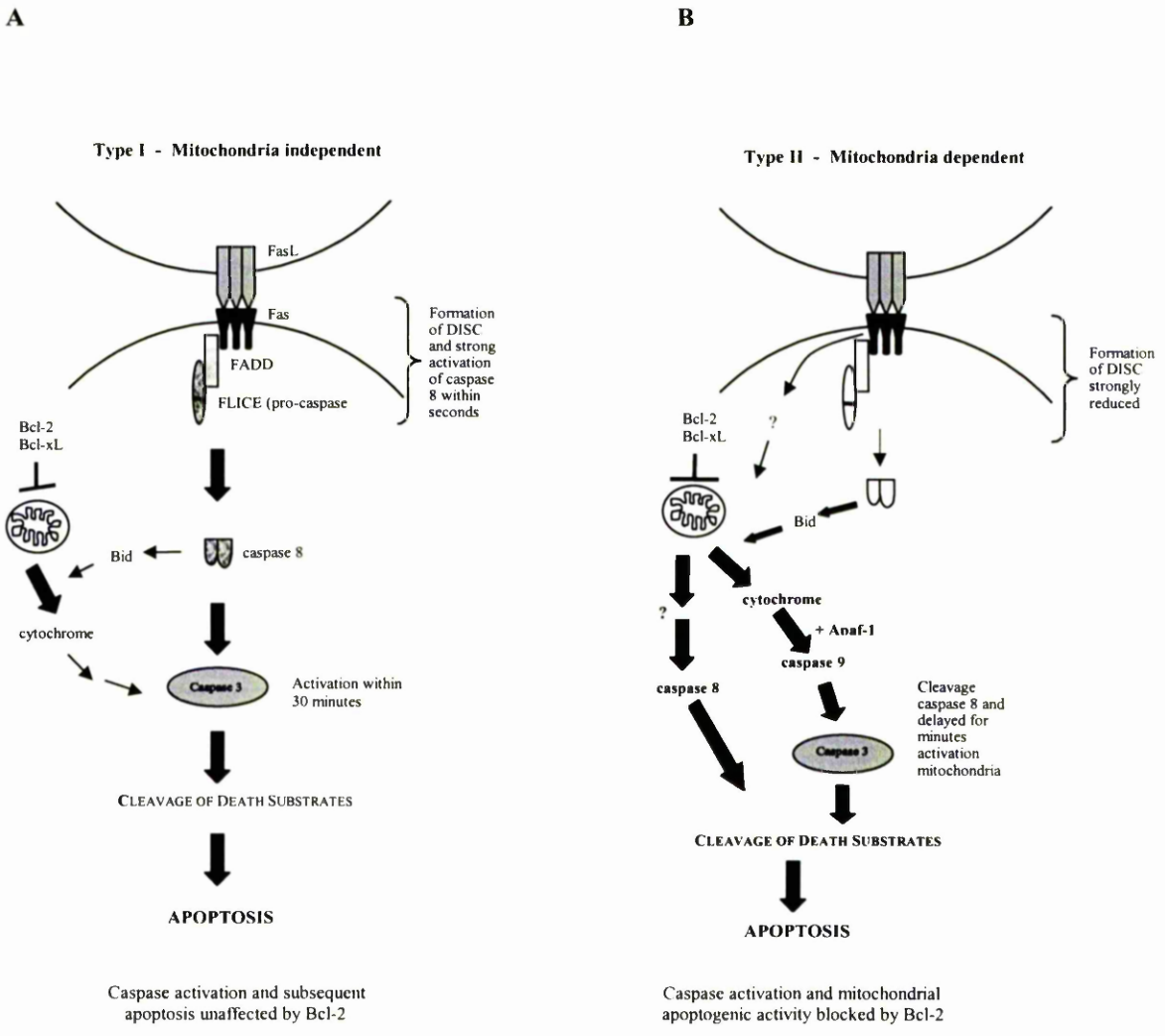


Figure 1.1 Two Fas Signalling Pathways

As mentioned previously however, there are proteins other than FADD which have been implicated in Fas signalling by direct interaction with Fas. These signalling pathways are independent of either the type I or type II classical death pathways. Yang and colleagues cloned and characterised a novel signalling protein which they called Daxx, which binds specifically to the Fas death domain but lacks a death domain of its own (Yang *et al.*, 1997b). Overexpression of Daxx enhances Fas-mediated apoptosis and leads to JNK activation (Yang *et al.*, 1997b). This pathway acts co-operatively with the FADD pathway and is inhibitable by Bcl-2, indicating a FADD independent apoptotic pathway downstream of Fas (Yang *et al.*, 1997b). The finding that in certain cells, for example HeLa cells, the JNK pathway is dispensable (Liu *et al.*, 1996; Yang *et al.*, 1997b), while other cells required the JNK pathway for Fas induced apoptosis (Yang *et al.*, 1997b) supports the involvement of Daxx and the JNK pathway in cell type specific Fas mediated apoptosis. However, comparison of the speed of activation of Jun kinases in type I and type II cells revealed no differences (Cahill *et al.*, 1996), so it remains to be seen how relevant the Daxx/JNK pathway is in mitochondria dependent apoptosis.

Caspase 10 is another protein that has been reported to be directly activated by Fas (Fernandes-Alnemri *et al.*, 1996), and is therefore a candidate for mitochondrial activation in type II cells. Scaffidi and colleagues however suggest that caspase 10 cleavage occurs downstream of mitochondrial activation and is therefore unlikely to be responsible for triggering mitochondrial apoptotic activity (Scaffidi *et al.*, 1998). Ligation of Fas has also been shown to result in activation of an apoptotic Ras/MAP kinase signalling pathway via sphingomyelinase mediated ceramide generation (Gulbins *et al.*, 1995). More recently, triggering of this pathway has been demonstrated to be regulated by caspases, since caspase inhibitors were able to block utilisation of sphingomyelinase and ceramide release (Brenner *et al.*, 1998). Caspase inhibitors were also able to block all Fas induced death suggesting that the ceramide pathway acts downstream of caspase activation. It may be that the ceramide pathway, caspase 10 activation and the Daxx/JNK pathway contribute to the spread of the intracellular death signal, and act as a signal amplifier for cell types with low caspase activity. The perceived importance of caspase 10 in the Fas signalling system was recently heightened with the discovery that inherited mutations in

caspase 10 are responsible for type II ALPS in humans, a condition similar to the classical disease associated with Fas mutations (Wang *et al.*, 1999a).

1.6.9 Fas:FasL Interactions in Tumourigenesis

The number of signalling systems that Fas can trigger underlines the importance of an intact Fas pathway. Unsurprisingly therefore, deregulation of Fas has been implicated in several physiological abnormalities. While we know that loss of function can lead to autoimmunity and lymphoproliferation, the importance of a defective Fas pathway in tumourigenesis is less well understood and has been the subject of much debate. Much of the confusion stems from the fact that even if loss of Fas is shown to accelerate tumourigenesis, it may not be because Fas is a bone fide tumour suppressor but rather due to the creation of an immunocompromised environment in which tumours may develop more readily. A number of studies however, have proposed that loss of Fas predisposes toward cancer.

Much of the doubt surrounding the role of Fas as a tumour suppressor resulted from the lack of malignancies in Fas deficient mice. To address the question of whether this was due to regulation by T-cells, Peng and colleagues generated T-cell deficient mice on a Fas^{lpr} background. These mice spontaneously developed malignant B cell lymphomas with significant associated mortality, while the non-T cell deficient Fas^{lpr} mice and the Fas^{wt} T cell deficient animals did not (Peng *et al.*, 1996). These results implicated Fas in the development of B cell lymphoma, while suggesting that T-cells are able to regulate B cell lymphomagenesis by a Fas independent mechanism. A report of outgrowth of monoclonal B cell populations in Fas deficient mice supported this theory (Davidson *et al.*, 1998). Although malignancies in Fas^{lpr} and Fas^{gld} mice had never previously been reported, Davidson and colleagues investigated the possibility that defective Fas-FasL interactions may lead to lymphoma later in life. They examined Fas^{lpr} and Fas^{gld} mice aged 6 to 15 months and found that a significant proportion had monoclonal B cell populations in spleen and lymph node, compared to none in the control groups (Davidson *et al.*, 1998). They also found that B cell populations from Fas^{gld} mice were transplantable into immunodeficient mice, where they grew and metastasised indicating the presence of malignant cells

(Davidson *et al.*, 1998). These data support a role for the Fas pathway in preventing development of B cell neoplasia.

Downregulation of Fas has also been reported in human tumours. Mutations in the Fas gene have been reported in gastric cancer and in T cell lymphoma (Zoi-toli *et al.*, 2000; Park *et al.*, 2001). In contrast however, a recent study of human haematological malignancies reported that Fas and Fas pathway components were not the targets of mutation (Rozenfeld-Granot *et al.*, 2001). Loss of FasL has also been described in murine tumours (Ouhtit *et al.*, 2000), however a significant positive correlation between malignancy and FasL expression has been observed in studies in human breast and gastric tumours in which FasL expression has been correlated with metastatic potential, tumour size and malignancy (Mottolese *et al.*, 2000; Liu *et al.*, 2001). The role of Fas:FasL interactions in human malignancy is still the subject of much debate.

If loss of Fas expression were a feature of tumour progression, it would be expected to allow acceleration of virally induced T cell lymphomagenesis. In Fas^{lpr} mice however, Moloney murine leukaemia virus induced lymphomagenesis was not accelerated in comparison with control C57BL/6 mice, and proviral insertion sites in Fas^{lpr} tumours were characteristic of wild type mice (Zornig *et al.*, 1995). If Fas represented a tumour suppressor, then it would be expected that genes that could act in synergy with loss of Fas, may represent preferential targets for proviral insertion, and that reduced latency may be observed for these tumours. This was not the case however, providing evidence against a role for Fas in tumour suppression.

Other groups have explored whether lack of a functional Fas pathway could represent a step in the multi-stage process of tumourigenesis, by investigating acceleration of transgene induced tumourigenesis on a Fas deficient background. In some cases, no acceleration has been seen. In Eμ-*pim-1* transgenic mice for example, which normally develop T-cell lymphoma with an incidence of about 10% and a latency period of 7-9 months, the Fas^{lpr} mutation does not predispose to tumourigenesis (Moroy *et al.*, 1993). The lymphoproliferation associated with the homozygous Fas^{lpr} mutation is accelerated however and this occurs through inhibition of apoptosis

by *Pim-1* overexpression. A similar acceleration of lymphoproliferative disease but not of lymphomagenesis was seen in *Trp53* null mice on a Fas^{lpr} background (Cameron *et al.* unpublished results).

Loss of either Fas or p53 may be important in tumourigenesis, because of their ability to induce apoptosis. Loss of both pathways together may be synergistic events during tumourigenesis, if these are independent of each other. The relationship between the two pathways has been studied however, and there may be some functional overlap. The relationship between p53 and Fas/FasL is complex since two distinct pathways, one transcription dependent, the other transcription independent, have been proposed for the effect of p53 on Fas. There is evidence to suggest that p53 regulates the expression of Fas in human tumour cell lines; introduction of wild-type p53 into p53 null cells can enhance expression of the Fas gene, and this does not require *de novo* protein synthesis, suggesting direct regulation of the Fas gene (Owen-Schaub *et al.*, 1995). In contrast Bennett and co-workers have described trafficking of Fas from the Golgi complex to the cell surface, and heightened sensitivity to Fas induced apoptosis on activation of regulatable p53 in human vascular smooth muscle cells (VSMCs, Bennett *et al.*, 1998). In these experiments, no difference was seen in Fas or FasL mRNA expression after p53 activation, but surface Fas was transiently increased (Bennett *et al.*, 1998). In a separate experiment, these authors also found that p53 induced apoptosis was reduced in Fas^{lpr} and Fas^{gld} MEFs, compared with wild type MEFs (Bennett *et al.*, 1998). They suggest that tumour cells lacking functional p53 will evade apoptosis induced by both p53 transcriptional targets and by FasL, or, more simply, that p53 loss results in loss of not only p53 mediated apoptosis, but also a reduction in Fas induced apoptosis. A later study however showed that while p53 dependent DNA damage-induced apoptosis required caspase 3 activation, this was independent of Fas:FasL interactions (Fuchs *et al.*, 1997). To confuse the relationship between Fas and p53 still further, while FADD-DN can inhibit activation induced proliferation in T cells from wild type mice it shows no inhibitory effect on proliferation of *Trp53* null cells (Zornig *et al.*, 1998). The reason for this has not been determined yet, but highlights a growth promoting role for TNF-R family members in addition to their apoptotic function, which may be mediated by inhibition of p53 dependent growth arrest.

Similarly, conflicting results have been generated from studies of the relationship between Fas and Bcl-2 in tumourigenesis. Deregulation of Bcl-2 expression has been found in many human cancers (reviewed by Reed *et al.*, 1996), however overexpression of Bcl-2 alone is relatively benign in transgenic mouse models (McDonnell *et al.*, 1989, Strasser *et al.*, 1990a). When coupled to additional lesions however, such as overexpression of the oncogene c-*MYC*, deregulated expression of Bcl-2 can rapidly lead to transformation (Strasser *et al.*, 1990b). The question of whether or not Bcl-2 overexpression and loss of functional Fas are overlapping events in tumourigenesis, if Fas has a role in preventing tumourigenesis at all, has been investigated in a number of studies. On a Fas^{lpr} background, Eμ-Bcl-2 transgenic mice showed no tumour formation but instead showed increased lymphoproliferation (Strasser *et al.*, 1990a; Strasser *et al.*, 1995), suggesting a Bcl-2 dependent mechanism for regulation of lymphocyte homeostasis in addition to the Fas-mediated pathway. These results would indicate that the two pathways (Fas loss and overexpression of Bcl-2) can work in an additive or complementary way to block cell death. This hypothesis is confirmed by studies using Bcl-2 transgenic mice with constitutive expression targeted to myeloid cells. In these mice, loss of Fas led to development of acute myeloblastic leukaemia, which did not occur in either control group (Traver *et al.*, 1998). These findings again suggest a synergy between defects in Fas and overexpression of Bcl-2. It is likely that in this model, enhanced Bcl-2 expression and loss of the Fas death pathway co-operate to facilitate survival of cells harbouring oncogenic mutations, which may otherwise be killed. Further, Bcl-2 overexpressing T cells are sensitive to anti-Fas antibody induced apoptosis (Van Parijs *et al.*, 1998) indicating a Fas mediated apoptotic pathway insensitive to Bcl-2, which is likely to operate through type I signalling. The extent of overlap in function between Fas and Bcl-2 may depend on the apoptotic stimuli and on the cell type. This theory is supported by the work of Scaffidi *et al.*, (1998) highlighting the existence of two distinct Fas signalling pathways in different cell types (Scaffidi *et al.*, 1998).

There also seems to be some synergy between oncogenic Ras and loss of Fas, as H-Ras has been reported to downregulate the expression of Fas and inhibit FasL mediated apoptosis via activation of the PI3 kinase pathway (Peli *et al.*, 1999). It

appears that Ras not only has the potential to promote proliferation, but also to inhibit FasL induced apoptosis. Ras has also been reported to inhibit c-MYC induced apoptosis through triggering of PI3 kinase (Kauffmann-Zeh *et al.*, 1997), but no mechanism by which Ras may link *MYC* and Fas induced apoptosis has been established thus far.

Regulation of FasL expression has also been observed in T-cells overexpressing the *AML1/Runx1/Cbfa2* gene (Fujii *et al.*, 1998), which is associated with human acute myeloid leukaemia. Overexpression of AML-1 induced resistance to anti-CD3 induced apoptosis, and inhibited expression of FasL (Fujii *et al.*, 1998). Induction of FasL transcription was also found recently to be dependent on expression of the full-length product of a newly discovered gene called apoptosis linked gene 4 (ALG-4, (Lacana' & D'Adamio, 1999). Since FasL is subject to such strict regulation, it is possible that it may have roles other than as a ligand for the Fas receptor, and may be involved in some Fas independent systems.

1.6.10 Fas and its Relationship with c-*MYC*

c-*MYC* is an oncogene frequently mutated in human tumours (Marcu *et al.*, 1992) and as such has been the subject of a great deal of research. Constitutive expression of c-*MYC* in lymphoid cells predisposes to lymphomagenesis (Adams *et al.*, 1985). Deregulation of *MYC* results in increased proliferation of cells through its activity as a transcription factor, but this proliferation is limited by MYC's other function as an inducer of apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992). In an environment of low serum concentration or hypoxia (Alarcon *et al.*, 1996), *MYC* upregulation results in apoptosis and this may restrain inappropriate cell growth. Delineation of the mechanisms used by MYC to induce apoptosis may be very important in establishing novel therapeutic targets.

The Fas pathway has been implicated in MYC induced apoptosis in some cell types (Hueber *et al.*, 1997), and a role for caspase 3 like proteases in c-MYC induced apoptosis has been demonstrated (Kagaya *et al.*, 1997), but the relationship between MYC and Fas has yet to be clearly established. Transgenic mice carrying the L-*MYC*

gene under the control of the immunoglobulin enhancer element E_μ show elevated expression of L-*MYC* in B and T-cells, and develop predominantly T-cell lymphoma (Moroy *et al.*, 1990). These mice were crossed onto a Fas^{lpr} background and showed an accelerated rate of tumour formation of both B and T-cell origin (Zornig *et al.*, 1995).

Generation of a regulatable form of c-*MYC*, made by linking c-*MYC* to a mutated form of the oestrogen receptor element which responds to 4-hydroxy tamoxifen (4-OHT) treatment (Hueber *et al.*, 1997), allowed examination of the role of Fas in c-MYC induced apoptosis. When cells stably expressing the c-MYC transgenic construct are treated with 4-OHT, they die by apoptosis (Hueber *et al.*, 1997). In immortalised S3T3 fibroblasts, blocking Fas:FasL interactions, or use of DN-FADD leads to reduced and delayed MYC-induced apoptosis (Hueber *et al.*, 1997). In addition, in mouse embryo fibroblasts (MEFs) from Fas^{lpr} mice transiently transfected with the construct, activation of c-MYC had no effect on the rate of apoptosis, while in wild type MEFs the rate of apoptosis increased (Hueber *et al.*, 1997). One hypothesis states that c-MYC utilises the Fas pathway at least in part, by sensitising the cell to Fas mediated apoptosis at some point downstream of Fas ligation (Juin *et al.*, 1999). Some evidence to support this theory is the lack of acceleration of FADD or pro-caspase 8 induced apoptosis by activated c-MYC in contrast to that normally seen in FasL induced apoptosis (Rohn *et al.*, 1998). In addition, suppression of c-MYC induced apoptosis by insulin like growth factor, IGF-1, and by Bcl2, occurs downstream of Fas ligation (Hueber *et al.*, 1997; Rohn *et al.*, 1998), and in cells expressing dominant negative FADD, c-MYC induced apoptosis is not inhibited (Yeh *et al.*, 1998).

Evidence from other groups suggests that *MYC* may collaborate with the Fas pathway through regulation of FasL expression. While no changes in Fas expression have been seen after c-*MYC* activation, regulation of FasL expression by c-*MYC* remains a possibility. A fungal metabolite, FR901228 that was shown to inhibit c-*myc* expression in lymphoid cells, was found to specifically inhibit activation (anti-CD3) induced apoptosis independently of Il-2 (Wang *et al.*, 1998). This was shown to be a result of inhibition of FasL expression which was reversed by ectopic c-*MYC*

expression, suggesting that inhibition of FasL is mediated through loss of c-*myc* (Wang *et al.*, 1998), and conversely that MYC is influencing FasL expression.

Further to these results, work by Thomas Brunner and Douglas Green in recent years has identified a link between *MYC* and FasL expression. Where previously, TGF-β was shown to enhance resistance to Fas induced apoptosis in T cells by an unknown mechanism (Cerwenka *et al.*, 1996), they have demonstrated that TGF-β1 inhibits activation (anti-CD3) induced apoptosis in human T-cells, by inhibition of FasL expression (Genestier *et al.*, 1999). Simultaneous inhibition of c-*MYC* expression was observed, and ectopic expression of c-*MYC* relieved the inhibition of FasL expression and of activation induced apoptosis (Genestier *et al.*, 1999). Recently, direct evidence of a regulatory role of c-Myc on transcription of FasL has been generated (Brunner *et al.*, 2000). Down regulation of c-*myc*, using anti-sense oligonucleotides blocked FasL mRNA expression in activated T cells (Brunner *et al.*, 2000). Subsequently, a binding site for the myc-max heterodimer was discovered in the FasL promoter which, when mutated abolished the transcriptional response of FasL to c-MYC overexpression (Kasibhatla *et al.*, 2000). Further work is required however to determine the reasons for the ability of c-MYC to upregulate FasL, and the relevance of this relationship in tumourigenesis.

1.6.11 Immune Privilege in Tumours

A further complication for those studying the role of Fas as a possible tumour suppressor is the fact that tumours may be able to evade the immune response by making use of the Fas pathway themselves (Walker *et al.*, 1998). In addition to down-regulation of the Fas receptor, it appears that in order to escape immune attack tumour cells can kill attacking lymphocytes by expression of FasL. In other words, tumours may become sites of immune privilege, similar to the eye. This has been demonstrated in co-culture experiments, in which tumour cells expressing FasL have killed Fas expressing Jurkat T lymphocytes (Strand *et al.*, 1996). For those tumours which have an intact Fas pathway but do not express FasL, another mechanism of immune evasion exists in the form of a decoy receptor, DcR3 (Pitti *et al.*, 1998). This is a soluble FasL receptor, which binds to FasL and restricts FasL mediated

apoptosis. The gene for this receptor has been found amplified in lung and colon cancers (Ohshima *et al.*, 2000), and represents another mechanism which may contribute to immune evasion by certain tumours. The fact that discovery of this receptor was only relatively recent, also gives weight to the possibility that there may be more, as yet undiscovered receptors for FasL.

1.6.12 Functional Redundancy in the TNF Family

The Fas pathway may not be critical in preventing tumourigenesis, because of functional redundancy between itself and other members of the TNF receptor family. The family includes two TNF receptors (TNF-R1 and TNF-R2), the NGF receptor, CD40, CD27, CD30, the lymphotoxin-β receptor, RANK (receptor activator of NF-κ B), DR-3, and the TRAIL (TNF related apoptosis inducing ligand / APO-2) receptors DR4, DR5, DcR1 and DcR2. This is not a complete list however. The family is still growing and more members are likely to be added in the future.

Many of these receptors are known primarily as regulators of proliferation. Interaction of RANKL (TRANCE/OPGL) on dendritic cells with its receptor on T cells stimulates naïve T cell proliferation in a mixed lymphocyte reaction and increases survival of $RANK^+$ T cells (Anderson *et al.*, 1997a). RANKL deficient mice exhibit defects in early differentiation of T and B lymphocytes and lack lymph nodes, indicating its importance as a regulator of lymphocyte development and organogenesis (Kong *et al.*, 1999).

CD40 is essential in activating antigen presenting cells (APCs) to process and present antigen effectively (Grewal & Flavell, 1998), and is required for the generation of CTLs (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). Cross-linking of CD40 on APCs is thought to activate them by improving antigen processing, upregulating cytokine production and increasing expression of co-stimulatory and adhesion molecules. When mice with lymphoma are treated with monoclonal activating anti-CD40 antibody there is a rapid cytotoxic T cell response resulting in a tenfold expansion of $CD8^+$ T cells over 5 days which eradicates the tumour (French *et al.*, 1999). Conversely, antigen specific T cell priming fails to

occur in mice lacking CD40L (Grewal *et al.*, 1995).

Among TNF family members, FasL and TRAIL share the highest homology and the ability to induce apoptosis, raising the possibility of functional redundancy between the two ligands. In support of this theory, Mariani and colleagues have recently demonstrated that FasL resistant cells may be sensitive to TRAIL induced apoptosis (Mariani *et al.*, 1997). Differential regulation of the two ligands has been shown on lymphoma cell lines with different cell types showing predominant expression of one or other ligand (Mariani & Krammer, 1998). The necessity for Fas to induce apoptosis in individual cell types may depend very much on the cell type and the relative abundance of these two ligands.

CHAPTER 2

MATERIALS & METHODS

2.1 ANIMALS

All animal work was carried out under Home Office regulations

2.1.1 Transgenic Mouse Stocks

The generation of CD2-*MYC*ER^{TM} transgenic mice has been described previously (Blyth *et al.*, 2000). A human c-*MYC* cDNA was fused to a mutated murine oestrogen receptor, which is responsive only to tamoxifen and not to oestradiol (Littlewood *et al.*, 1995), and cloned into the multiple cloning region of the CD2 minigene vector VA hCD2 (Zhumabekov *et al.*, 1995) in order to target the c-*MYC* gene to the T-cell lineage, as described in Blyth *et al.*, (2000). This work was carried out by Dr. M. Stewart. The CD2-*MYC*ER^{TM} construct was then microinjected into C57Bl/6J x CBA/Ca F2 (B6/CBA) fertilised eggs according to standard protocols (Hogan *et al.*, 1986) by Dr. E. Cameron and Mrs. M. Bell. Two transgenic lines were established, the CD2-*MYC*ER^{TM}15 and the CD2-*MYC*ER^{TM}2 lines. The CD2-*MYC*ER^{TM}15 line (hereafter referred to as CD2-*MYC*ER^{TM}) was selected for use in this study, except where stated otherwise, and animals were maintained as heterozygotes on an indiscriminate B6/CBA background.

Fas^{lpr} mice deficient for a functional Fas gene were obtained from Harlan, UK, and maintained as homozygotes. These mice are derived from a spontaneous congenic mutation in the Fas gene, originally found in MRL strain mice (Watanabe-Fukunaga *et al.*, 1992). Control MRL mice were also supplied by Harlan, UK.

Mice deficient for a functional *Trp53* tumour suppressor gene were derived using homologous recombination in murine ES cells by L. Donehower and co-workers (1992). Briefly, a null mutation with an inserted *pol*II-*neo* expression cassette and a 106bp deletion in exon 5 of the p53 gene was introduced into the germ line of 129/Sv

ES cells. Chimaeric mice, generated by introducing the targeted ES cells into C57Bl/6 blastocysts, were crossed onto a C57Bl/6 background. The genetic background of the mice used in the studies described here were an indeterminate mix of C57Bl/6, 129/Sv and NIH strains. The use of the p53-deficient mice (hereafter referred to as *Trp53* null or *Trp53*$^{-/-}$) has been with the kind permission of Dr. Larry Donehower.

2.1.2 *In Vivo* Induction of CD2-*MYC*ER^{TM} Transgene

To induce activity of the CD2-*MYC*ER^{TM} transgenic construct *in vivo*, animals carrying the CD2-*MYC*ER^{TM} transgene were administered tamoxifen by continuous oral dosing in drinking water, made up every 7 days. Tamoxifen citrate (Sigma-Aldrich) was dissolved in absolute ethanol and made up in ultra-pure water, to a concentration of 100μg/ml in 1% ethanol. Control animals were treated with 1% ethanol in ultra-pure water.

2.1.3 Challenge of Mice with MoMuLV

MoMuLV clone 1A supernatant was isolated from virus infected 3T3 cells (a gift of Dr. A. Berns) during the log phase of growth and filter sterilised through a 0.45μm filter (Gelmain Sciences) to remove cell debris. Aliquots of 1ml of filtered supernatant were frozen at -70°C until required. Neonatal animals were inoculated intraperitoneally with 0.1ml of supernatant, estimated at 10^4-10^5 infectious units of virus, within 24 hours of birth, by Dr. K. Blyth.

2.1.4 Tumour Transplantation

Tumour cells were transplanted into Fas^{lpr}, MRL and MF1-nude host mice (Harlan, UK). Suspension tumour cells were prepared as described in 2.4.2. Cells were prepared for transplantation by centrifugation at 1500rpm for 5 minutes, the supernatant removed, and the cells resuspended in RPMI, by Dr. Karen Blyth. 2 x 10^7 tumour cells in 0.5ml volume were injected intraperitoneally into 6-8 week old Fas^{lpr} and MRL recipient mice by Dr. E. Cameron and Dr. K. Blyth. Frozen cells

were thawed and then prepared as described above for transplant into nude mice. 2 x 10^7 tumour cells in 0.5ml volume were injected intraperitoneally into 6-8 week old female nude mice, by Dr. K. Blyth.

2.1.5 Mouse Genotype Analysis

Positive CD2-*MYC*ER^{TM} and *Trp53* null transgenic animals were identified by screening tail DNA for the presence of the transgene/null mutation by Southern blot hybridisation. The status of the Fas gene was examined by PCR analysis of tail DNA.

Potential transgenic animals were weaned from the breeding stocks at 3-4 weeks of age and separated by sex. At 4-6 weeks of age, animals were anaesthetised by inhalation of fluothane (Schering-Plough) anaesthetic vapour in oxygen gas, and a 1-2cm biopsy of tail taken. The tail wound was then cauterised. Animals were tagged by an ear nicking identification scheme and allowed to recover. DNA extraction and Southern blot analysis on the tail tissue was carried out as described in section 2.2.

2.1.6 Clinical Examination and Post-mortem Investigation

Experimental cohorts and breeding stocks of mice were maintained for defined periods of time and the health of animals checked at least three times weekly. The development of lymphoid neoplasia in the transgenic animals presented as cachexia and tachypnoea. The development of lymphoproliferative disorders in Fas^{lpr} animals presented as swellings at the sites of subcutaneous lymph nodes, and arteritis, characterised by skin abrasions. Sarcomas and carcinomas, which often developed in *Trp53* null mice, presented as visible swellings, paresis or cachexia depending on the anatomical site of the tumour. Animals were humanely sacrificed by cervical dislocation when clinical signs first became evident.

Post-mortem was routinely undertaken immediately after euthanasia, or as near to the time of death as possible, and the pathology recorded. Particular attention was paid to the lymphoid organs, specifically the thymus, spleen, mesenteric and subcutaneous

lymph nodes. Evidence of metastasis or lymphocytic infiltration to non-lymphoid organs was also noted. Tissues were frozen in cryotubes (Gibco BRL) in liquid nitrogen (BOC) for Southern analysis; fixed in 10% neutral buffered formalin for histopathological examination; and placed in RPMI medium (Gibco BRL) for cell biology experiments.

2.2 SOUTHERN HYBRIDISATION ANALYSIS

2.2.1 Isolation of Genomic DNA from Mouse Tail Tissue

Fresh tail tissue was placed in an eppendorf tube and 0.5ml of lysis buffer (100mM Tris-HCl, pH 8.5; 5mM EDTA, pH 8.0 [both Sigma]; 0.2% SDS [BDH]; 200mM NaCl [Fisher]) added. Proteinase K (Invitrogen) was added to a final concentration of 0.5mg/ml, and the samples incubated at 55°C for approximately 16 hours. Samples were then centrifuged in a microfuge at 13,000rpm for 10 minutes to obtain a firm pellet. The supernatant was added to 0.5 ml isopropanol (BDH), in an eppendorf tube, and the tube inverted several times until precipitation was complete. DNA was removed by lifting the aggregated precipitate from solution and allowing excess isopropanol to evaporate. DNA was resuspended in 100µl of ultra-pure water and left for several hours to dissolve.

In order to clean the DNA for PCR analysis, samples were made up to 200µl in ultra-pure water, and an equal volume of phenol:chloroform (Sigma-Aldrich) added. Samples were centrifuged in a microfuge at 13,000rpm for 10 minutes, and the top layer carefully removed and transferred to a fresh eppendorf tube. Approximately 0.1 x volume of 3M CH_3COONa (Fisher) and 2.5 x volume of ethanol (BDH) were added, and the DNA precipitated out by gentle inversion of the tube. The DNA was removed, and excess ethanol air dried, and then redissolved in 50µl of ultra-pure water. DNA concentration was determined by measuring the optical density of the sample at 260nm, on a DU 640 spectrophotometer (Beckman). The DNA sample was then stored at 4°C.

2.2.2 Isolation of Genomic DNA from Mouse Tumour Tissue

Genomic DNA was isolated from mouse tumour tissue using the Nucleon^{TM} II method (Scotlab Bioscience). Approximately 100mg of frozen tissue was ground to a powder in liquid nitrogen (BOC) using a chilled mortar and pestle (BDH). The tissue was resuspended in 2ml of Reagent B (400mM Tris-HCL at pH 8.0, 60mM EDTA, 150mM NaCl and 1% SDS) in a 15ml centrifuge tube by vortexing briefly. RNAse (Sigma) was added to a final concentration of 400ng/ml, and the samples were incubated at 37°C for 30 minutes. Deproteinisation was carried out by adding 500µl of Nucleon sodium perchlorate and rotary mixing for 15 minutes at room temperature. The sample was then incubated in a shaking water bath at 65°C for 25 minutes. DNA was extracted by adding 2ml of chloroform (BDH), which had been stored at -20°C. The sample was rotary mixed for 10 minutes at room temperature and then centrifuged at 3200rpm for 1 minute. 300µl of Nucleon silica suspension was added and the sample centrifuged at 4300rpm for 3 minutes. The DNA phase above the Nucleon silica suspension layer was transferred to a clean 15ml centrifuge tube and centrifuged briefly at 4200rpm to pellet residual Nucleon silica. The supernatant was carefully decanted into a clean 15ml centrifuge tube and two equal volumes of cold ethanol, at 4°C, added. The tube was inverted gently to precipitate the DNA. The precipitated DNA was spooled out into a 1.5ml eppendorf tube and left to dry. The DNA was resuspended in 200 - 400µl of ultra pure water by leaving the sample overnight at room temperature. The DNA concentration was determined by measuring the optical density of the sample at 260nm, on a DU 640 spectrophotometer (Beckman). The DNA sample was stored in an eppendorf tube at 4°C.

2.2.3 Restriction Analysis of Genomic DNA

To detect the presence of the transgene, or the *Trp53* null mutation, or to analyse the integrity of the endogenous *myc* gene, restriction analysis was carried out. DNA digestion with restriction enzymes, separation by agarose gel electrophoresis, transfer to Hybond^{TM} N membranes (Amersham International plc.), hybridisation and

washing of blots were all carried out as described in Sambrook *et al.*, (1989). Briefly, 10ng of the sample to be tested was incubated with 3 units of restriction enzyme (all Gibco BRL), and digestion carried out at 37°C overnight. *Bgl*-II restriction enzyme was used to generate fragments to identify the presence of the CD2-*MYC*ER^{TM} transgene, and *Bam*-HI restriction enzyme was used to examine the *Trp53* gene. *Kpn*-I was used to cut the endogenous *myc* gene, in order to analyse gene rearrangements.

5µl of 5 x TBE loading dye (50% glycerol [BDH], 50% TBE, and bromophenol blue [Sigma-Aldrich]) was added to restriction digested samples. DNA fragments were separated on a 0.8% agarose (Gibco BRL) gel overnight, in 1 x TBE buffer (108g Tris base, 55g Boric Acid, 40ml 0.5M EDTA [all Sigma], pH 8.0 in 1L of de-ionised water) at 24V. DNA gels were subsequently stained with 50mg/L ethidium bromide (Sigma) in TBE buffer for 20 minutes, and destained with de-ionised water for 20 minutes. Gels were viewed on an ultraviolet transilluminator (UVP) and photographed using an MWG-Biotech system to confirm the presence of DNA in the lanes.

The DNA was transferred to Hybond^{TM} N membrane (Amersham International plc.) in 10 x SSC (83.3g NaCl, 41.7g Citric Acid, in 1L water) by the Southern Blotting technique, as described by Sambrook *et al.*, 1989. DNA was immobilised to the nylon membrane by UV crosslinking using a UV Stratalinker XL1500 (Spectronics Corporation). Fragments of interest were detected by hybridisation with radiolabelled probes.

2.2.4 Radiolabelled Probes

CD2-*MYC*ER^{TM} transgene sequences were detected using a human c-*MYC* exon 3 probe (1.38kb *Eco*R1/*Cla*1 fragment made by Ms. J. Irvine). The 260bp *Trp53* exon 4 probe was generated by Dr. E. Baxter, from a plasmid subclone pLTRp53cG (Eliyahu *et al.*, 1985) by polymerase chain reaction (PCR) using oligo primers 5'-CCA TCA CCT CAC TGC ATG G-3' and 5'-CGT GCA CAT AAC AGA CTT GGC-3'. Rearrangements of the endogenous c-*myc* gene were detected using a probe

against exon 3 and part of exon 2 of murine *myc* (0.7kb *Pst*I/*Eco*RI fragment made by Mrs A. Terry).

Using a High Prime™ DNA labelling kit (Boehringer-Mannheim), c-*MYC* and *Trp53* fragments were radiolabelled by nick end labelling to generate dsDNA probes. 20ng of fragment was made to 11µl with ultra pure water, boiled for 10 minutes and chilled on ice. 4µl of High Prime™ and 5µl of [α32P] dCTP (>3000 Ci/mmol, Amersham International plc) were added and incubated at 37°C. The labelled fragment was then eluted through a nick column (Pharmacia) with TE buffer (10mM Tris, pH 8.0, 1mM EDTA, pH 8.0 [Sigma]) and stored at -20°C for up to 2 weeks.

2.2.5 Hybridisation of DNA Blots

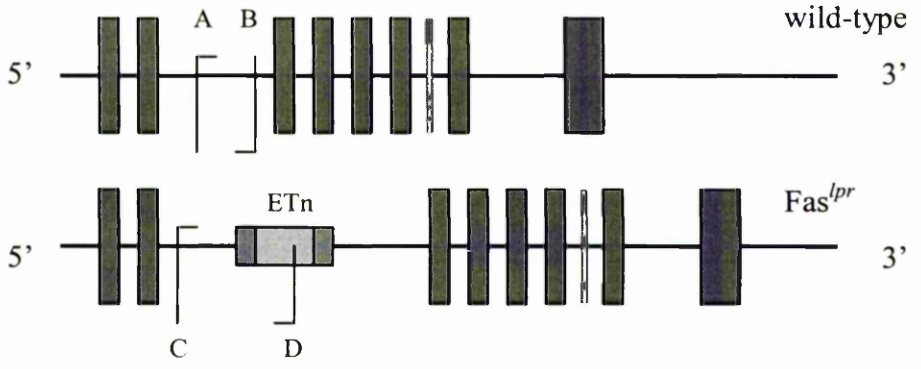
Before hybridisation, DNA blots were pre-incubated with 10-20ml of RapidHyb™ (Amersham International) hybridisation solution in a roller bottle (Hybaid) at 65°C. Radiolabelled dsDNA probe was boiled for 5 minutes and chilled on ice prior to addition of 100-150µl to the pre-hybridised blots. 500µl of Genebloc (Immunogen International) was also added. The blots were hybridised for 2-3 hours at 65°C using RapidHyb™ (Amersham International plc) hybridisation solution as per supplier's instructions. Blots were washed twice at 65°C with 2 x SSC; 0.1% SDS (Sigma) for 15 minutes and twice at 65°C with 0.2 x SSC; 0.1% SDS for 15 minutes and set up for autoradiography with Hyperfilm™-MP (Amersham International plc.).

2.3 PCR Analysis of Genomic DNA – Determination of Fas Status

Polymerase Chain Reaction (PCR) was used to determine the genotype of Fas^{lpr} animals. Genomic DNA was isolated from mouse tail tissue as described in section 2.2.1. Four primers were designed as shown in Figure 2.1. Amplification of genomic DNA was carried out in a 50µl reaction mix which consisted of 2ng DNA, 2 units of Taq polymerase (Perkin Elmer), 200µM of each deoxynucleoside triphosphate in 10mM Tris/50mM KCl/1.5mM $MgCl_2$ buffer. Either primers A and

B, or C and D were added to a final concentration of 0.5μM. Thermal cycling was performed on a Hybaid PCR Express, and conditions used were denaturation at 94°C for 5 minutes followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, ending with a final extension phase of 7 minutes at 72°C and 4°C soak indefinitely. Primers A and B together detect the wild-type allele and primers C and D together detect the Fas^{lpr} mutation of the allele.

PCR products were subjected to gel electrophoresis on a 1.5% agarose (Gibco BRL) gel for 3 hours at 100V in TBE buffer (108g Tris base, 55g Boric Acid, 40ml o.5M EDTA [all Sigma], pH 8.0 in 1L de-ionised water). Gels were stained with ethidium bromide (Sigma) and photographed under ultraviolet transillumination (MWG Biotech system).



Primer Design

A	5' –GGT-TAC-AAA-AGG-TCA-CCC-AT- 3'
B	5' –GGT-CAG-TGA-GTA-ATG-GGC-TC- 3'
C	5' -CAC-TTT-ACT-CAT-TGA-CTT-AT- 3'
D	5' –CGT-TGC-TCC-GAT-GTC-CGA-TA- 3'

Figure 2.1 PCR Probe Design for Fas^{lpr} Diagnosis

2.4 TISSUE CULTURE TECHNIQUES

2.4.1 Media

Complete RPMI

Media was prepared using aseptic techniques. RPMI 1640 medium (Gibco BRL) was supplemented with 10% heat inactivated foetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine (all from Gibco BRL) and 5 x 10^{-5}M 2-mercaptoethanol (BDH).

Complete DMEM

Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) was supplemented with 10% heat inactivated foetal calf serum, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine (all from Gibco BRL) and 5 x 10^{-5}M 2-mercaptoethanol (BDH).

Cell Freeze Down Medium

10% dimethyl sulphoxide (Sigma-Aldrich) and 20% foetal calf serum (Gibco BRL) was made up in RPMI 1640 medium and filter sterilised through a 0.45μm filter (Gelmain Sciences) in aseptic conditions.

2.4.2 Single Cell Tumour Preparation

Tumour/thymus tissue was disaggregated aseptically in complete RPMI medium using sterile scalpel blades (Fisher Scientific UK) in a 60mm tissue culture petri dish (Gibco BRL). Lymphocytes were isolated on a Ficoll-Paque (Pharmacia) density gradient at 3000rpm for 10 minutes in a 15ml centrifuge tube (Falcon). The interphase layer containing the live lymphocytes was washed in 10ml complete RPMI medium and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 5-10ml of complete RPMI medium. A viable cell count was carried out by trypan blue (Gibco BRL) exclusion on a haemocytometer (Sigma-Aldrich).

Cells were frozen to -70°C in 1.5ml Nunc cryotubes (Gibco BRL) at a concentration

of 5-10 x 10^6 cells/ml in freeze down medium, using isopropanol filled controlled rate freeze down tubs (Sigma-Aldrich) and stored in liquid nitrogen (BOC).

2.4.3 Establishment of Tumour Cell Lines

Tumour cells were prepared as in section 2.4.2. Cells at a concentration of 2.5 x 10^6 cells/ml in complete RPMI medium were cultured in 25cm^2 or 80cm^2 tissue culture flasks (Gibco BRL) at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures were passaged every 4-7 days into fresh medium and maintained at a density of 5 x 10^5 - 5 x 10^6 cells/ml. Cell lines established for a time greater than 6 months were frozen as described above.

2.4.4 Fibroblast Cell Culture

Mouse embryo fibroblasts (MEFs, prepared and supplied by Dr. M. Hu) and NIH/3T3 fibroblasts (ATCC) were cultured in complete DMEM in 25cm^2 or 80cm^2 tissue culture flasks (Gibco BRL) at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures were passaged every 5-7 days before cells became completely confluent. Culture medium was removed and discarded, and the cell layer briefly rinsed with Trypsin/EDTA solution (Gibco BRL). 1 - 3 ml of Trypsin/EDTA solution was added to the flask and the cells observed until the cell layer was dispersed (5 to 10 minutes). Cells were washed in 10ml complete DMEM medium and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the cells resuspended in an appropriate volume of complete DMEM.

For co-culture experiments fibroblasts were detached from culture flasks as described above, and a viable cell count carried out by trypan blue (Gibco BRL) exclusion on a haemocytometer (Sigma-Aldrich). Fibroblasts were seeded in 24 well plates (Nunc) at a concentration of 1-2 x 10^5 cells/ml in 1.0ml complete DMEM, and incubated at 37°C in an atmosphere of 5% CO_2 in air. Following adhesion of the fibroblasts to the culture wells, the culture medium was removed, and suspension cells added at a concentration of 1.0 x 10^6 cells/ml in complete RPMI medium.

2.5 T Cell Activation Assay

Activation induced cell death was studied *in vitro*. This was stimulated by plate-bound anti-CD3 antibody (Pharmingen). 96 well flat-bottomed plates (Nunc) were coated with anti-CD3 antibody or isotype matched control antibody (Pharmingen) as follows. Antibodies were prepared in an appropriate volume of sterile PBS (Gibco BRL) at a concentration of 10μg/ml. Each well was coated with 30μl of antibody in PBS, and the plates were incubated at 37°C for 90 minutes. Antibody was poured out of the wells, and the excess removed by blotting with absorbent paper. The wells were washed 3 times with ice-cold PBS, and the excess PBS removed by blotting. Suspension cells at 1.5 x 10^6 cells/ml were added into the antibody-coated wells.

2.6 Cell Biology Experiments

2.6.1 *In Vitro* Apoptosis Studies

MYC induced apoptosis was studied in CD2-*MYC*ER^{TM} cell lines. Activation of the transgene was induced by addition to the cell culture of 4-hydroxy-tamoxifen (RBI, Sigma-Aldrich). A stock solution of 4-hydroxy-tamoxifen was made up at 0.1mM in ethanol. From this stock, 4-OHT was added to cell cultures to a final concentration of 250nM (Blyth *et al.*, 2000) unless otherwise stated. Ethanol at equivalent volumes was added to controls. Table 2.1 shows the inhibitors and activators that were used to investigate related pathways during the project. Further details of these are given in the text.

Total cell numbers, and viability of cells in culture was usually assessed by trypan blue exclusion, in the first instance, using 0.4% trypan blue solution (Gibco BRL). Cell viability assays were carried out in 24 well or 96 well plates (Nunc) in complete RPMI medium with 1.0 x 10^6 cells/ml for established cell lines, or 2 x 10^6 cells/ml for primary tumour cells, and viable cell numbers determined at 24 hour intervals. All cultures were performed in triplicate or quadruplicate and viability curves were based on the average number of live cells expressed as a percentage of the average

total. Statistical analysis was carried out using Students *t*-test. P values quoted refer to results of Students *t*-test unless otherwise stated. Error bars on graphs refer to standard deviation unless otherwise stated.

Table 2.1 Regulators of *MYC* Related Pathways

	Clone/Product Name	Concentration	Supplier
anti-CD3ε	145-2C11	10μg/ml	Pharmingen
anti-CD28	37.51	10μg/ml	Pharmingen
anti-Fas	Jo2	2ng/ml	Pharmingen
anti-FasL	MFL-3	2ng/ml	Pharmingen
cyclosporin A	-	100ng/ml	Sigma-Aldrich
caspase 3 inhibitor	Ac-DEVD-CHO	10μM	Alexis Biochemicals
caspase 8 inhibitor	Z-IETD-FMK	10μM	TCS Biologicals
PI3 kinase inhibitor	Ly294002	10μM	Sigma-Aldrich

2.6.2 Annexin V Staining

To confirm apoptosis in cell cultures, fluorescence conjugated Annexin V staining was determined using flow cytometry. Treatment with 10 μM dexamethasone (Sigma-Aldrich) was usually used as an internal control for induction of apoptosis.

Labelling solution was prepared by diluting 20μl Annexin-V-Fluos labelling reagent (Roche) in 1.0ml of incubation buffer (10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 5mM $CaCl_2$). 10^6 cells were washed with sterile PBS (Gibco BRL), and centrifuged at 1500rpm for 5 minutes. Supernatant was removed and discarded and the cell pellet resuspended in 100μl of labelling solution. Cells were incubated at 4°C in darkness, for 15 minutes as per manufacturer's instructions. Cells were transferred to sterile test tubes (Falcon) in 400μl of incubation buffer, and analysed on a Coulter Epics Elite using the manufacturers recommended protocol.

2.6.3 [^{3}H] Thymidine Incorporation

To determine the proliferative index of activated suspension T cells, a [^{3}H] thymidine incorporation assay was used. Cells were seeded in 96 well flat-bottomed plates (Nunc), at a concentration of 1.5 x 10^6 cells/ml, in a volume of 200µl complete RPMI. Cell cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. After 48 hours, each well was pulsed with 1µCi/ml per well of [^{3}H] thymidine (Amersham) for 16 hours. The micro-plate with bonded filter (Packard) was rinsed once with water, and the samples transferred onto the filter using a cell harvester (Packard). Wells were then rinsed out 5 times with water, onto the filter, and the filter was dried (1hour, 37°C). The underside of the filter was sealed, and 25µl of scintillation fluid (National Diagnostics) added onto each well. The top of the plate was then sealed, and the plate passed through a plate reader (Packard) according to the manufacturer's instructions. Counts were performed in quadruplicate, and the results shown are based on an average of the results. Statistical analysis was carried out using Student's *t* test, and error bars on graph refer to standard deviation.

2.7 IMMUNOPHENOTYPE ANALYSIS

Single cell lymphocyte suspensions were prepared as described in section 2.4.2 and washed in cold phosphate-buffered saline (PBS, Gibco BRL) containing 0.1% BSA (Sigma) and 0.01% sodium azide (BDH). Cells at 1-2 x 10^6 per ml were centrifuged at 7000rpm in a microfuge for 3 minutes and the supernatant discarded. The cells were labelled by resuspending them with rat monoclonal anti-mouse antibody (Table 2.2) for 30 minutes at 4°C. 1-2µg of antibody was used to label 1-2 x 10^6 cells. Samples were centrifuged at 7000rpm for 3 minutes and washed with 200µl of cold PBS/BSA/sodium azide, as described above. Two further rounds of this washing procedure were carried out. Cells were resuspended in 500µl of cold PBS, containing 0.1% BSA and 0.01% sodium azide, in sterile test tubes (Falcon) and analysed on a Coulter Epics Elite using the manufacturers recommended protocol. For each fluorochrome used, irrelevant isotype matched antibody controls were used

to set a region to exclude background staining. Fluorescence above this threshold was considered as positive fluorescence.

Table 2.2 Rat Monoclonal Antibodies to Mouse Surface Antigens

Antibody	Clone	Isotype	Supplier
anti-mouse CD8a FITC conjugate	KT15	Rat IgG2a	Serotec
anti-mouse CD4 R-phycoerythrin conjugate	YTS191.1	Rat IgG2b	Serotec
anti-mouse CD3 Quantum Red conjugate	29B	Rat IgG2b	Sigma
anti-mouse CD45R FITC conjugate	RA3-6B2	Rat IgG2a	Pharmingen/Sigma

2.8 WESTERN IMMUNOBLOTTING

2.8.1 Preparation of Protein Extracts

Protein was extracted from approximately 1 x 10^6 cells. Cells were washed in 1ml PBS (Gibco BRL), centrifuged at 1500rpm for 3 minutes, the supernatant removed and discarded, and the cells washed again. After the second wash, cells were resuspended in 100µl RIPA buffer (150mM NaCl [Fisher], 1.0% NP-40, 0.5% DOC [both Sigma], 0.1% SDS [BDH], 50mM Tris, pH8.0 [Sigma]) and 1:100 protease inhibitors (Sigma-Aldrich), and incubated on ice for 30-60 minutes. Samples were centrifuged at 14,000rpm for 5 minutes, at 4°C to remove debris. The supernatant was transferred to 100µl of 2 x loading buffer (2% SDS [BDH], 100mM dithiothreitol, 60mM Tris, pH6.8, 0.01% bromophenol blue [all Sigma]), and the protein extracts stored at -20°C.

2.8.2 Separation of Proteins and Western Transfer

Samples were electrophoretically separated on denaturing SDS polyacrylamide (Biorad) gels in electrophoresis buffer (25mM Tris [Sigma], 250mM glycine [Fisher], pH 8.3, 0.1% SDS [BDH]) using the BioRad minigel system. Gels were run at 150V for 1 hour. Samples were then transferred to Immobilon polyvinylidene diflouride membranes (Millipore) preactivated in methanol (BDH). The Biorad minigel system was used, and transfer was carried out in transfer buffer (0.192M glycine, 25mM Tris) at 100V for 45 minutes. Completion of transfer was assessed by prestained molecular weight markers (BioRad).

2.8.3 Detection of Proteins

Following transfer of proteins onto membrane, membranes were incubated in blocking buffer (1 x PBS, 0.1% Tween-20 [BDH] with 5% non-fat dry milk) for 1 hour at room temperature. Membranes were then incubated with primary antibody (at the manufacturers recommended dilution) in 2ml blocking buffer with gentle agitation overnight at 4°C. For antibodies used, see Table 2.3. Membranes were then washed for 3 x 10 minutes with wash buffer (1 x PBS, 0.1% Tween-20), and then incubated with the appropriate HRP-conjugated secondary antibody (anti-mouse, anti-rabbit or anti-goat, all from Sigma), at the recommended dilution in 10ml blocking buffer, at room temperature for 1 hour. Membranes were washed again for 3 x 10 minutes. Detection of proteins was carried out by electrochemical luminescence (ECL), using the ECL-plus detection kit (Amersham) according to the manufacturer's instructions. Membranes were exposed to Hyperfilm^{TM} (Amersham) and exposed for 10 – 60 seconds.

Table 2.3 Antibodies Used in Western Immunoblotting

Antibody	Clone	Isotype	Supplier
p53 (normal and mutant)	Pab 240	mouse $IgG1_{\iota}$	Santa-Cruz Biotechnology
β-actin	I-19	goat IgG	Santa-Cruz Biotechnology
phospho-Akt (Ser 473)	-	rabbit IgG	New England Biolabs
Akt	-	rabbit IgG	New England Biolabs

CHAPTER 3

MYC INDUCED T CELL LYMPHOMAGENESIS IS NOT ACCELERATED IN FAS^{lpr} MICE

3.1 INTRODUCTION

3.1.1 The CD2-*MYC*ER^{TM} Transgenic Mouse Model

The c-MYC transcription factor promotes cell cycle progression (reviewed by Evan *et al.*, 1994), and it is likely that this is the reason that the c-*MYC* oncogene is frequently mutated in human tumours (Spencer & Groudine, 1991). In conditions in which growth is restricted however, overexpression of *MYC* may drive cells towards apoptosis rather than proliferation (Evan *et al.*, 1992), and it is this function of *MYC* which may act as a brake to tumour development. Recently, a role for the Fas pathway as a mediator of MYC induced apoptosis in fibroblasts was reported (Hueber *et al.*, 1997). To investigate the effects of the Fas pathway on MYC induced lymphomagenesis, a CD2-*MYC*ER^{TM} transgenic mouse model was studied. These mice express a hybrid protein of the human c-*MYC* oncogene under the control of a modified oestrogen receptor. The construct is inducible by the oestrogen agonist/antagonist 4-hydroxytamoxifen (4-OHT), but not by oestradiol (Littlewood *et al.*, 1995). Transgene expression is targeted to the T-cell lineage by the CD2 dominant control region (Zhumabekov *et al.*, 1995). MYC activity can be modulated in CD2-*MYC*ER^{TM} mice by administration of tamoxifen in drinking water however there is some residual transgene activity without activation, and animals not treated with tamoxifen show a background tumour incidence of 23% by 300 days of age (Blyth *et al.*, 2000). Tumour incidence is significantly increased to 68% following tamoxifen treatment (Blyth *et al.*, 2000). Further, activation of the *MYC*ER^{TM} construct in thymocytes *in vivo* has been shown to induce both proliferation and apoptosis (Blyth *et al.*, 2000), suggesting that tamoxifen treatment results in activation of functional MYC *in vivo.*

Transformation is a multi-step process however, and more than one mutation is required before tumourigenesis occurs. CD2-*MYC*ER^{TM} transgenic animals develop tumours of a clonal nature with a relatively wide latency range (Blyth *et al.*, 2000), suggesting that additional genetic lesions are required for development and progression of transgene induced lymphomas. Events that collaborate with overexpression of c-*MYC* in the multi-step process of tumourigenesis may be studied by breeding these mice together with transgenic mice carrying other genetic lesions. Acceleration of tumourigenesis in these animals can then be monitored. Synergy between a number of different genes has been demonstrated in this way (reviewed by Macleod & Jacks, 1999). For example crossing Eμ-*MYC* and Eμ-Bcl-2 transgenic mice revealed synergy between *MYC* and Bcl-2 in promoting lymphomagenesis (Strasser *et al.*, 1990b). Studies of *MYC* transgenic mice on a *Trp53* null background have also revealed a collaboration between deregulated MYC and loss of *Trp53* (Blyth *et al.*, 1995; Hsu *et al.*, 1995; Prasad *et al.*, 1997).

3.1.2 Relationship Between c-*MYC* and Fas in Lymphomagenesis

Genetic lesions that result in blockage of apoptosis have been shown to represent key events in multi-stage tumourigenesis. Since the ability of c-MYC to induce apoptosis as well as cellular proliferation may act to restrict tumour development, the loss of apoptotic pathways is particularly important in tumours with c-*MYC* involvement. A dependence on Fas in c-MYC mediated apoptosis has been shown recently in fibroblasts (Hueber *et al.*, 1997), and various studies have demonstrated loss of sensitivity to Fas induced apoptosis in tumour cells (Falk *et al.*, 1992; Shima *et al.*, 1995). Evidence from other groups has shown that *myc* may collaborate with loss of the Fas pathway because of the ability of Myc to upregulate FasL expression (Cerwenka *et al.*, 1996; Wang *et al.*, 1998; Genestier *et al.*, 1999; Brunner *et al.*, 2000). Despite this, Fas^{lpr} cells do not appear to be prone to transformation, and the role of the Fas pathway in tumourigenesis is not yet clear.

3.1.3 Experimental Aims

The aim of this series of experiments was to investigate the relationship between loss of Fas and deregulation of c-*MYC* in T-cell lymphoma development using a transgenic mouse model. Mice which overexpressed a regulatable c-*MYC* gene and lacked a functional Fas pathway would provide a valuable model for studying the combined effects of these two genetic lesions *in vivo*. Because untreated CD2-*MYC*ER^{TM} animals show a relatively low background tumour incidence, they are a useful model for studying acceleration of tumourigenesis. The aim of the first experiment was to investigate whether lymphomagenesis was accelerated in untreated CD2-*MYC*ER^{TM} mice on a Fas^{lpr} background. It was also possible to activate the transgene in these mice, to investigate whether greater expression of *MYC* might collaborate with loss of Fas, since the level of *MYC* expression may affect the balance between induction of proliferation or apoptosis in the cells in which the transgene is expressed.

3.2 RESULTS

3.2.1 Generation of Experimental Animals

In order to investigate any collaboration *in vivo* between c-*MYC* overexpression and loss of the Fas pathway, CD2-*MYC*ER^{TM} transgenic mice on a Fas^{lpr} background were generated. To achieve this, CD2-*MYC*ER^{TM} mice were crossed with Fas^{lpr} mice to generate CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ offspring. These animals were then backcrossed with Fas^{lpr} mice to generate a cohort consisting of 4 equally represented populations: CD2-*MYC*ER^{TM}Fas^{lpr}, CD2-*MYC*ER^{TM}Fas$^{lpr/-}$, Fas^{lpr} and Fas$^{lpr/-}$ mice. DNA was extracted from tail biopsy sections for genotypic analysis. Presence of the *MYC* transgene was detected using a human c-*MYC* exon 3 probe against Bgl 2 fragments on Southern blots. PCR was used to determine the Fas^{lpr} status of the mice (see section 2.3 of Materials and Methods).

3.2.2 Lymphomagenesis in CD2-*MYC*ER^{TM}Fas^{lpr} Mice

CD2-*MYC*ER^{TM} mice homozygous for the *lpr* mutation were generated in expected numbers as predicted by Mendelian genetics, signifying that these mice were viable, and that the combination of these two genetic lesions did not result in embryonic lethality. Further, these mice exhibited no obvious developmental abnormalities. Animals were not treated with tamoxifen, so there was only residual transgene activity in the mice in this cohort.

The numbers of animals in each cohort are shown in Table 3.1, and the overall survival of each group in Figure 3.1. It is important to note that lymphomagenesis in these mice is driven by deregulation of c-*MYC* activity, since 46 out of 63 (73%) CD2-*MYC*ER^{TM} animals developed thymic lymphoma by the age of 12 months, compared with 1 out of 63 (2%) non-transgenic littermate controls. The one incident of lymphomagenesis in a non-transgenic control was of longer latency, and likely to be an example of a spontaneous tumour in an older mouse.

Table 3.1 Lymphomagenesis and Lymphoproliferative Disease in CD2-*MYC*ER^{TM}Fas^{lpr} Mice

Genotype	No. of Animals Total	No. of Animals (%) TL*	Latency (days) +/- S.D. TL	No. of Animals (%) *lpr*†	Latency (days) +/- S.D. *lpr*
CD2-*MYC*ER^{TM}Fas^{lpr}	39	26 (67%)	117 +/- 29.0	13 (33%)	152 +/- 45.6
CD2-*MYC*ER^{TM}Fas$^{lpr/-}$	24	20 (83%)	118 +/- 27.6	0	-
Fas^{lpr}	42	1 (2%)	326	41 (98%)	161 +/- 54.7
Fas$^{lpr/-}$	21	0	-	0	-

* thymic lymphoma † lymphoproliferative disease

Comparison of tumour-free survival between CD2-*MYC*ER^{TM} mice homozygous and heterozygous for the Fas^{lpr} mutation is complicated by the ongoing exit of mice from the cohort due to development of lymphadenopathy and autoimmune disease. There

was however no significant difference in overall survival between CD2-*MYC*ER^{TM} transgenic mice homozygous and heterozygous for the Fas^{lpr} mutation (Figure 3.1). Further, CD2-*MYC*ER^{TM} mice on a homozygous Fas^{lpr} background did not develop lymphoma at a significantly higher incidence than those heterozygous for Fas^{lpr}. Of the CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ heterozygous animals, 20 from 24 (83%) developed thymic lymphoma, compared with 26 from 39 (67%) of the CD2-*MYC*ER^{TM}Fas^{lpr} homozygous animals. Neither was the latency of *MYC* induced tumours significantly altered by the Fas^{lpr} status of the animals. CD2-*MYC*ER^{TM} induced tumours had a latency of 117 days (+/- 29.0 days) in the CD2-*MYC*ER^{TM}Fas^{lpr} group compared to 118 days (+/- 27.6 days) in CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice. The data also show that presence of the transgene does not significantly affect lymphoproliferative disease associated with the Fas^{lpr} genotype, since the latency of development was not significantly different between transgenic and non-transgenic Fas^{lpr} mice. The results presented here suggest that the Fas pathway does not act to inhibit *MYC* induced lymphoma development or progression.

3.2.3 Pathology of Experimental Animals

Transgenic animals homozygous for the *lpr* mutation developed either thymic lymphoma or lymphoproliferative disease whereas transgenic animals heterozygous for *lpr* developed only thymic lymphomas, consistent with the targeting of the transgene to the T-cell compartment. Animals were monitored over a twelve-month period and sacrificed when clinical signs of neoplasia or lymphoproliferative disease were present. In the case of lymphoma, affected animals exhibited some or all of the following clinical signs: increased respiratory rate; cachexia; reduced movement and an abnormal high-stepping gait, indicative of a neoplastic mass in the thoracic cavity. Indicators of lymphoma in CD2-*MYC*ER^{TM}Fas^{lpr} and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice were similar. Moribund Fas^{lpr} mice affected by severe lymphadenopathy showed enlarged lymph nodes, and skin lesions and abrasions especially around the ears and neck, caused by pruritis.

Most of the CD2-*MYC*ER^{TM}Fas^{lpr} mice which became ill and were sacrificed due to development of thymic lymphoma also showed clinical signs associated with the *lpr*

phenotype. Diagnosis of thymic lymphoma or lymphoproliferative disease was made on the basis of gross pathology at post-mortem. In mice in which a tumour had developed, the usual bi-lobed structure of the thymus was lost, and on most occasions the lymphoma filled the entire thoracic cavity. In some animals metastatic spread to lymph nodes was observed since some lymph nodes appeared slightly enlarged. In the cases of metastatic spread, enlargement of lymph nodes was non-uniform, for example, subcutaneous lymph nodes on one side of the animal only may be affected. In animals exhibiting severe symptoms associated with the Fas^{lpr} phenotype, all lymph nodes were abnormally enlarged, and splenomegaly with an expanded white pulp was observed. In some mice with advanced lymphoproliferative disease the kidneys were also enlarged and had a pale, mottled appearance, suggesting lymphocytic infiltration. In severe cases of lymphoproliferative disease, the thymus was slightly enlarged, but retained its bi-lobed structure.

3.2.4 The Remaining Wild Type Fas Allele is not a Target for Deletion in CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice

Investigation of loss of heterozygosity is useful in studying collaboration between genetic lesions. If the Fas pathway has an essential role in c-MYC induced apoptosis in T cells, then it would follow that loss of the functional Fas pathway may be a critical step in *MYC* induced lymphomagenesis. In *MYC* induced tumours on a Fas^{lpr} heterozygous background, loss of heterozygosity at the Fas gene locus would represent a key event in tumourigenesis, and would be expected to occur frequently. To explore this concept in this system, tumours were taken from CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice that developed thymic lymphomas. Tumour samples were snap frozen, and DNA was extracted. The integrity of the remaining wild type Fas allele was analysed using PCR (Figure 3.2). In all tumours examined an amplified fragment consistent with presence of the wild type Fas allele was still detected (7 from 7), although the presence of point mutations and deletions outside the amplified gene sequence cannot be excluded using this approach. The results are reinforced by *in vitro* assays of Fas function in explanted CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumour cells, which are described in detail in Chapter 5. In combination these data support the evidence for a lack of synergy between *MYC* deregulation and loss of Fas.

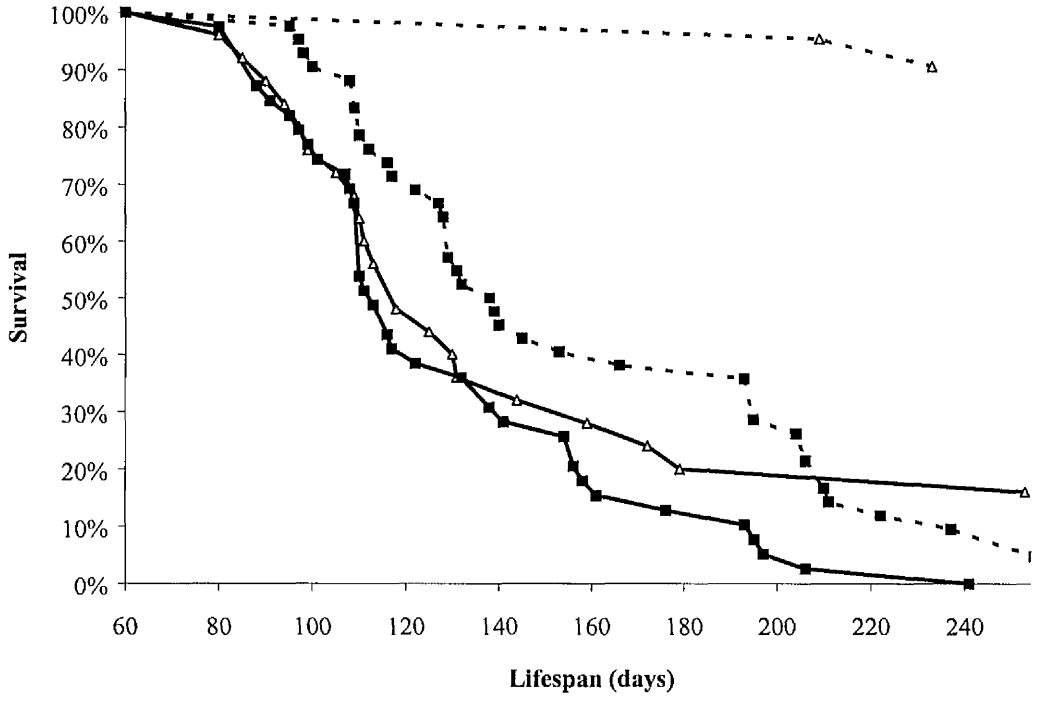


Figure 3.1 Disease Free Survival of Untreated CD2-*MYC*ER^{TM}Fas^{lpr} Mice and Control Littermates.

CD2-*MYC*ER^{TM}Fas^{lpr} mice (solid line, filled squares, n=39), CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice (solid line, open triangles, n=24), Fas^{lpr} mice (broken line, filled squares, n=42), Fas$^{lpr/-}$ mice (broken line, open triangles, n=21). These data represent overall survival. Deaths in the CD2-*MYC*ER^{TM}Fas^{lpr} group were due to either thymic lymphoma or severe lymphoproliferative disease.

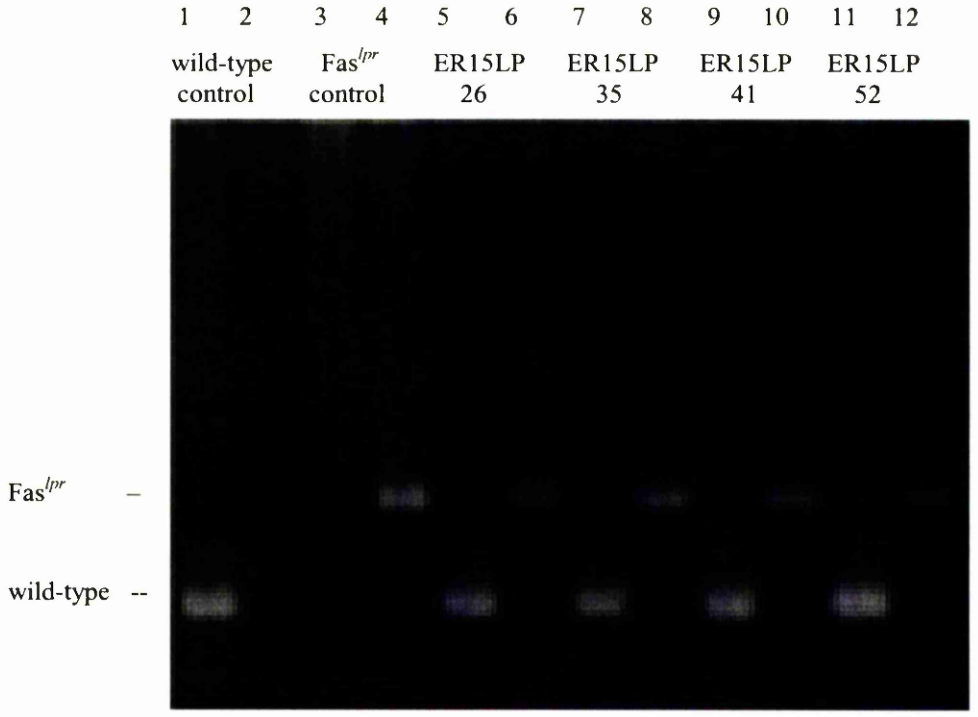


Figure 3.2 No Loss of Heterozygosity at the Fas Locus in c-*MYC* Induced Tumours

PCR products run on Ethidium Bromide stained agarose gel. For each sample two PCR reactions were performed using primers against either wild type (lanes 1, 3, 5, 7, 9 & 11) or Fas^{lpr} sequence (lanes 2, 4, 6, 8, 10 & 12). Lanes 1 & 2 show wild type control. Lanes 3 & 4 show Fas^{lpr} control. PCR products from CD2-*MYC*ER^{TM}$Fas^{lpr/-}$ tumour samples are shown in lanes 5-12.

3.2.5 The Phenotype of CD2-*MYC*ER^{TM} Thymic Lymphomas was not Altered by the Fas^{lpr} Genotype

While there was no significant difference in the tumour incidence or latency between CD2-*MYC*ER^{TM} mice homozygous or heterozygous for Fas^{lpr}, it was necessary to establish whether loss of the Fas pathway might alter the phenotype of *MYC* induced tumours, or affect the lineage of the cells which become transformed. As well as giving an indication of the cell lineage of lymphomas, investigation of the cell surface markers expressed on explanted tumour cells should give an indication of whether the phenotype of a tumour has been altered by the status of the Fas pathway. Transformed cells from CD2-*MYC*ER^{TM}Fas^{lpr} and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumours were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD8 and CD45R (B220), and analysed by flow cytometry to assess their phenotype. To exclude non-specific signal, cells were also labelled with isotype control antibodies conjugated to each fluorochrome. These are species and class-matched non-specific antibodies which should allow background fluorescence to be gated.

In all of the CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumours tested, the majority of cells were of the CD4^{+}/CD8^{+} phenotype (8/8) (Figure 3.3B). This phenotype did not appear to be altered by *lpr* genotype, since all of the CD2-*MYC*ER^{TM}Fas^{lpr} tumours tested were also of the double positive phenotype (8/8) (Figure 3.3C). These results agree with previous extensive analyses of CD2-*MYC*ER^{TM} tumours in which all tumours tested belonged to the CD4^{+}/CD8^{+} phenotype (Blyth *et al.*, 2000). In addition, all tumours tested for the T cell specific marker, CD3, were found to be of T cell origin, since all stained positive. The majority of cells from these tumours also stained negative for the B cell marker CD45R.

If the *lpr* mutation was important in *MYC* induced lymphomagenesis it might be expected that tumours arising in CD2-*MYC*ER^{TM}Fas^{lpr} animals would reflect the Fas^{lpr} genotype, however this is not the case. The phenotype of CD2-*MYC*ER™ tumours was not altered by the Fas^{lpr} genotype. Although Fas^{lpr} animals have no functional Fas pathway and consequently have aberrant T cell homeostasis, there was no shift in the phenotype of cell types susceptible to CD2-*MYC*ER™ induced

tumourigenesis in these mice. A recognised diagnostic feature of Fas^{lpr} induced lymphadenopathy is an enlarged population of CD4-/CD8- double negative T cells, expressing the B cell marker CD45R/B220 in the lymph nodes of these mice. Despite the massive accumulation of abnormal T cells in the lymph nodes of Fas^{lpr} mice however, there is no evidence that this expanded population of cells is susceptible to transformation, since no tumours of this phenotype were observed. These results provide support for the evidence already presented that loss of the Fas pathway is not important in *MYC* induced lymphoma development or progression.

3.2.6 Induction of the Transgene by Administration of Tamoxifen in CD2-*MYC*ER^{TM}Fas^{lpr} Mice

Previously it was found that although untreated CD2-*MYC*ER^{TM} animals show a background tumour incidence, the incidence of tumours in CD2-*MYC*ER^{TM} mice could be significantly increased by tamoxifen treatment (Blyth *et al.*, 2000). Tamoxifen activates the transgene by binding to and inducing the modified oestrogen receptor which is linked to human c-*MYC* gene. It was important to investigate the effects of transgene induction in Fas^{lpr} mice since activation of the transgene might shift the balance between the ability of c-*MYC* to induce proliferation or apoptosis. If increased transgene induction resulted in increased MYC induced apoptosis then loss of the Fas pathway might allow accelerated lymphomagenesis in CD2-*MYC*ER^{TM} animals. To assess the effects of inducing the transgene on a Fas^{lpr} background, a cohort of CD2-*MYC*ER^{TM}Fas^{lpr} mice were administered tamoxifen. As in the previous experiment, CD2-*MYC*ER^{TM} mice were crossed with Fas^{lpr} mice to generate CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ offspring. These animals were then backcrossed with Fas^{lpr} mice to generate 4 equal populations of mice which were homozygous or heterozygous for the *lpr* mutation, with approximately half of each group carrying the transgene. Animals were genotyped as described previously in section 3.2.1. From one week of age these mice were treated continuously with tamoxifen, by oral dosing in drinking water. Since tamoxifen must be solubilised in ethanol before being added to drinking water, control mice were treated with ethanol in their drinking water. Optimal transgene induction in CD2-*MYC*ER^{TM} animals is achieved when treated

with tamoxifen from birth (Blyth *et al.*, 2000), however mice in this cohort were less tolerant to ethanol in drinking water, so treatment was started at one week of age.

As before, animals were monitored and sacrificed when clinical signs were present. Due to the intolerance of the animals to tamoxifen and ethanol treatment, which was characterised by cachexia and general malaise, the experiment was stopped at 8 months. Clinical signs of thymic lymphoma and lymphoproliferation were similar to those seen in mice from the untreated CD2-*MYC*ER^{TM}Fas^{lpr} cohort, described in section 3.2.3. Diagnosis of thymic lymphoma or lymphoproliferative disease was made on the basis of gross pathology at post-mortem. In keeping with the previous experiment, transgenic animals homozygous for the *lpr* mutation developed either thymic lymphoma or lymphoproliferative disease whereas transgenic mice animals heterozygous for *lpr* developed only lymphomas. The gross pathology of lymphomas arising in these mice was more severe since in all of the CD2-*MYC*ER^{TM}Fas^{lpr} animals harbouring a lymphoma, the thymus was enlarged to such an extent that the tumour filled the entire thoracic cavity. In addition, fewer of the tamoxifen treated CD2-*MYC*ER^{TM}Fas^{lpr} mice had to be sacrificed due to lymphoproliferative disease in this experiment (1 from 12, Table 3.2), compared with untreated mice of the same genotype (13 from 39). This was presumably due to the reduced latency of lymphoma development in CD2-*MYC*ER^{TM} mice treated with tamoxifen, which is discussed below.

Table 3.2 Lymphomagenesis and Lymphoproliferative Disease in Tamoxifen Treated CD2-*MYC*ER^{TM}Fas^{lpr} Animals

Genotype	No. of Animals Total	No. of Animals TL*	Average Latency (days) +/- S.D.	No. of Animals *lpr*†
Tamoxifen treated				
CD2-*MYC*ER^{TM}Fas^{lpr}	12	11 (92%)	94 +/- 39.5	1
CD2-*MYC*ER^{TM}Fas$^{lpr/-}$	11	11 (100%)	86 +/- 8.9	0
Fas^{lpr}	9	0	-	8
Fas$^{lpr/-}$	16	0	-	0
Ethanol treated				
CD2-*MYC*ER^{TM}Fas^{lpr}	8	6 (75%)	123 +/- 5.0	2
CD2-*MYC*ER^{TM}Fas$^{lpr/-}$	8	6 (75%)	125 +/- 20.9	0

* thymic lymphoma

† lymphoproliferative disease

3.2.7 Survival of CD2*MYC*ER^{TM}Fas^{lpr} mice on Tamoxifen Treatment

Previous results have shown that increased *MYC* activity in tamoxifen treated CD2-*MYC*ER^{TM} animals results in increased tumourigenesis. In this experiment the overall survival of CD2-*MYC*ER^{TM} mice administered tamoxifen from 7 days old was found to be significantly reduced compared to the same group of mice following treatment with ethanol (Figure 3.4B). Tumour incidence in the tamoxifen treated group of CD2-*MYC*ER^{TM} mice was increased to 96% (22 from 23) by age 8 months, compared to 75% (12 from 16) of the ethanol treated group. Further, the average latency of tumours in this group was significantly reduced (90 +/- 28.3 days in tamoxifen treated CD2-*MYC*ER^{TM} animals, compared with 124 +/- 14.6 days in the ethanol treated control group, $P<0.01$). The figures for the ethanol treated CD2-*MYC*ER^{TM} animals are supported by the larger cohort of untreated CD2-*MYC*ER^{TM} animals described in section 3.2.2, which develop thymic lymphoma with an overall incidence of 73%, and an average latency of 118 +/- 28.1 days. These data confirm that despite the observation that transgene activation is leaky and tumours develop in

untreated CD2-*MYC*ER^{TM} animals, transgene activity can be further enhanced by tamoxifen, and this increased activity is reflected by reduced tumour latency. Therefore levels of *MYC* activity correlate with tumour incidence and survival.

Although increased *MYC* activity may alter the kinetics of tumourigenesis in tamoxifen treated mice, absence of the Fas pathway still had no discernible effect on lymphoma development in these mice. While tamoxifen treatment resulted in significantly increased tumour incidence and reduced tumour latency in CD2-*MYC*ER^{TM} mice, there was no significant difference in overall survival between CD2-*MYC*ER^{TM}Fas^{lpr} and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice following tamoxifen treatment (Figure 3.4A). Tumour incidence in CD2-*MYC*ER^{TM} mice was not significantly increased by the Fas^{lpr} genotype even following long-term transgene induction. The incidence of thymic lymphoma in CD2-*MYC*ER^{TM}Fas^{lpr} mice was 11 from 12 (92%), compared with 11 from 11 (100%) in CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ animals. Finally, the average latency of *MYC* induced tumours in tamoxifen treated animals was not significantly increased by the Fas^{lpr} status of the animals (94 +/- 39.5 days in CD2-*MYC*ER^{TM}Fas^{lpr} mice compared with 86 +/- 8.9 days in the CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ group, P>0.1). These results confirm that the Fas pathway does not act to inhibit *MYC* induced lymphoma development or progression.

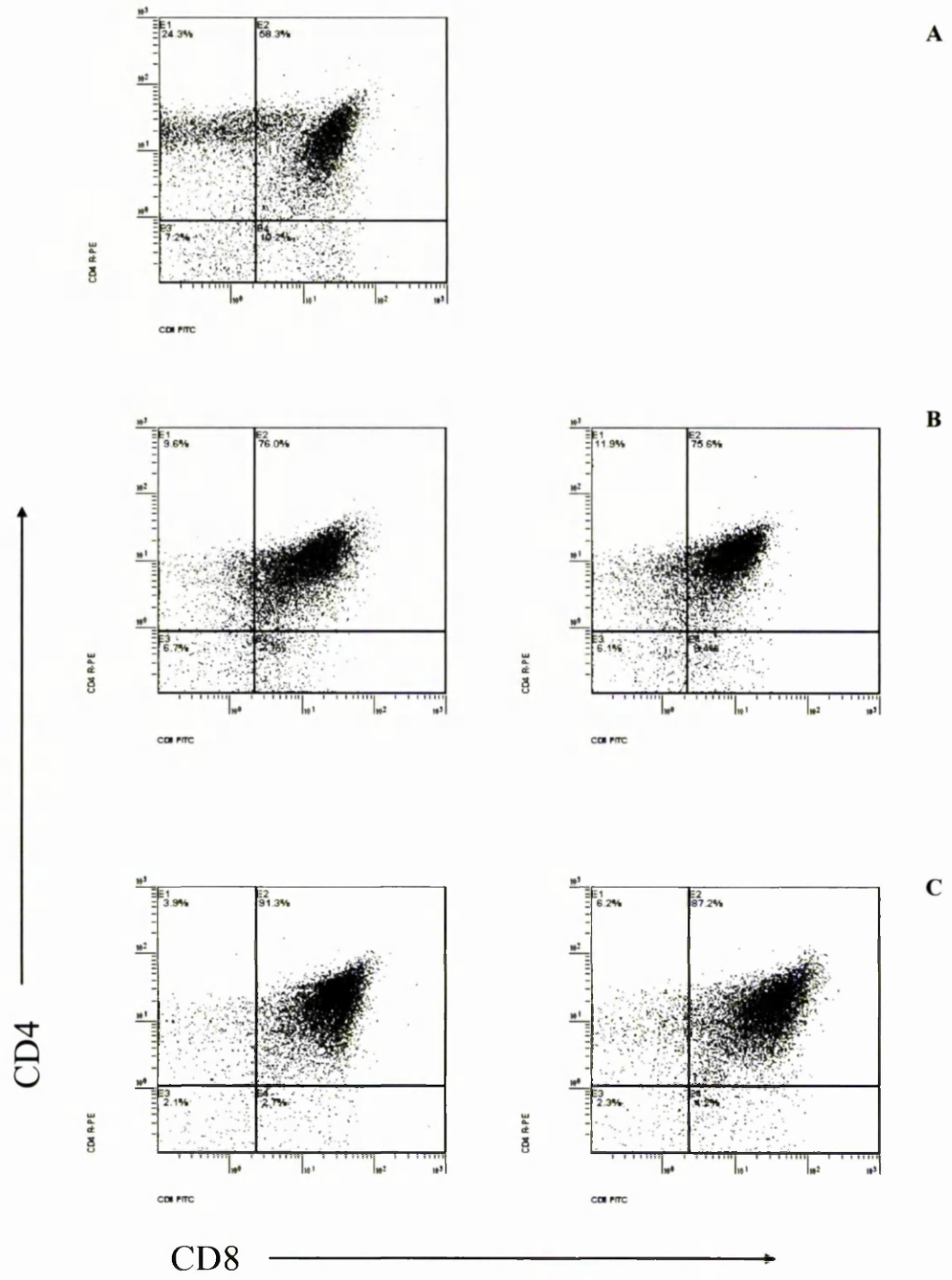


Figure 3.3 Analysis by Flow Cytometry of Cell Surface Phenotype in CD2-*MYC*ER^{TM}Fas^{lpr} Tumours.

CD4 and CD8 surface marker expression in CD2-*MYC*ER^{TM} induced thymic lymphomas. The majority of tumours tested of both Fas^{lpr} and Fas$^{lpr/-}$ genotype were double positive for these markers. **A,** Control thymocytes **B,** Examples of thymic lymphoma cells from CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice. **C,** Examples of thymic lymphoma cells from CD2-*MYC*ER^{TM}Fas^{lpr} mice.

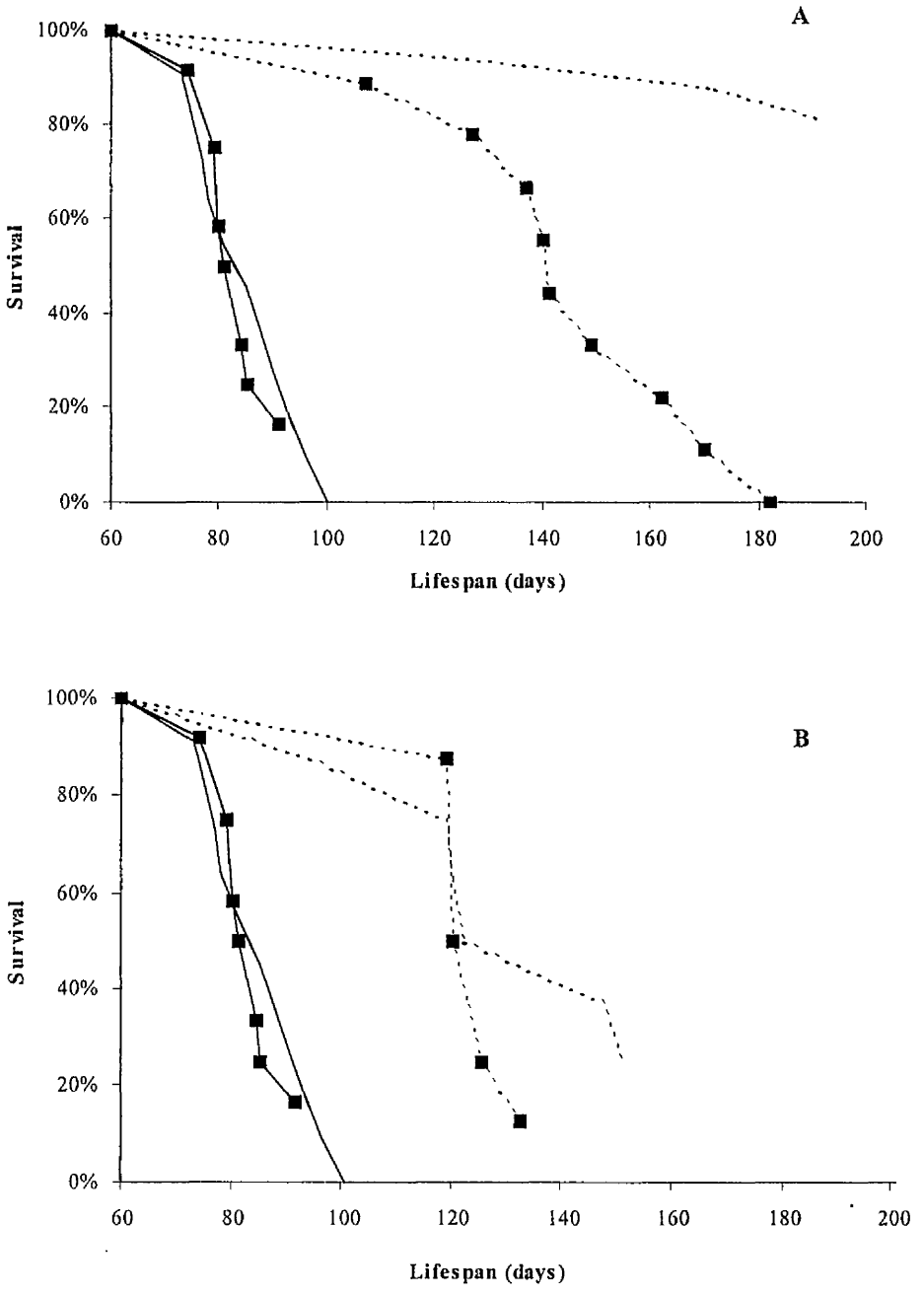


Figure 3.4 Disease Free Survival of Tamoxifen Treated CD2-*MYC*ER^{TM}Fas^{lpr} Mice.

A, Disease free survival of tamoxifen treated CD2-*MYC*ER^{TM}Fas^{lpr} mice. CD2-*MYC*ER^{TM}Fas^{lpr} mice (solid line, filled squares, n=12), CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice (solid line, no symbols, n=11), Fas^{lpr} mice (broken line, filled squares, n=9), Fas$^{lpr/-}$ mice (broken line, no symbols, n=16). **B,** Disease free survival of tamoxifen and ethanol treated CD2-*myc*ER^{TM} mice. Tamoxifen treated CD2-*MYC*ER^{TM}Fas^{lpr} (solid line, filled squares, n=12) and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice (solid line, no symbols, n=11); Ethanol treated CD2-*MYC*ER^{TM}Fas^{lpr} (broken line, filled squares, n=8) and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice (broken line, no symbols, n=8). These data represent overall survival. Tamoxifen treatment was given in drinking water continuously from 7 days of age.

3.2.8 *MYC* Induced Lymphomagenesis is Affected by Background Strain

From the survival figures of both untreated and tamoxifen treated CD2-*MYC*ER^{TM} animals described in sections 3.2.2 and 3.2.7, it was noted that the average latency of tumours in CD2-*MYC*ER^{TM}Fas^{lpr} and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice was less than previously reported in CD2-*MYC*ER^{TM} mice (Blyth *et al.*, 2000). This data is shown in Table 3.3. One concern arising from these experiments therefore, was that although the Fas^{lpr} mutation is recessive, Fas^{lpr} heterozygous mice might have some previously undescribed phenotype, possibly a reduction in levels of Fas, which could collaborate with overexpression of *MYC* in lymphomagenesis, or may compromise the immune system to allow lymphoma development. Another possible reason for the increased rate at which lymphomas develop in these animals is the difference in background mouse strain. Strain differences have already been reported to be important modifiers of cancer development (Festing, 1993). Since CD2-*MYC*ER^{TM} mice are on a mixed C57/CBA strain, whereas Fas^{lpr} and Fas$^{lpr/-}$ mice are MRL strain it was essential to investigate any possible influence strain may have on acceleration of tumourigenesis. CD2-*MYC*ER^{TM} mice were crossed with MRL mice to create CD2-*MYC*ER^{TM} transgenic mice which were 50% MRL strain (F1). These mice were then backcrossed onto MRL mice to generate transgenic mice which were 75% MRL strain (F2), and corresponded to the original experimental cohort of untreated CD2-*MYC*ER^{TM}Fas^{lpr} mice and their CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ littermate controls.

Animals were monitored for background tumour incidence and sacrificed when clinical signs were observed. Clinical signs were consistent with those seen in the untreated CD2-*MYC*ER^{TM}Fas^{lpr} and CD2-*myc*ER^{TM}Fas$^{lpr/-}$ animals which developed thymic lymphoma, as described previously in section 3.2.3. In this experiment CD2-*MYC*ER^{TM} mice developed thymic lymphomas exclusively, diagnosis made on the basis of gross pathology at post mortem, while non-transgenic animals remained disease free for the duration of the experiment. The overall survival of CD2-*MYC*ER^{TM} mice was significantly reduced by MRL background (Figure 3.5), and the incidence of thymic lymphoma was significantly increased ($P<0.01$). 80% (8 from 10) F2 MRL mice developed thymic lymphoma by age 12 months, compared to 57% (26/46) of F1 animals. Incidence in both cohorts is significantly increased compared

to the incidence of 23% (20 from 88) in untreated CD2-*MYC*ER^{TM} ($P<0.01$, see Table 3.3 and Blyth *et al.*, 2000). As shown in Figure 3.5, the latency of tumours in CD2-*MYC*ER^{TM} mice was also significantly affected by strain difference, 106 +/- 25.1 days in F_2 MRL mice compared to 137 +/- 30.0 days in F_1 MRL mice ($P<0.05$). Together these data confirm that strain difference rather than the effect of *lpr* heterozygosity is responsible for the high incidence of thymic lymphoma in these mice.

Table 3.3 Tumour Incidence in Cohorts of CD2-*MYC*ER^{TM} Animals

	Incidence of TL
*CD2-*MYC*ER^{TM} (untreated)	20/88 (23%)
*CD2-*MYC*ER^{TM} (treated)	16/26 (62%)
CD2-*MYC*ER^{TM} x Fas^{lpr} (untreated)	46/63 (73%)
CD2-*MYC*ER^{TM} x Fas^{lpr} (treated)	22/23 (96%)
CD2-*MYC*ER^{TM} x MRL (F1)	27/47 (57%)
CD2-*MYC*ER^{TM} x MRL (F2)	8/10 (80%)

* Blyth *et al.*, 2000

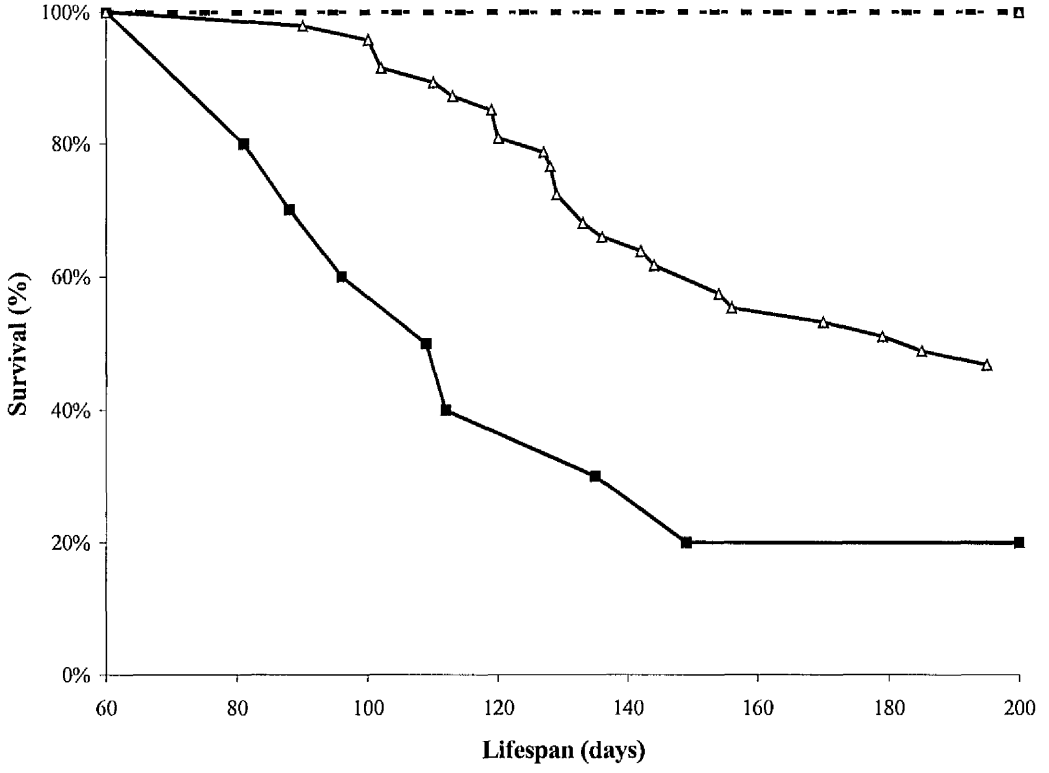


Figure 3.5 Tumour Free Survival of Untreated CD2-*MYC*ER^{TM} Mice on an MRL Background.

CD2-*MYC*ER^{TM}/75% MRL strain mice (solid line, filled squares, n=10), CD2-*MYC*ER^{TM}/50% MRL strain mice (solid line, open triangles, n=47), 75% MRL strain mice (broken line, filled squares, n=20), 50% MRL strain mice (broken line, open triangles, n=49). These data represent overall survival.

3.3 DISCUSSION

3.3.1 No Synergy Between c-*MYC* and Loss of Fas in T Cell Lymphomagenesis

The ability of MYC to induce apoptosis in certain conditions, as well as proliferation, led to the hypothesis that apoptosis may act as a brake to *MYC* mediated tumourigenesis. Genetic events that occur during tumourigenesis to block apoptotic pathways such as the Fas or p53 pathways, or to stimulate survival signals, for example Bcl-2, may be essential for *MYC* mediated oncogenesis. Fas signalling represents an important apoptotic pathway in T cells (Russell *et al.*, 1993; Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995, reviewed by Nagata, 1997). T cell homeostasis achieved through down-regulation of the immune system by activation induced suicide of T cells, and deletion of autoreactive B and T cells, is dependent on Fas:FasL interactions (reviewed by Nagata, 1997). Since Fas signalling is clearly significant in T cell apoptosis, the possibility of synergy between deregulated *MYC* and loss of Fas in T cell lymphomagenesis was explored.

The putative tumour restricting properties of Fas in c-*MYC* induced lymphomagenesis were investigated in an *in vivo* tumour model. Transgenic mice carrying the CD2-*MYC*ER^{TM} transgene and homozygous null for the Fas receptor were generated by crossing CD2-*MYC*ER^{TM} mice with Fas^{lpr} mice that lack functional Fas. CD2-*MYC*ER^{TM} animals are predisposed to development of T cell lymphoma (Blyth *et al.*, 2000). On a Fas^{lpr} background, the incidence of lymphoma development was not increased, and there was no difference in the latency of tumours compared with CD2-*MYC*ER^{TM} animals heterozygous for the *lpr* mutation. It was concluded from these observations that loss of Fas signalling and deregulation of *MYC* expression do not synergise in T cell lymphomagenesis. Conflicting results had been reported, of increased tumour formation in Eμ-L-*MYC* mice on a Fas^{lpr} background (Zornig *et al.*, 1995). In this study, L-*MYC* expression was targeted to B and T cells by the immunoglobulin enhancer element, Eμ. On a Fas^{lpr} background, tumour incidence in these animals was increased, and latency was reduced (Zornig *et al.*, 1995). There may be several reasons for the conflicting data. Tumours arising in Eμ-*MYC* animals were of both B and T cell origin (Zornig *et al.*, 1995), whereas

100% of tumours in CD2-*MYC*ER^{TM} animals were T cell tumours. It may be that the importance of Fas mediated MYC induced apoptosis, or the loss of, in lymphomagenesis depends on the cell type in which *MYC* is deregulated. Further experiments, which will be described later, however, confirm the lack of synergy between deregulation of *MYC* and loss of Fas at least in our system.

3.3.2 Tumour Phenotype is Unaffected by Fas^{lpr} Genotype

Previous studies in CD2-*MYC*ER^{TM} mice have shown that even without transgene induction, these mice spontaneously develop tumours at a relatively low incidence, which can be increased with transgene induction (Blyth *et al.*, 2000). Tumours were exclusively thymic lymphomas of moderate latency. These characteristics make the model ideal for studying *MYC* collaborating genes *in vivo*. In this study, tumour incidence and latency were unaffected by Fas^{lpr} background and, synonymous with CD2-*MYC*ER^{TM} animals, tumours in CD2-*MYC*ER^{TM}Fas^{lpr} mice were exclusively thymic lymphomas. Lymphocytes with an abnormal CD4-/CD8- phenotype typically accumulate in the lymph nodes and spleen of Fas^{lpr} mice (Cohen & Eisenberg, 1991), and lymphadenopathy was observed in Fas^{lpr} animals in this study, irrespective of whether they carried the transgene or not. However, no cases of generalised multicentric lymphoma occurred despite the vastly expanded pool of T cells in the lymph nodes of these mice.

Further, the surface phenotype of CD2-*MYC*ER^{TM} induced thymic lymphomas was not altered by *lpr* genotype. Analysis by flow cytometry revealed that all tumours, regardless of *lpr* background expressed the T cell marker, CD3, but not CD45R, a B cell marker, indicating that all tumours were of T cell origin. The majority of tumours whether homozygous or heterozygous for the Fas^{lpr} mutation, were of the CD4^{+}/CD8^{+} double positive phenotype. These results agree with previous, more extensive analysis of CD2-*MYC*ER^{TM} tumours which showed that the majority were of the CD4+/CD8+ double positive phenotype (Blyth *et al.*, 2000). This is in direct contrast to the population of cells which accumulate in the lymphoid organs of Fas^{lpr} mice. These cells express CD45R, and are double negative for CD4/8 (Morse *et al.*, 1982; Budd *et al.*, 1992) however the results show that these abnormal cells do not

appear to be susceptible to transformation. It is possible that the abnormal phenotype of these cells renders them incapable of proliferation, or at least confers a proliferative disadvantage on them. Although they lack a death pathway and may have an impaired apoptotic response to certain stimuli, these cells may also exhibit an impaired response to proliferative signals. Further, absence of Fas does not appear to affect the phenotype of cells in which transformation occurs. These observations are consistent with the theory that the events leading to *MYC* induced lymphomagenesis are not altered in Fas^{lpr} mice.

3.3.3 No Loss of Heterozygosity in CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ Mice

The littermate controls used in these experiments were heterozygous for the Fas^{lpr} mutation, and the possibility remained that although the mutation is recessive, Fas^{lpr} heterozygosity might enhance *MYC* induced lymphomagenesis. If *MYC* deregulation and loss of Fas really did represent synergistic events in lymphoma development, then deletion of the remaining wild-type Fas allele in $Fas^{lpr/-}$ T cells may occur early during transformation, resulting in accelerated lymphomagenesis. For this reason, the integrity of the remaining wild type Fas allele in transgene positive *lpr* heterozygotes was examined using a PCR based technique. No gross mutations were detected in any of the Fas^{lpr} heterozygous tumours tested. While this technique does not detect point mutations in the Fas gene, the results suggest that the remaining wild type Fas allele is not a target for mutation during *MYC* induced lymphoma development. This provides further evidence that loss of Fas and *MYC* do not represent synergistic events in the process of lymphomagenesis. Absence of the Fas pathway does not accelerate *MYC* induced lymphomagenesis. Moreover, there is no pressure for *MYC* transformed cells to lose the Fas pathway. Studies of cell lines, which will be reported in Chapter 5, show that the Fas pathway is still functional *in vitro* in lymphoma cells from CD2-*MYC*ER^{TM}$Fas^{lpr/-}$ mice, providing supporting evidence that loss of heterozygosity at the Fas locus does not occur in *MYC* induced tumours.

3.3.4 CD2-*MYC*ER^{TM} Transgene Activity Represents Driving Force Behind Tumourigenesis

In untreated CD2-*MYC*ER^{TM} animals, lymphomagenesis was not accelerated by loss of the Fas pathway. The possibility remains that following transgene induction, MYC induced apoptosis is enhanced, and loss of apoptotic signals such as the Fas pathway may be required to drive the cells towards proliferation, rather than death. To confirm that deregulated *MYC* and loss of Fas do not collaborate in the formation of T cell tumours, CD2-*MYC*ER^{TM}Fas^{lpr} and their littermate controls were treated with tamoxifen from one week of age in order to achieve high activation of the transgenic construct. Although leaky, activity of the transgene is regulatable, and transgene activation results in an increased rate of tumour formation in transgenic mice (Blyth *et al.*, 2000). In this experiment, tumour development was significantly accelerated in CD2-*MYC*ER^{TM} animals treated with tamoxifen, compared to untreated CD2-*MYC*ER^{TM} animals, and tumours were of significantly reduced latency. On a Fas^{lpr} background, however, there was no further increase in tumourigenesis, and latency of tumours was unaffected. The CD4+/CD8+ double positive phenotype of tumours in tamoxifen treated CD2-*MYC*ER^{TM} animals was the same as that observed in untreated CD2-*MYC*ER^{TM} mice. It is reasonable therefore, to conclude from these data that tumours arising in CD2-*MYC*ER^{TM} animals are induced by deregulated *MYC* expression, and that their development and progression are independent of Fas status.

Clearly, tumours in CD2-*MYC*ER^{TM} are driven by *MYC* overexpression. Loss of the Fas apoptotic pathway however has no effect on the incidence or latency of lymphomas in these mice. Further studies in CD2-*MYC*ER^{TM} transgenic animals have revealed that not only does long term induction of the transgene increase tumour incidence, but short term transgene induction by 4-OHT injection results in both proliferation and apoptosis of non-transformed T cells *in vivo* (Blyth *et al.*, 2000). In addition, in tumour cells explanted from CD2-*MYC*ER^{TM} mice, MYC induced apoptosis can be stimulated by 4-OHT treatment (Blyth *et al.*, 2000, see also Chapter 5). It appears therefore that the apoptotic function of MYC is not lost, but may be overcome during transformation. These results are supported by studies using an

on/off conditional transgenic model which demonstrated the potency of *MYC* as an oncogene. In this model, *MYC* expression could be activated and inactivated using a transgene under tetracycline control (Felsher & Bishop, 1999). Sustained transgene expression led to tumour formation which could be reversed upon inactivation of the transgene (Felsher & Bishop, 1999). Further, apoptosis was observed in regressing tumours, indicating that apoptotic pathways were still active in transformed cells. Although the ability of MYC to induce proliferation must undoubtedly counteract the induction of apoptosis during tumourigenesis, it appears that this does not happen through loss of apoptotic pathways. Rather *MYC* may rely on survival signals to overcome apoptosis in order for tumour outgrowth to occur.

3.3.5 Fas:FasL Interactions in Tumourigenesis

Although the Fas pathway does not appear to play an important role in restriction of *MYC* induced T cell lymphomagenesis at least in our system, Fas signalling may well be important in restricting tumour formation in other cell types. Zornig *et al.* found that loss of the Fas pathway caused an increased incidence of tumourigenesis in Eμ-*MYC* transgenic mice in which expression of the transgene was targeted to B and T cells (Zornig *et al.*, 1995). Similar results have been generated in studies of Bcl-2 transgenic mice. In Bcl-2 transgenic mice where expression is targeted to myeloid cells, loss of Fas led to development of acute myeloblastic leukaemia (Traver *et al.*, 1998). In contrast, when expressed in lymphoid tissues, the Eμ-Bcl-2 transgene collaborates with the Fas^{lpr} mutation to accelerate lymphoproliferative disease, but tumour incidence is not increased (Strasser *et al.*, 1995; Tamura *et al.*, 1996). The reasons for the differences between the results of Zornig *et al.* are not clear, however it is likely that Fas mediated apoptosis is of more importance in some cell types than in others. It is possible that Fas mediated apoptosis may be more strictly controlled in cells of lymphoid origin, but particularly T cells, since the pathway is crucial for maintaining T cell homeostasis (Lynch *et al.*, 1995). It might be expected that loss of Fas would be particularly detrimental to T cells, however it is also possible that T cells may more able to deal with loss of the Fas pathway, because they may be able to recruit other pathways to mediate cell death which are not active in other cell types.

If this were the case, Fas signalling, or lack of, may not be involved in T cell lymphomagenesis.

In some other cases, a functional Fas pathway may certainly act to restrain tumour development. Spontaneous plasmacytoid tumours are reported to occur with a significantly higher incidence in older Fas^{lpr} and Fas^{gld} animals (lacking FasL) (Davidson *et al.*, 1998). 32% and 28%, of Fas^{lpr} and Fas^{gld} animals respectively, between 11 and 15 months of age, harboured B cell malignancies (Davidson *et al.*, 1998). Parallel results have been shown in T cell deficient mice, which are more susceptible to B cell tumours when the Fas pathway is absent (Peng *et al.*, 1996). Mice lacking both αβ and γδ T cells on a Fas^{lpr} background developed B cell lymphoma at an incidence of 60-70% by 7 months, compared to ~10% of T cell deficient, Fas positive animals (Peng *et al.*, 1996). And in Rag-1 null mice, which are T cell deficient, transgenic expression of DN-FADD resulted in thymic lymphoma in later life (> 16 weeks), whereas no tumours were observed in Rag-1 null, Fas^{lpr} animals (Newton *et al.*, 2000). It may be that the B cell tumours which are seen in T cell deficient Fas^{lpr} mice originate in a mature B cell population which is not present in Rag-1 null mice. Increased incidence of B cell lymphomagenesis has also been reported in cases of autoimmunity or immunosuppression in humans (Penn, 1986; Magrath *et al.*, 1992) suggesting that perhaps the deregulated immune system in Fas^{lpr} and Fas^{gld} animals, rather than the lack of Fas mediated apoptosis *per se*, is responsible for increased tumourigenesis when Fas:FasL interactions are blocked.

Downregulation of Fas has been reported in various types of tumours, however, and a recent study in transformed fibroblasts suggested that re-expression of Fas could increase the latency of tumour outgrowth or abolish tumour development completely (Schroter *et al.*, 2000). In humans, mutations of the Fas gene have been reported in gastric cancer (Park *et al.*, 2001), and in aggressive types of cutaneous T cell lymphoma (Zoi-Toli *et al.*, 2000). In other conditions, Fas signalling may be irrelevant. A recent analysis of human haematological malignancies reported that Fas and Fas pathway components were not the targets of mutation (Rozenfeld-Granot *et al.*, 2001). If Fas pathway components were lost during tumourigenesis, it might

be expected that the efficacy of cytotoxic anti-cancer therapies might be reduced in these tumours. Absence of Fas signalling however, has no effect on radiation and drug induced apoptosis in lymphocytes, although Bcl-2 is involved in regulation of apoptosis in these circumstances, and p53 is required (Villunger *et al.*, 1997; Newton & Strasser, 2000). Drug induced apoptosis in lymphocytes has recently been reported to be independent of signalling from any of the death receptors, but dependent on post-mitochondrial caspase 3 activation (Wieder *et al.*, 2001).

Deregulation of *MYC* and loss of Fas have been implicated as collaborating events because of the suggestion that Fas:FasL interactions were required for MYC induced apoptosis (Hueber *et al.*, 1997). However, Myc has been shown to interact with the Fas pathway through upregulation of FasL expression (Wang *et al.*, 1998; Genestier *et al.*, 1999; Brunner *et al.*, 2000; Kasibhatla *et al.*, 2000). If MYC required Fas to mediate apoptosis, it might be expected that Fas pathway components be downregulated in *MYC* induced tumours, but no reports exist of this.

FasL expression is frequently upregulated in tumours. Increased FasL expression has been correlated with increased tumour size and metastasis in cases of breast cancer (Mottolese *et al.*, 2000), and with malignancy and mitotic index in gastric smooth muscle tumours (Liu *et al.*, 2001). Further, upregulation of FasL on neoplastic cells in Hodgkin's Lymphoma has been reported (Verbeke *et al.*, 2001). There may be several reasons why selection of FasL expressing cells occurs during tumourigenesis, the most likely being that FasL expressing cells may be better equipped to evade the immune response. Selection events during tumourigenesis appear to favour FasL expression. It is reasonable to speculate that MYC may upregulate FasL as a mechanism of immune evasion. If this is the case however, it seems unlikely that Fas signalling is a major mechanism for MYC induced apoptosis, since outgrowth of FasL bearing cells occurs. It is also possible that Fas:FasL induced apoptosis can be blocked in cells in which MYC is upregulated, by the upregulation of survival signals. The ability of FasL to confer immune privilege in tumours will be discussed further in Chapter 4.

3.3.6 Predisposition to *MYC* Induced Tumours in MRL Strain Mice - Involvement of Modifier Genes

Although the absence of a functional Fas pathway did not influence *MYC* induced lymphomagenesis, tumour formation was significantly accelerated in MRL strain animals. CD2-*MYC*ER^{TM} mice are generated on a C57/CBA background, but when crossed with MRL mice, the offspring carry 50% MRL genes. When these F1 progeny are crossed again with MRL mice, the resulting F2 animals carry 75% MRL genes. The results presented here show that *MYC* induced tumourigenesis is significantly enhanced in F2 MRL animals in comparison to mice which are 50 % MRL strain. This acceleration is independent of Fas function, since MRL control animals are wild type for Fas and FasL. Clearly however, there are features in the genome of MRL mice which predispose towards cancer, or at least *MYC* induced tumourigenesis. The data provide compelling evidence of the importance of modifier genes in the formation of tumours.

The genetic background of an animal often influences the effect of oncogenic mutations. Investigation of over 430 different inbred laboratory mouse strains has identified 38 different strains with a predisposition to spontaneous tumour formation (Festing, 1993; Festing *et al.*, 1994), and studies on strain dependent phenotypes in transgenic mouse models of cancer have led to the identification of tumour modifier genes or loci (reviewed by Balmain & Nagase, 1998). Modifier genes are not oncogenes however. Rather they convey resistance or susceptibility to environmentally induced 'spontaneous' mutations in oncogenes or tumour suppressor genes, which then promote or support tumourigenesis by directly influencing cellular proliferation or survival (reviewed by Balmain & Nagase, 1998).

Modifier genes may control response to DNA damage or the metabolism of mutagenic agents that cause it. Alternatively they may influence later events in the process of tumourigenesis, such as tumour growth rate or vascularisation, or even metastasis (Lifsted *et al.*, 1998). An example of a tumour modifier gene is *Mom-1*. This was the first tumour modifier gene to be cloned, and was identified in *Min* mice, which develop intestinal and colonic adenomas, due to a mutation in the *Apc* gene

(Moser *et al.*, 1990; Moser *et al.*, 1992; Su *et al.*, 1992). Tumour incidence varied significantly depending on the strain background (Moser *et al.*, 1992). The locus responsible for this variation was identified and named *Mom-1* for modifier of *Min-1* (Dietrich *et al.*, 1993). Further analysis revealed this locus held the secreted phospholipase 2a (*Pla2g2*) gene, mutations in which predisposed to the *Min* mutation (MacPhee *et al.*, 1995). It has been suggested that this gene may reduce the incidence of intestinal tumours by preventing damage caused by dietary fatty acids.

From the results generated in the MRL strain, it seems that one or more tumour modifier genes exists in these mice, which may predispose to *MYC* induced oncogenesis. Several loci encoding candidate modifier genes which predispose to, or accelerate *lpr* associated disease have already been identified in MRL mice (Wang *et al.*, 1997). MRL-Fas^{lpr} mice develop lymphadenopathy and splenomegaly, and autoimmunity, and die of glomerulonephritis or arthritis, however other strains of Fas^{lpr} mice develop lymphoproliferative disease, but not nephritis or arthritis. The functions of the modifier genes encoded in MRL mice are not yet known, although each has varying degrees of linkage to different symptoms of the disease (reviewed by Theofilopoulos & Kono, 1999). Although loss of Fas signalling in Fas^{lpr} mice is the essential mechanism underlying the *lpr* phenotype, manifestation of this phenotype and the clinical picture is affected by genetic background.

To date, no tumour susceptibility loci have been identified in MRL animals; indeed no reports of predisposition to tumourigenesis in MRL animals exist. The search for modifier genes may be of assistance in future studies of oncogenic gene mutations, and in particular mimicking the effects of modifier genes may be useful in addition to the more traditional approaches to tumour therapy.

CHAPTER 4

LYMPHOMAGENESIS IN FAS^{lpr} MICE INFECTED WITH MURINE LEUKAEMIA VIRUS

4.1 INTRODUCTION

4.1.1 Murine Leukaemia Virus

Moloney Murine Leukaemia Virus (MoMuLV) is a slow transforming, replication competent retrovirus, that induces murine lymphomagenesis when inoculated into newborn mice (reviewed by Fan, 1997). It induces typically T cell lymphomagenesis in 100% of infected animals, with a latency range of between three and nine months depending on background strain and viral titre (Asjo *et al.*, 1981). Upon infection, viral DNA is randomly integrated into the DNA of the host cell, and may cause activation of genes, or their promoters, into which it is inserted. Tumourigenesis in MoMuLV infected mice depends on the eventual insertional activation of proto-oncogenes by the virus, leading to deregulated cell proliferation and transformation. In addition, cell survival may be permitted if tumour suppressor genes, and genes coding for components of apoptotic pathways become inactivated by retroviral insertion (Jahner & Jaenisch, 1985; Ben-David *et al.*, 1990; Lander & Fan, 1997).

4.1.2 Retroviral Mutagenesis and Identification of Co-operating Oncogenes

Retroviral insertional mutagenesis has become a widely used strategy to identify genes involved in oncogenesis (reviewed by Jonkers & Berns, 1996). The first oncogene to be identified by this method was *pim-1*. *pim-1* was discovered as a region frequently rearranged in MuLV induced lymphomas (Cuypers *et al.*, 1984), and insertions at this site resulted in enhanced transcription of the *pim-1* gene (Selten *et al.*, 1985). Further studies revealed that the gene encoded a serine threonine kinase (Selten *et al.*, 1986; Meeker *et al.*, 1987a; Meeker *et al.*, 1987b; Saris *et al.*, 1991), overexpression of which led to lymphomagenesis in *pim-1* transgenic mice (van Lohuizen *et al.*, 1989). Several other genes have been identified as preferential

targets for proviral integration, and subsequently as important mediators of transformation (reviewed by van Lohuizen & Berns, 1990; Jonkers & Berns, 1996). For example, frequent activation of c-*myc* and N-*myc* oncogenes by insertional mutagenesis in MuLV induced lymphomas underlines the role of these as powerful effectors of lymphomagenesis (Selten *et al.*, 1984; van Lohuizen *et al.*, 1989).

Synergy between oncogenes can also be uncovered using insertional mutagenesis in transgenic mouse models. For example, in *pim-1* transgenic mice, in which MuLV infection results in significantly reduced latency of T cell lymphomagenesis, insertional activation of c-*myc* or N-*myc* was observed in 100% of lymphomas, suggesting strong collaboration between these two genes (van Lohuizen *et al.*, 1989). This was confirmed by studies crossing *pim-1* and *MYC* transgenic mice, in which tumour development was significantly faster in double transgenic mice (Moroy *et al.*, 1991). Genes other than *pim-1*, for example *bmi-1*, have also been identified as synergistic partners for c-*MYC*, by MuLV infection of Eμ-*MYC* transgenic mice (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991). Many studies have shown that tumour development in certain oncogenic transgenic mouse models is greatly accelerated when animals are inoculated with MuLV neonatally (reviewed by Berns, 1991). In this way, genes that collaborate with the transgene can be identified, by identifying loci at which proviral insertion occurs more frequently than in non-transgenic littermate controls.

4.1.3 Experimental Aims

In the previous chapter, studies of transgenic mice demonstrated that deregulated *MYC* and loss of Fas do not collaborate in T cell lymphomagenesis, at least in the CD2-*MYC*ER^{TM} model. In the following chapter, the possible role of Fas as a tumour suppressor was studied, by investigating whether Fas^{lpr} mice were more susceptible to MuLV induced tumourigenesis. The aim was also to prove that the lack of synergy observed in Chapter 3 was confirmed in a different model of lymphomagenesis, and not due to some idiosyncrasy of the previous model which may arise as an insertional effect of the transgene. If deregulation of c-*myc* and loss of Fas signalling represent synergistic events in lymphomagenesis, the frequency of

proviral insertions at c-*myc* would be expected to be significantly increased in MuLV infected Fas^{lpr} mice.

4.2 RESULTS

4.2.1 MuLV Infection of Experimental Animals

To test the susceptibility of Fas^{lpr} animals to lymphomagenesis induced by MuLV, a cohort of 60 mice homozygous for the Fas^{lpr} mutation, and 60 strain-matched MRL mice were infected. Neonatal mice were inoculated intraperitoneally with MuLV within 24 hours of birth, and monitored over a twelve-month period. Animals were sacrificed when signs of tumour development became evident. Gross pathological examination revealed that the majority of animals in both groups developed thymic lymphoma with varied involvement of subcutaneous lymph nodes. A small number of animals in both groups showed signs of more generalised multicentric lymphoma, with gross enlargement of the spleen, mesenteric and subcutaneous lymph nodes, some lymphocytic infiltration in the liver and kidneys, and involvement of the thymus to a much lesser extent than that seen in thymic lymphoma. A proportion of Fas^{lpr} mice showed clinical signs associated with the *lpr* phenotype, as described in Chapter 3, in addition to lymphoma, while others developed severe lymphadenopathy and autoimmunity, and were sacrificed before any signs of tumour development appeared.

4.2.2 Survival of MuLV Infected Fas^{lpr} Mice

As shown in Figure 4.1, disease free survival of MuLV infected Fas^{lpr} mice was unchanged compared to MRL control mice. Both MRL-Fas^{lpr} and their MRL control counterparts developed tumours, the latency of which were not significantly different. Between the ages of 60 and 150 days, 88% (53/60) of MRL-Fas^{lpr} mice were found to develop tumours, compared to 80 % (48/60) of control MRL animals in the same period. The average latency of tumour development was also unaffected by *lpr* status (95 days in Fas^{lpr} animals compared to 92 days in controls). These results are

consistent with data from another laboratory (Zornig *et al.*, 1995), however the overall rate of tumour development is increased in this experiment, presumably due to strain differences, and variation in the number of viral units with which mice were inoculated.

4.2.3 c-*myc* is not a Preferential Target for Proviral Insertions in Fas^{lpr} Mice

Investigation of the possibility of proviral insertions or other rearrangements at the c-*myc* locus was carried out to compare insertion rates between MRL-Fas^{lpr} and MRL control animals. In those animals in which thymic lymphoma had occurred, DNA was extracted from snap frozen tumour samples for further analysis. Rearrangements and insertions were detected by hybridisation analysis using a murine *myc* probe against *Kpn*I fragments of tumour DNAs (Figure 4.2). Of the non-thymic lymphomas, incidence in both cohorts was so low that no further analysis was carried out, however latency of these tumours was not significantly different between the two cohorts (108 +/- 22.9 days in Fas^{lpr} mice compared with 94 +/- 17.1 days in MRL control strain mice).

Rearrangements at the c-*myc* locus were observed in both mouse cohorts however the number of tumours containing insertions at c-*myc* in MRL-Fas^{lpr} mice was not significantly increased compared to MRL controls (see Table 4.1). Of 53 Fas^{lpr} tumours tested, 10 were found to have insertions at c-*myc* compared to 15 from 42 MRL control tumours tested. These data suggest that c-*myc* is not a preferential target for insertion in Fas^{lpr} tumours. In addition, there was no correlation between tumour latency and insertions at c-*myc* in either cohort. In Fas^{lpr} animals, the average latency of MuLV induced tumours carrying insertions at c-*myc* was 103 days, equal to the latency of tumours in which no insertions were detected. Similarly, in control MRL mice, the average latency of tumours with and without c-*myc* insertions was 90 and 95 days respectively (Table 4.1). These findings confirm the lack of synergy between deregulation of c-*myc* and loss of Fas in the development and progression of T cell lymphomas.

Table 4.1 MuLV Infection of Fas^{lpr} and Control Mice - Tumour Latency and Rate of Insertion at c-*myc*

FREQUENCY OF C-*MYC* INSERTIONS[†]		
MRL-Fas^{lpr} mice		10/53*
MRL mice		15/42*
AVERAGE TUMOUR LATENCY		
Fas^{lpr} mice	c-*myc* rearranged	103 days
	no rearrangement at c-*myc*	103 days
MRL mice	c-*myc* rearranged	90 days
	no rearrangement at c-*myc*	95 days

[†] in thymic lymphomas for which DNA was available

* the difference between the two cohorts is not statistically significant

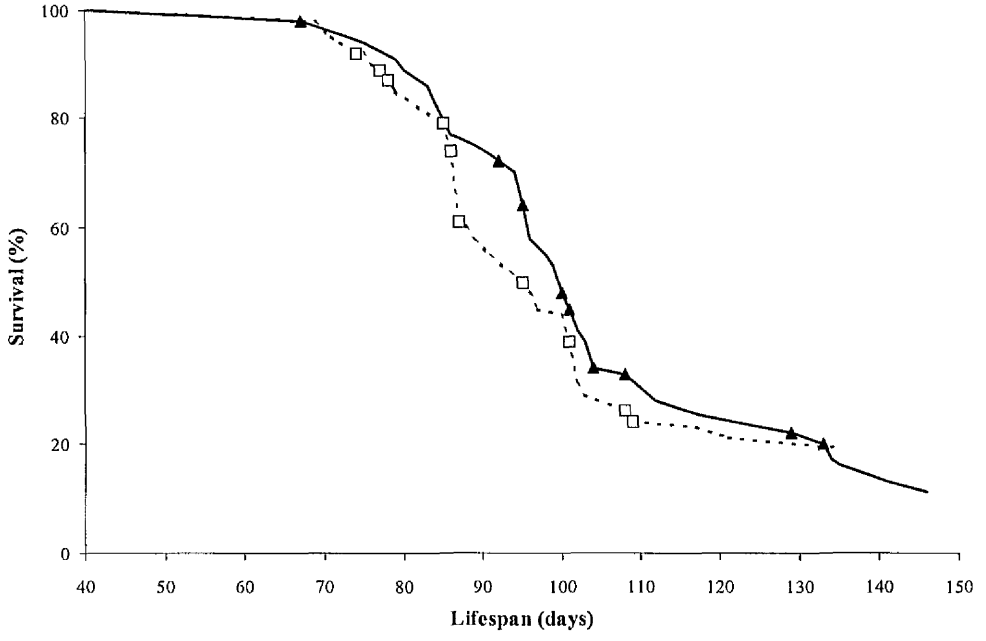


Figure 4.1 Disease Free Survival of MuLV Infected MRL-Fas^{lpr} and Control MRL Mice

Survival of MuLV infected Fas^{lpr} (solid line, filled triangles, n=60) and MRL control mice (broken line, open squares, n=49). Symbols represent animals harbouring tumours in which proviral insertion at c-*myc* had occurred. These data represent overall survival.

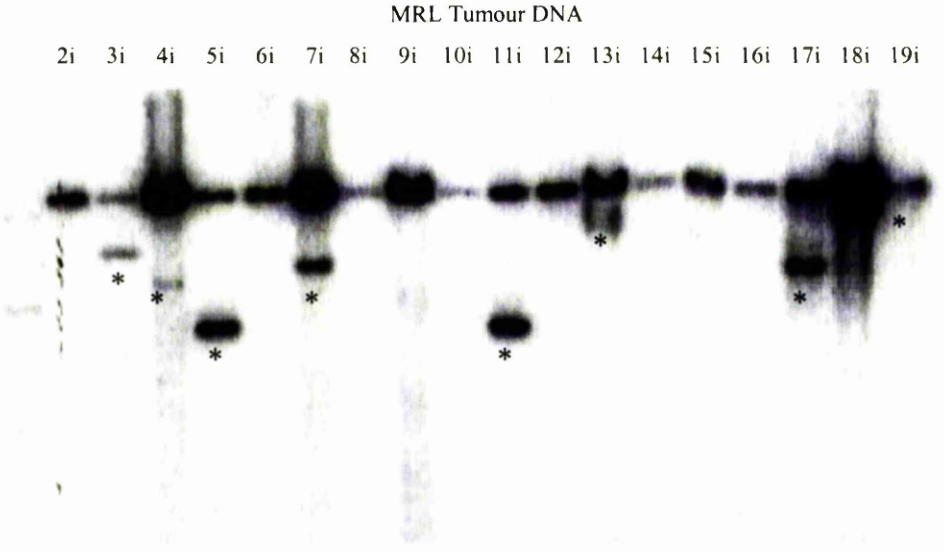


Figure 4.2 Hybridisation Analysis of Proviral Insertions at c-*myc* in Fas^{lpr} Mice

DNA from tumours that arose in MuLV infected Fas^{lpr} and MRL animals was analysed by Southern blotting with a murine *myc* probe. This figure shows examples of proviral integration, or lack of, at the c-*myc* locus in a series of MuLV induced lymphomas in the MRL cohort. Asterisks mark the positions of proviral integration sites. The upper band present in all lanes represents the germline c-*myc* gene.

4.2.4 Establishment of Transplanted Tumours in Fas^{lpr} and Control MRL Mice

Malignant or virally transformed cells express tumour or viral antigens bound to major histocompatibility complex (MHC) class I molecules on the cell surface (Vanky *et al.*, 1987; Rotzschke *et al.*, 1990; Hom *et al.*, 1991). This allows immune recognition of these transformed cells and a response is then elicited by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (Vanky *et al.*, 1987; Rotzschke *et al.*, 1990; Hom *et al.*, 1991). Fas:FasL interactions are known to be an important component of the CTL and NK cell mediated immune response (Rouvier *et al.*, 1993; Ju *et al.*, 1994; Kagi *et al.*, 1994b; Lowin *et al.*, 1994; Arase *et al.*, 1995; Oshimi *et al.*, 1996), and have been reported to have a role in clearance of tumour cells and virally infected cells (Zajac *et al.*, 1996; Komada *et al.*, 1997; Kashii *et al.*, 1999). In fact, expression of Fas on tumour cells has been shown to result in increased activity of anti-tumour CTLs and enhanced host survival (Bradley *et al.*, 1998). Because Fas^{lpr} mice do not express Fas on the surface of their cells, it might be expected that any tumour cells or virally infected cells would be protected to some degree from the CTL and NK cell mediated immune response, since they can no longer initiate apoptosis by Fas:FasL interactions. Therefore it is interesting that in Fas^{lpr} mice, MuLV induced lymphomagenesis is not accelerated compared to their control counterparts.

With the aim of investigating further the role of Fas:FasL interactions in tumour establishment, tumours arising in MuLV infected Fas^{lpr} and MRL control mice were transplanted to Fas^{lpr} and MRL hosts. Thirty lymphomas selected from each of the Fas^{lpr} and control MRL virally infected cohorts were explanted. Cells were prepared from disaggregated tumours, and 2 x 10^7 cells from each tumour in 500µl volume were transplanted intraperitoneally into an age-matched Fas^{lpr} and MRL host, as shown in Figure 4.3.

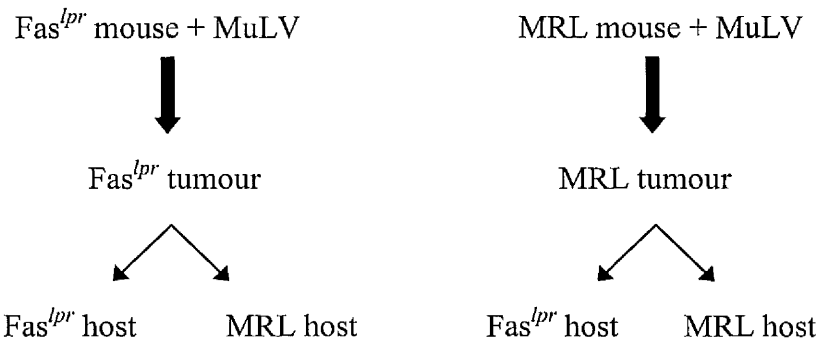


Figure 4.3 Transplantation of MuLV Transformed Cells into Fas^{lpr} and MRL Control Mice

Animals were monitored over a 100-day period, and sacrificed when clinical signs were present. Those mice in which tumours established, displayed enlarged abdomens, consistent with intraperitoneal transplantation of tumour cells. In all these mice diagnosis was made on the basis of gross pathology at post mortem. Every affected animal presented with a mass in the abdominal cavity, either as an abnormally enlarged mesenteric lymph node, or as a separate tumour mass. In some cases involvement of the other lymphoid organs was observed.

4.2.5 Host and Tumour Factors are Involved in Establishment of Transplanted Tumours

Nearly all Fas^{lpr} tumours grew rapidly in Fas^{lpr} hosts (93%), whereas only 7% of MRL tumours established in MRL hosts. In addition, only 21% of MRL hosts transplanted with Fas^{lpr} transformed cells succumbed to tumours. The results for this experiment are shown in Table 4.2. These results are not complicated by incidence of lymphoproliferative disease because although most Fas^{lpr} animals that did not harbour tumours developed lymphoproliferative disease, tumours established long before any signs of lymphoproliferation (Figure 4.4), which normally arise at around 5 months of age. Initially these results would suggest that immunosurveillance was

minimal in Fas^{lpr} mice, and that in MRL mice, there was a strong immune response to transplanted tumours cells. However, only 10% of MRL tumours grew when transplanted into Fas^{lpr} hosts, suggesting that there is still an effective immune response in these animals. Neither are these differences due to histocompatibility based rejection, as these were allogenic transplants. Moreover, MRL animals reject MRL tumours to the same extent. In view of the fact that even in MRL hosts, Fas^{lpr} tumour establishment appeared higher compared to growth of MRL tumours, it may be that tumour specific factors are involved to a greater extent, than host factors.

Table 4.2 Establishment of Transplanted Fas^{lpr} and MRL Control Tumours

	Fas^{lpr} tumour		MRL tumour	
	Fas^{lpr} host	MRL host	Fas^{lpr} host	MRL host
Tumour Establishment	26/28 (93%)	6/28 (21%)	3/30 (10%)	2/29 (7%)

* Animals succumbing to unrelated illness or as a result of intolerance to the transplantation procedure are not included in these figures.

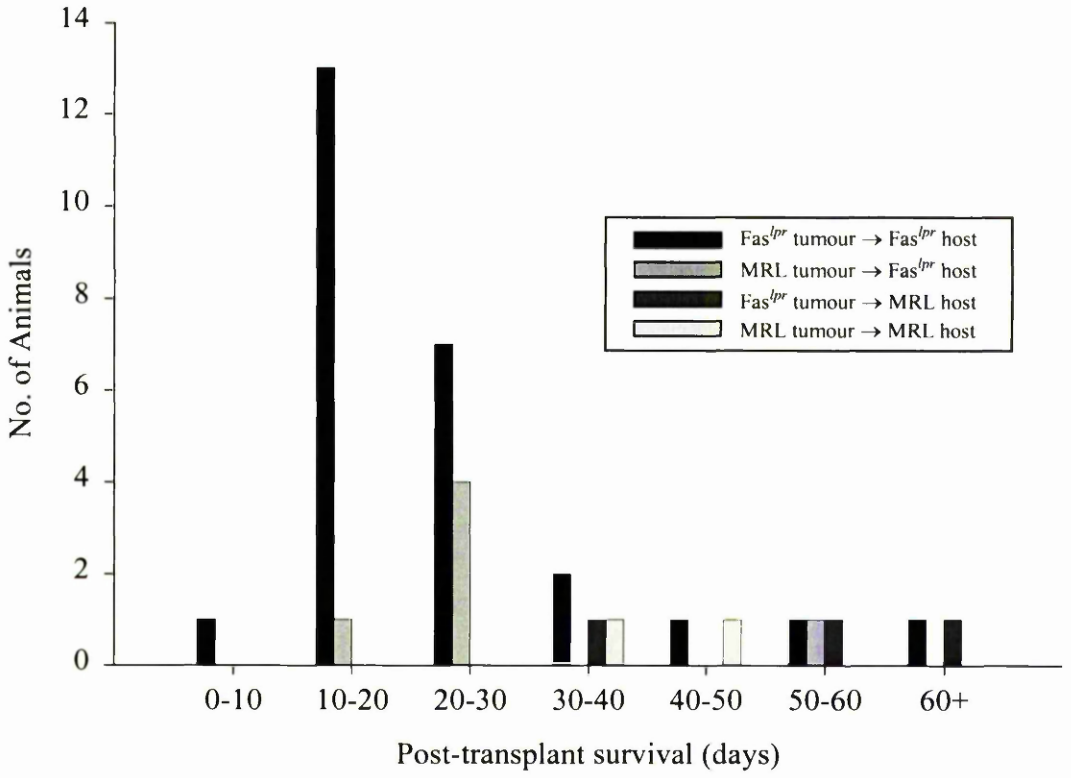


Figure 4.4 Latency of Transplanted Tumours in Fas^{lpr} and MRL Mice

Survival of Fas^{lpr} mice transplanted with Fas^{lpr} (black bars) or MRL (mid-grey bars) tumours, and of MRL mice transplanted with Fas^{lpr} (dark grey bars) or MRL (light grey bars) tumours. Chart shows number of mice in each cohort sacrificed during the indicated time-points due to tumour development.

4.2.6 Establishment of Transplanted Tumours in Athymic Mice

To investigate why so many tumours fail to establish in these experiments, selected tumours of both genotype were transplanted into athymic nude mice. These mice have a defect which results in hairlessness and failure of the thymus to develop, and consequently are incapable of producing mature T cells (Pantelouris, 1968). The defect is caused by a mutation in the gene encoding a member of the winged-helix family of transcription factors named winged-helix nude (whn, Nehls *et al.*, 1994). This mutation results in the expression of a truncated protein which lacks the DNA binding domain and is presumably unable to regulate transcription of other genes involved in development (Nehls *et al.*, 1994; reviewed by Reth, 1995). The absence of mature T cells in these mice gives rise to an inability to reject allogenic transplants, and a decreased response to T dependent antigens, although these animals are not especially susceptible to spontaneous tumourigenesis (Sharkey & Fogh, 1979). These animals were housed in a class II isolator unit.

Eight tumours from each of the original MuLV infected Fas^{lpr} and MRL cohorts were selected and matched with respect to latency in the original host, and presence or absence of proviral integration at c-*myc*. Tumour cells (2×10^7) were transplanted intraperitoneally into individual age matched nude mice, which were then monitored over a four-week period. Animals were sacrificed when clinical signs were apparent. Since these host mice lack mature T cells, the hypothesis was that tumours would not be rejected because of expression of tumour or virus specific antigen on the surface of transplanted cells, thereby revealing any disparity in ability to survive between tumour genotypes.

A summary of the results of this experiment is shown in Table 4.3. Transplanted lymphoma cells from both cohorts established rapidly in nude mice, and there was no significant difference in incidence or latency between Fas^{lpr} and MRL tumours. The latency of tumour development was not significantly altered by the integrity of the c-*myc* gene in either the Fas^{lpr} or MRL transplanted lymphomas. The experimental cohort was small however, and it is difficult to draw reliable conclusions from these figures. Although there was no significant difference in the survival of Fas^{lpr}

tumours, with an intact or rearranged c-*myc* gene, it was not possible to confirm that no synergy exists between *myc* and loss of Fas. The fact that all transplanted tumours were so readily established means the question of whether loss of the Fas pathway in transformed cells allows increased tumour survival remains unanswered, although host factors would appear to play a role in tumour cell clearance. Possible models to explain these results will be discussed later.

Table 4.3 Establishment of Fas^{lpr} and Control MRL Tumours in Athymic Hosts

Host	Tumour	Incidence	Mean Latency	S.D.
NuNu	Fas^{lpr}	100% (8/8)	19 days	+/- 4.9
	insertion at c-*myc*		16 days	+/- 2.9
	no rearrangement		21 days	+/- 5.0
NuNu	MRL	100% (8/8)	20 days	+/- 6.5
	insertion at c-*myc*		23 days	+/- 7.8
	no rearrangement		19 days	+/- 5.9

4.3 DISCUSSION

4.3.1 No Acceleration of Lymphomagenesis in MuLV Infected Fas^{lpr} Mice

As Chapter 3 demonstrated, no acceleration of c-*MYC* induced T cell lymphomagenesis was observed on a Fas^{lpr} background. MuLV infection of Fas^{lpr} mice has revealed that neither is there collaboration between viral infection and loss of Fas. MuLV infected Fas^{lpr} mice developed lymphoma at the same rate as control MRL counterparts, despite the lack of functional Fas. This is in agreement with other reports which showed that lymphomagenesis was not accelerated in Fas^{lpr} mice compared to infected control animals (Zornig *et al.*, 1995).

Recent studies have attempted to elucidate the role of Fas and FasL in MuLV induced tumours (Choe *et al.*, 1998; Bonzon & Fan, 2000). MuLV induced lymphomagenesis involves both early and late events. Preneoplastic changes occur in infected mice, one of which is thymic atrophy due to enhanced thymocyte apoptosis (Bonzon & Fan, 1999). Fas has been implicated in this enhanced apoptosis since MuLV infected cells show elevated expression of cell surface Fas (Choe *et al.*, 1998; Bonzon & Fan, 2000). If these cells remained susceptible to Fas induced apoptosis, this would limit the capacity for tumourigenesis. In order for MuLV infected cells to survive, and for outgrowth of tumour cells to occur, it is therefore reasonable to suggest that MuLV may ultimately confer on infected cells, the ability to overcome Fas induced apoptosis. It is possible that MuLV infection and proviral integration could affect either downstream effectors of apoptotic pathways, or may upregulate survival pathways. If MuLV is able to induce events that cause the Fas pathway to be blocked or inhibited, then that may explain why tumour latency is not significantly greater in MRL mice compared to Fas^{lpr} animals. The results also argue against a role for Fas as a *bona fide* tumour suppressor gene, since they highlight the lack of collaboration between loss of Fas, and other oncogenic mutations. MuLV infection has been successfully used as a method for identifying collaborating genes in transgenic mice (reviewed by Berns *et al.*, 1991). If proviral insertions arose in Fas^{lpr} cells to deregulate oncogenes which could collaborate with loss of Fas, then it would be expected that lymphomagenesis would be accelerated in these mice. There

was however, no acceleration of tumourigenesis in Fas^{lpr} animals compared to control MRL animals. Results in Chapter 3 demonstrated that loss of Fas and deregulation of c-*MYC* were not synergistic events in lymphomagenesis. The results in this chapter extend those results and suggest that Fas is not a tumour suppressor, and loss of Fas does not confer susceptibility to tumours.

These findings are somewhat surprising given that Fas^{lpr} mice have an impaired immune system. Antigen responsiveness has been studied in control MRL and Fas^{lpr} mice, and shown to be defective in Fas^{lpr} responding T cells, compared to control cells (Fischbach, 1984). In addition, Fas has been shown to be involved in mediating cytotoxicity of CTLs and NK cells (Kagi *et al.*, 1994b; Lowin *et al.*, 1994; Arase *et al.*, 1995; Braun *et al.*, 1996). These findings would suggest that the immune system in Fas^{lpr} animals might be less efficient in the response to viral infection than that of control animals. It is probable that the response to MuLV infection of T cells is mediated by CTLs and NK cells, and that T lymphoma cells might express tumour and viral specific antigens which make them susceptible to CTL and NK cell killing. Yet virally induced tumourigenesis is not accelerated in Fas^{lpr} mice which lack the Fas pathway. It is likely then that other apoptotic mechanisms can be recruited in Fas deficient mice, to mediate clearance of virally transformed cells.

4.3.2 c-*myc* is not a Preferential Target for Proviral Insertion in Fas^{lpr} Mice

Insertional activation events are essential for MuLV induction of tumour formation. Activation and overexpression of proto-oncogenes leads to uncontrolled proliferation and transformation of host T cells but apoptotic pathways may also need to be blocked to allow survival of transformed cells. Insertions at c-*myc* are frequently observed in MuLV induced lymphomas (Selten *et al.*, 1984; van Lohuizen *et al.*, 1989). If a functional Fas pathway was capable of restricting *myc*'s role in oncogenesis, then the proportion of MuLV induced lymphomas arising in Fas^{lpr} animals expected to contain proviral integrations at c-*myc* would be higher still. This phenomenon has been seen in previous studies in which animals transgenic for a *myc* collaborating gene have been infected with MuLV. In Eμ-*pim*-1 transgenic animals

infected with MuLV for example, all tumours have insertions at either c-*myc* or at N-*myc* (van Lohuizen *et al.*, 1989). In Fas^{lpr} mice however, *myc* did not represent a preferential target for proviral mutagenic insertion.

The results of the transgenic studies in Chapter 3 showed that cells with sustained c-*MYC* expression are not more susceptible to lymphomagenesis in the absence of Fas. The lack of collaboration between *MYC* and loss of Fas in CD2-*MYC*ER^{TM}Fas^{lpr} transgenic mice was confirmed in MuLV infected Fas^{lpr} and control mice. There was no difference in survival between the infected Fas^{lpr} and control cohorts, and c-*myc* did not represent a preferential target for proviral insertion in Fas^{lpr} mice. More importantly perhaps, even in tumours arising in Fas^{lpr} mice, in which MuLV integration had deregulated *myc*, the latency of those tumours was not significantly altered compared to either Fas^{lpr} tumours in which c-*myc* was not rearranged, or to MRL tumours regardless of the status of c-*myc*. These results clearly show that in this system at least, loss of Fas and deregulation of c-*myc* do not represent synergistic events in lymphomagenesis. This finding is in contrast to the results of Zornig *et al.* (1995), who reported acceleration of tumourigenesis in Eμ-L-*MYC* mice on a Fas^{lpr} background, although no acceleration of MuLV induced tumourigenesis in Fas^{lpr} animals. The reason for this difference is unclear, but may reflect the existence of more than one physiological outcome of Fas ligation, which may be tissue and context dependent. The Fas pathway may be a critical mediator of apoptosis in one cell lineage, but have some functional redundancy in other cell types. For example other TNF family members might be recruited in T cells in the absence of the Fas pathway. In addition, Fas:FasL interactions may not always result in death of the tumour cell, depending on the cell type and the levels of expression (see Chapters 7 & 8).

4.3.3 Tumour and Host Factors are Involved in Survival of Fas^{lpr} or Control Tumour Transplants

Transplantation studies of MuLV transformed Fas^{lpr} or MRL control cells, into Fas^{lpr} or MRL hosts, was carried out to determine the importance of Fas:FasL interactions in preventing tumour establishment. Lack of Fas on tumour cells occurring in Fas^{lpr}

animals may protect the tumour cells from FasL mediated killing by cytotoxic T cells or NK cells. However Fas^{lpr} animals may also be susceptible to increased tumour incidence because they may be generally immunocompromised (Fischbach, 1984). The transplantation experiments were carried out to investigate these possibilities, and the consequences of loss of Fas on tumour cells, and also in the host. The results generated however were complex (see Figure 4.5), and open to several different interpretations.

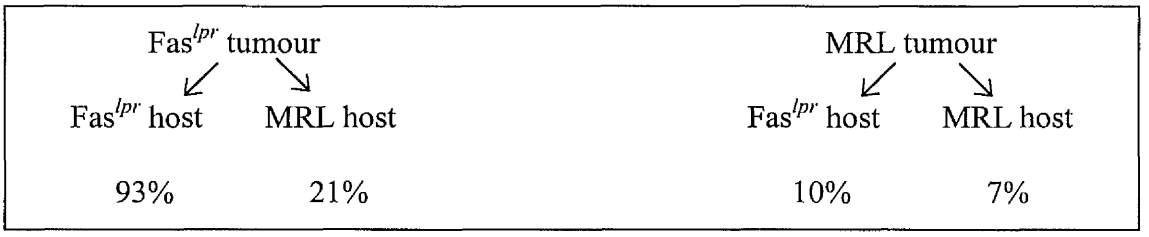


Figure 4.5 Survival of MuLV Transformed Cells Transplanted into Fas^{lpr} and MRL Hosts

Firstly it was noted that the survival of Fas^{lpr} tumours in Fas^{lpr} hosts was greatly enhanced compared to establishment of MRL tumours in MRL hosts (93% compared to 7%). This could be explained either by the lack of Fas on the tumour cells from Fas^{lpr} animals, which may render them resistant to clearance by CTLs and NK cells (Rouvier *et al.*, 1993; Kagi *et al.*, 1994b; Lowin *et al.*, 1994), or because Fas^{lpr} hosts could be generally immunocompromised (Fischbach, 1984). It may be that clearance of transplanted MRL tumour cells in MRL hosts occurs by a specific T cell response to viral and tumour antigens which is mediated by Fas:FasL interactions, and/or that the immune system and immune surveillance in MRL animals is much more efficient compared to that of Fas^{lpr} animals.

The results of transplantation of Fas^{lpr} tumours into Fas^{lpr} or control MRL hosts, suggested that the latter explanation might be more likely, since the rate of establishment for Fas^{lpr} tumours was 93% in Fas^{lpr} hosts and only 21 % in MRL mice. The clearance of Fas^{lpr} tumours in MRL mice cannot be mediated by the Fas pathway however, since the tumours lack functional Fas. Instead, the immune

system in MRL mice must induce death of tumour cells through a Fas independent pathway. These results suggest that Fas^{lpr} animals have generally poor immune function, since they are unable to clear tumours as well as the MRL strain controls. If this is the reason for the high establishment rate of tumours in Fas^{lpr} mice however, it leaves the question why virally or transgene induced tumours do not arise in Fas^{lpr} mice with greater frequency, and reduced latency. There may be another reason that explains why MRL mice are better able to clear Fas^{lpr} tumours than are Fas^{lpr} hosts. Although these mice are strain matched, it may be that Fas^{lpr} tumours are not truly histocompatible with MRL hosts, and are recognised as foreign and therefore killed more easily in MRL animals than in like hosts. The 100% establishment rate of tumours transplanted into nude athymic mice also suggests that host environment and efficacy of immune surveillance must be critical in controlling the events leading to tumourigenesis and tumour cell survival.

From comparison of other data from the transplantation studies, it appears that host-independent factors associated with the tumour are also important in establishment or clearance of the tumour. Fas^{lpr} tumours appear to survive better in MRL hosts than can MRL tumours. When Fas^{lpr} tumours and MRL tumours were transplanted into MRL hosts, only 7% of MRL tumours survived, while 21% of the Fas^{lpr} tumours established. These results suggest that the lack of Fas expression on the surface of the Fas^{lpr} tumours renders them more resistant to immune attack. From these data, it appears that survival of transplanted tumours is enhanced when the Fas pathway is absent. This is not completely unexpected; Fas^{lpr} tumours do lack a major death pathway used by CTLs and NK cells in tumour cell clearance (Rouvier *et al.*, 1993; Kagi *et al.*, 1994b; Lowin *et al.*, 1994).

Thus far, the results seem to suggest that tumour establishment in Fas^{lpr} mice would be affected by both lack of Fas expression on tumour cells, and by an innate immune deficiency in Fas^{lpr} mice. Comparison of another set of transplants complicate the conclusions drawn so far however. The rate of establishment of MRL tumours was not significantly different in MRL hosts compared to Fas^{lpr} hosts (7% compared to 10%). These results would argue against a general immunodeficiency in Fas^{lpr} animals, in terms of tumour immunity, since there was no significant difference

between these mice and control MRL mice in response to MRL tumours. It may be that in this situation, there is a lack of histocompatibility between the Fas^{lpr} hosts and MRL tumours, which results in enhanced clearance of the MRL tumours. It is also possible that Fas^{lpr} mice are more effective at destroying MRL tumours, because FasL is expressed at higher levels in these mice (Chu *et al.*, 1995; Watanabe *et al.*, 1995), and Fas expressing tumours might be more susceptible to immune attack in these mice. Both of these factors may counteract an innate immunodeficiency in Fas^{lpr} mice.

The results have shown that Fas^{lpr} tumours have a growth advantage when transplanted. Another reason for the increased survival of Fas^{lpr} tumours in like hosts, compared to MRL hosts might be the immune deficiency associated with the Fas^{lpr} host. However, the growth of MRL tumours in Fas^{lpr} hosts appears to be reduced somewhat compared to Fas^{lpr} tumours in MRL hosts (10% compared to 21%). Since these results can be neither fully explained by the level of immune surveillance in the host, or by the presence or absence of Fas on the tumour cells, it is necessary to consider other factors which may also affect tumour establishment. From these results it appears that Fas^{lpr} hosts may have some advantage over MRL animals in certain circumstances, at least in terms of tumour clearance.

One explanation for this may be that tumours act as sites of immune privilege to evade the immune response. High levels of FasL have been demonstrated on a number of malignancies of both haematopoietic (Ohshima *et al.*, 1997; Perzova & Loughran, 1997; Villunger *et al.*, 1997; Xerri *et al.*, 1997; Mariani & Krammer, 1998) and non-haematopoietic origin (Hahne *et al.*, 1996; O'Connell *et al.*, 1996; Strand *et al.*, 1996; Niehans *et al.*, 1997; Saas *et al.*, 1997; Shiraki *et al.*, 1997). It has been proposed that reminiscent of sites of immune privilege such as the eye and testis (Griffith *et al.*, 1995), FasL expression on tumour cells could allow them to evade immune attack by inducing apoptosis of invading Fas-bearing CTLs (Greil *et al.*, 1998; Gastman *et al.*, 2000; Zeytun *et al.*, 2000). Recently, MuLV infected cells were shown to evade immune attack by expression of FasL and engagement of Fas on the surface of antiviral T cells (Rich & Green, 1999).

In Fas^{lpr} cells, FasL expression is significantly higher than in control cells (Chu *et al.*, 1995; Watanabe *et al.*, 1995) and when transplanted into control mice, Fas^{lpr} tumour cells may grow well and escape immune attack by inducing apoptosis in invading Fas-bearing CTLs. Although cells from MRL animals may express less surface FasL than those from Fas^{lpr} animals, they should also have the ability to induce apoptosis by ligation of Fas on invading CTLs. In Fas^{lpr} hosts however, a tumour could express very high levels of FasL, but would be unable to counterattack infiltrating lymphocytes, which would express no surface Fas receptor (see Figure 4.6). This suggests that effectively, immune privilege does not exist in Fas^{lpr} mice, and allows speculation that at least some tumour types may have a growth disadvantage in Fas^{lpr} mice. This was demonstrated in Fas^{lpr} mice in which a delay in growth of a melanoma was observed (Hahne *et al.*, 1996).

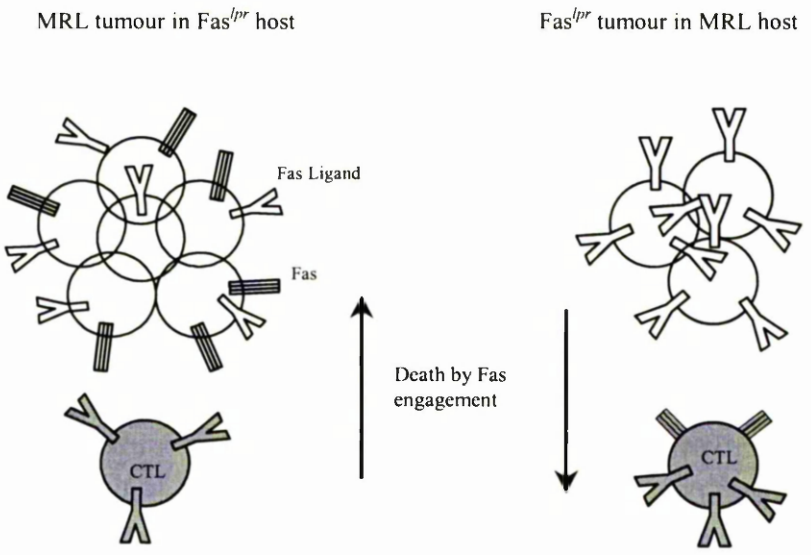


Figure 4.6 Tumour Rejection in Fas^{lpr} Mice?

If the Fas^{lpr} background were to offer enhanced ability to reject some tumours, through the lack of Fas on invading immune cells, it would clearly depend on the context of FasL expression in the tumour, and on the tumour cell type. Although in the past, studies of FasL expression on tumour cells have focused on the possible role of Fas:FasL interactions in restricting tumour development (Ouhtit *et al.*, 2000),

more recent studies on immune privilege have re-evaluated the consequences of FasL expression on tumours since greater defence from immune attack has been observed in some models as a result of increased surface FasL (reviewed by Walker *et al.*, 1998; O'Connell *et al.*, 1999; Restifo, 2000). This may be due to the reported reverse signalling through FasL, which is believed to augment proliferation of FasL expressing cells under certain circumstances (Suzuki & Fink, 1998). Whatever the reason, the conditions governing whether FasL expression confers immune privilege or enhanced tumour growth are not yet clear. When immune privilege as a method of tumour escape is fully understood, further insight may be gained into why c-*MYC* and MuLV induced T cell lymphomagenesis are not accelerated in Fas^{lpr} mice.

CHAPTER 5

FAS INDEPENDENT APOPTOSIS IN CD2-*MYC*ER^{TM} T CELLS

5.1 INTRODUCTION

5.1.1 The CD2-*MYC*ER^{TM} Transgene *In Vitro*

CD2-*MYC*ER^{TM} transgenic mice provide a valuable model for the study of *MYC* induced lymphomagenesis *in vivo*. The capacity of the transgene to be induced *in vitro* is also important for studying the effects of MYC upregulation. Activation of MYC has been demonstrated in explanted thymocytes from transgenic mice. Although normal thymocytes die within 3-5 days in culture, treatment of explanted CD2-*MYC*ER^{TM} thymocytes with 4-OHT results in significantly increased cell death compared to untreated transgenic thymocytes, and also compared to treated controls (Blyth *et al.*, 2000). This feature of the transgene makes it possible to study individual pathways in c-MYC induced apoptosis. It should be possible to block distinct death signals, and investigate the effects on MYC induced apoptosis.

Despite their transformed status, MYC activity can still be regulated with 4-OHT treatment in CD2-*MYC*ER^{TM} tumour cells (Blyth *et al.*, 2000). Treatment of CD2-*MYC*ER^{TM} T lymphoma cells with 4-OHT *in vitro* has been previously shown to induce significantly increased apoptosis (Blyth *et al.*, 2000). Analysis of explanted CD2-*MYC*ER^{TM} lymphoma cells can reveal whether or not the apoptotic function of MYC has been lost during the process of tumourigenesis and may offer elucidation of the mechanisms involved in MYC induced apoptosis.

5.1.2 The Role of Fas Signalling in MYC Induced Apoptosis

In common with other oncogenes, c-*MYC* is capable of mediating cell proliferation, or cell death by apoptosis. The ability of MYC to induce apoptosis may act as a brake to tumourigenesis, and genetic events that prevent apoptosis would represent

significant events in tumour development or progression. Studies have shown that in immortalised fibroblast lines, and in mouse embryo fibroblasts c-MYC induced apoptosis is mediated by, and dependent on Fas:FasL interactions (Hueber *et al.*, 1997). Further, induction of caspase 3 like activity, which is part of the Fas signalling pathway, has been detected and shown to be essential in MYC induced apoptosis in fibroblasts (Kagaya *et al.*, 1997). Recently, the assertion that Fas was necessary for MYC induced apoptosis has been tempered, with the hypothesis that MYC may induce apoptosis through release of cytochrome *c*, which is independent of Fas signalling (Juin *et al.*, 1999). It has been suggested instead that MYC may act by sensitising cells to death induced by Fas ligation (Prendergast, 1999).

Although the *in vivo* studies have shown no role for the Fas pathway in MYC induced lymphomagenesis, *in vitro* studies of apoptosis in CD2-*MYC*ER^{TM}Fas^{lpr} and CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ cells were carried out, to ascertain whether or not Fas signalling was required for MYC to induce apoptosis in T cells, and to establish if the apoptotic function of MYC was susceptible to loss during lymphomagenesis.

5.1.3 Experimental Aims

The aim of the following experiments was firstly to examine whether or not Fas was required for MYC to induce apoptosis in T cells, by upregulating MYC in non-transformed cells from CD2-*MYC*ER^{TM}Fas^{lpr} mice. It was also necessary to determine if the ability of MYC to effect apoptosis was retained during transformation, in CD2-*MYC*ER^{TM} lymphoma cells on both a Fas^{lpr} and Fas$^{lpr/-}$ background.

5.2 Results

5.2.1 MYC Induced Apoptosis in Non-Transformed CD2-*MYC*ER^{TM}Fas^{lpr} Thymocytes

For the purpose of investigating the role of Fas in c-MYC induced apoptosis, thymocytes from CD2-*MYC*ER^{TM} mice, homozygous or heterozygous for the *lpr*

mutation, were studied *in vitro*. CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ mice were crossed with Fas^{lpr} animals to generate offspring which were CD2-*MYC*ER^{TM}Fas^{lpr}, CD2-*MYC*ER^{TM}Fas$^{lpr/-}$, Fas^{lpr} or Fas$^{lpr/-}$. Healthy littermate animals were sacrificed at 3 weeks old and their thymii removed. Thymocyte suspension cultures were prepared for each thymus and cultured *in vitro* in quadruplicate, with 4-OHT to induce the CD2-*MYC*ER^{TM} transgene, or with ethanol control. Cells were cultured in RPMI medium as described in Chapter 2. Thymocytes were cultured alone, and no stimulatory or survival factors were added. Cell viability in these cultures was assessed by a live/dead trypan blue exclusion assay, and expressed as a percentage of the number of live cells over the total cell number.

Although normal thymocytes do not survive for long in culture without survival factors, and there is a high level of background death, a significant increase in cell death was seen in 4-OHT treated CD2-*MYC*ER^{TM} thymocytes compared to 4-OHT treated non-transgenic controls (Figure 5.1A, $P<0.01$), and compared to ethanol treated CD2-*MYC*ER^{TM} thymocytes (Figure 5.1A, $P<0.01$), after 24 hours. There was a slight but significant difference in background death between ethanol treated CD2-*MYC*ER^{TM} and non-transgenic thymocytes ($P<0.05$). This may be due to some residual transgene activity in these cells, due to the leaky nature of the transgenic construct.

Background death of CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ thymocytes was however significantly increased compared to CD2-*MYC*ER^{TM}Fas^{lpr} cells (Figure 5.1B, $P<0.01$). The reason for this is not clear. It is possible however that there is some *in vivo* selection event which results in survival of CD2-*MYC*ER^{TM}Fas^{lpr} thymocytes that are more resistant to apoptosis following explantation, or that the ability to undergo apoptosis generally is reduced in Fas^{lpr} thymocytes. Enhanced MYC activity may result in selection of Fas^{lpr} thymocytes which are less anergic, and better able to survive *in vitro*. More important however, was the observation that significant induction of death was still observed following 4-OHT treatment in CD2-*MYC*ER^{TM}Fas^{lpr} thymocytes, despite the lack of Fas signalling ($P<0.01$). There was no significant difference in the rate of cell death between 4-OHT treated CD2-*MYC*ER^{TM} transgenic thymocytes on a Fas^{lpr} homozygous or heterozygous background after 24 hours (Figure 5.1B). Average

induction of death in 4-OHT treated CD2-*MYC*ER^{TM}Fas^{lpr} thymocytes was 4.9% compared to 4.6% in heterozygote controls. These studies show that MYC induced apoptosis can occur in healthy thymocytes independently of Fas. Neither is the level of MYC induced apoptosis in T cells diminished by lack of Fas signalling.

5.2.2 Explanted CD2-*MYC*ER^{TM}Fas^{lpr} Tumour Cells Retain the Ability to Undergo MYC Induced Apoptosis

The results in the previous section demonstrate that MYC activity can be regulated in non-transformed CD2-*MYC*ER^{TM} cells in *vitro*. During transformation however, cells may come under pressure to lose apoptotic pathways in order to survive. Despite their transformed status however, MYC activity can still be regulated with 4-OHT treatment in explanted CD2-*MYC*ER^{TM} tumour cells (Blyth *et al.*, 2000). This feature of the transgenic model allowed investigation of MYC induced apoptosis in neoplastic CD2-*MYC*ER^{TM} cells in the presence or absence of a functional Fas pathway.

A series of 6 of each CD2-*MYC*ER^{TM} lymphoma cell cultures homozygous or heterozygous for the Fas^{lpr} mutation, were incubated with or without 4-OHT, and cell viability measured at 24 hour intervals by the trypan blue exclusion assay. Significant induction of apoptosis was observed in all CD2-*MYC*ER^{TM}Fas^{lpr} tumour cell cultures, and the mean induction of death in these tumours was not significantly different from that observed in CD2-*MYC*ER^{TM} tumours heterozygous for the Fas^{lpr} mutation: 25.9 +/- 13.0% in CD2-*MYC*ER^{TM}Fas^{lpr} tumour cells compared to 33.2 +/- 19.9% in CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ cells after 48 hours 4-OHT treatment. Figure 5.2 shows representative survival curves of CD2-*MYC*ER^{TM}Fas^{lpr} tumour cells. Control CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumour cells are shown in Figure 5.3. In all CD2-*MYC*ER^{TM} cell cultures tested, significant apoptosis was induced when treated with 4-OHT (Figures 5.2, 5.3). Induction of death was due to activation of the CD2-*MYC*ER^{TM} transgene, since 4-OHT treatment does not affect the survival of cells not carrying the transgene (Cameron *et al.*, 2000). These results indicate that the ability of MYC to induce apoptosis is not eliminated during transformation.

MYC induced apoptosis was not blocked in Fas^{lpr} cells indicating that at least in T lymphoma cells, Fas was not required for MYC induced apoptosis *in vitro*. T lymphoma cell lines were established successfully from CD2-*MYC*ER^{TM}Fas^{lpr} mice and cultured with 4-OHT. Significant induction of apoptosis was observed in all cell lines tested (3/3, $P<0.01$), even after extended passage. A representative cell line is shown in Figure 5.2F. Apoptosis following 4-OHT treatment of these cell lines was confirmed using Annexin V staining and flow cytometric analysis (Figure 5.4). Together these data demonstrate that MYC induced apoptosis can occur independently of Fas in these cell lines, and that loss of Fas does not predispose cells to loss of MYC induced apoptosis in the long term.

5.2.3 *MYC* Induced Tumours Have an Intact Fas Signalling Pathway

The results presented so far indicate that loss of the Fas pathway does not accelerate *MYC* induced T cell lymphomagenesis, and that the Fas pathway is not required for MYC induced apoptosis. It is possible however that the Fas pathway plays some role in MYC induced apoptosis in T cells, and selection events during tumourigenesis may result in loss of the Fas pathway, particularly in cells heterozygous for the Fas^{lpr} mutation, if Fas is an important mediator of MYC induced apoptosis. For this reason it was important to investigate the integrity of the Fas pathway in normal untransformed thymocytes and in tumours induced by the CD2-*MYC*ER^{TM} transgene. The efficacy and specificity of the agonistic anti-Fas antibody, Jo2, were confirmed in untransformed thymocytes from control and Fas^{lpr} animals. Treatment with Jo2 antibody resulted in significantly increased cell death in control thymocytes (Figure 5.5A, $P<0.01$), but had no effect on Fas^{lpr} thymocytes (Figure 5.5B). Cell viability was assessed by trypan blue exclusion.

Transformed cells from CD2-*MYC*ER^{TM} tumours homozygous or heterozygous for the *lpr* mutation were explanted and incubated in the presence of Jo2. Treatment with Jo2 antibody resulted in significantly increased cell death in all CD2-*MYC*ER^{TM} *lpr* heterozygous cultures tested (6 from 6). Mean induction of death in these cells 48 hours after treatment with anti-Fas antibody ranged from 8.0 +/- 4.7% to 47.6 +/- 0.7%. A representative survival curve is shown in Figure 5.5C. The anti-Fas

antibody did not significantly increase cell death in any of the cultures from CD2-*MYC*ER^{TM} tumours arising in Fas^{lpr} animals however, confirming the lack of a functional Fas pathway in these cells. A representative survival curve is shown in Figure 5.5D. These findings indicate that at least in the CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumours tested, the Fas pathway was intact and there had been no selection for events that blocked Fas induced death. These data confirm the results of earlier experiments which showed no loss of heterozygosity at the Fas locus in *MYC* induced tumours. Further, the data indicate that the Fas pathway is not functional in Fas^{lpr} cells, at least at the level of our detection.

5.2.4 MYC Activation Does Not Sensitise Fas^{lpr} Cells to Fas Mediated Apoptosis

Juin *et al* (1999) have discussed the possibility that MYC activation leads to cells becoming sensitised to apoptosis induced by Fas and p53 through triggering of cytochrome *c* release into the cytosol. Since the *lpr* mutation is leaky, Fas^{lpr} cells are not completely lacking in surface Fas, although functional Fas is below detectable levels in these cells (Watanabe-Fukunaga *et al.*, 1992a). To exclude the possibility that increased levels of MYC could sensitise Fas^{lpr} cells to Fas induced apoptosis, CD2-*MYC*ER^{TM}Fas^{lpr} T lymphoma cells were cultured with 4-OHT and the agonistic anti-Fas antibody, Jo2. If MYC were able to sensitise Fas^{lpr} cells to apoptosis induced by Fas, it would be expected that Jo2 would induce death of cells in which MYC was upregulated. Figure 5.6A shows that Jo2 has no effect on the survival of primary CD2-*MYC*ER^{TM}Fas^{lpr} lymphoma cells. This survival curve is representative of another 5 primary CD2-*MYC*ER^{TM}Fas^{lpr} tumours. Figure 5.6B shows a representative survival curve of an established cell line. Similar results were obtained for 3 cell lines in total. The results show that even following MYC upregulation, the negligible activity of the Fas pathway in Fas^{lpr} cells is not sufficient to be sensitised to induce apoptosis by upregulated MYC.

5.2.5 Modulation of MYC Activity in Lymphoma Cells from Tamoxifen Treated Mice

Results shown so far have indicated that MYC induced apoptosis is not abrogated during spontaneous tumourigenesis in untreated CD2-*MYC*ER^{TM} mice, and that it can occur in the absence of a functional Fas pathway. As discussed in Chapter 3, activation of the transgene in CD2-*MYC*ER^{TM} mice treated with tamoxifen results in increased tumour incidence, and reduced latency. It was necessary to investigate whether or not it was possible to induce the transgenic construct further *in vitro*, by addition of 4-OHT, and upregulate MYC in transformed cells from these animals. Further, it was necessary to establish whether MYC could still induce apoptosis in these cells independently of the Fas pathway. It may be that when MYC activity is upregulated *in vivo*, stimulation of apoptosis is also enhanced, and thus there is more pressure to lose apoptotic pathways so that proliferation exceeds cell death.

Tumour cells were explanted from these mice for the purpose of investigating MYC induced apoptosis *in vitro*. Analysis of a series of 5 tumours showed that MYC activity could still be induced in explanted tumour cells from CD2-*MYC*ER^{TM} animals in which transgene activation had already occurred, and that MYC induced apoptosis was not blocked in Fas^{lpr} cells. Figure 5.7 shows significant induction of apoptosis in cultures from four tumours arising in tamoxifen treated CD2-*MYC*ER^{TM}Fas^{lpr} mice. The range of induction of cell death in CD2-*MYC*ER^{TM}Fas^{lpr} tumours 72 hours following *in vitro* 4-OHT treatment was 12.2 +/- 9.1 %. This was significantly reduced compared with 4-OHT treated tumours from untreated mice of the same genotype ($P<0.01$), and this is likely to be due to the transgene being previously activated. Nevertheless significant induction of death was observed in all tumours tested. In addition, significant induction of cell death was observed by trypan blue exclusion and Annexin staining in a cell line that was established from a CD2-*MYC*ER^{TM}Fas^{lpr} tumour (ERLPTW 32, shown in Figure 5.4). These results show that although tumourigenesis was accelerated in these mice, the apoptotic function of MYC remained intact, and confirm the lack of requirement for the Fas pathway in MYC induced apoptosis.

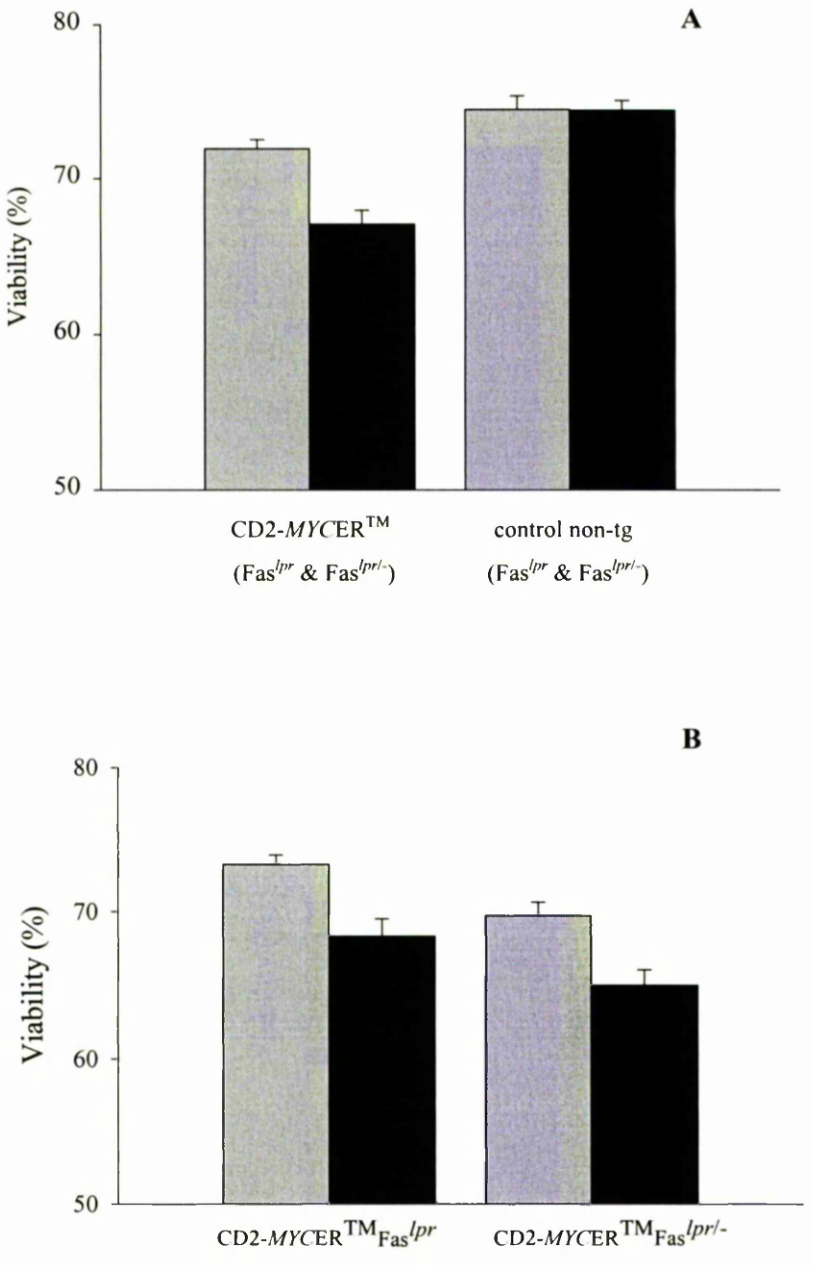


Figure 5.1 *In Vitro* Survival of 4-OHT Treated CD2-*MYC*ER™ Thymocytes

A, Explanted thymocytes from CD2-*MYC*ER™ and non-transgenic control mice and **B,** Explanted thymocytes from CD2-*MYC*ER™Fas^{lpr} and CD2-*MYC*ER™Fas$^{lpr/-}$ mice were cultured with (black bars) and without (grey bars) the addition of 4-OHT, and viability assessed by trypan blue exclusion. Results show live:dead counts as a percentage of live over total, performed in quadruplicate at 24 hours following incubation with 4-OHT. Results represent an average of eight CD2-*MYC*ER™ mice (4 Fas^{lpr} and 4 Fas$^{lpr/-}$) and four non-transgenic controls.

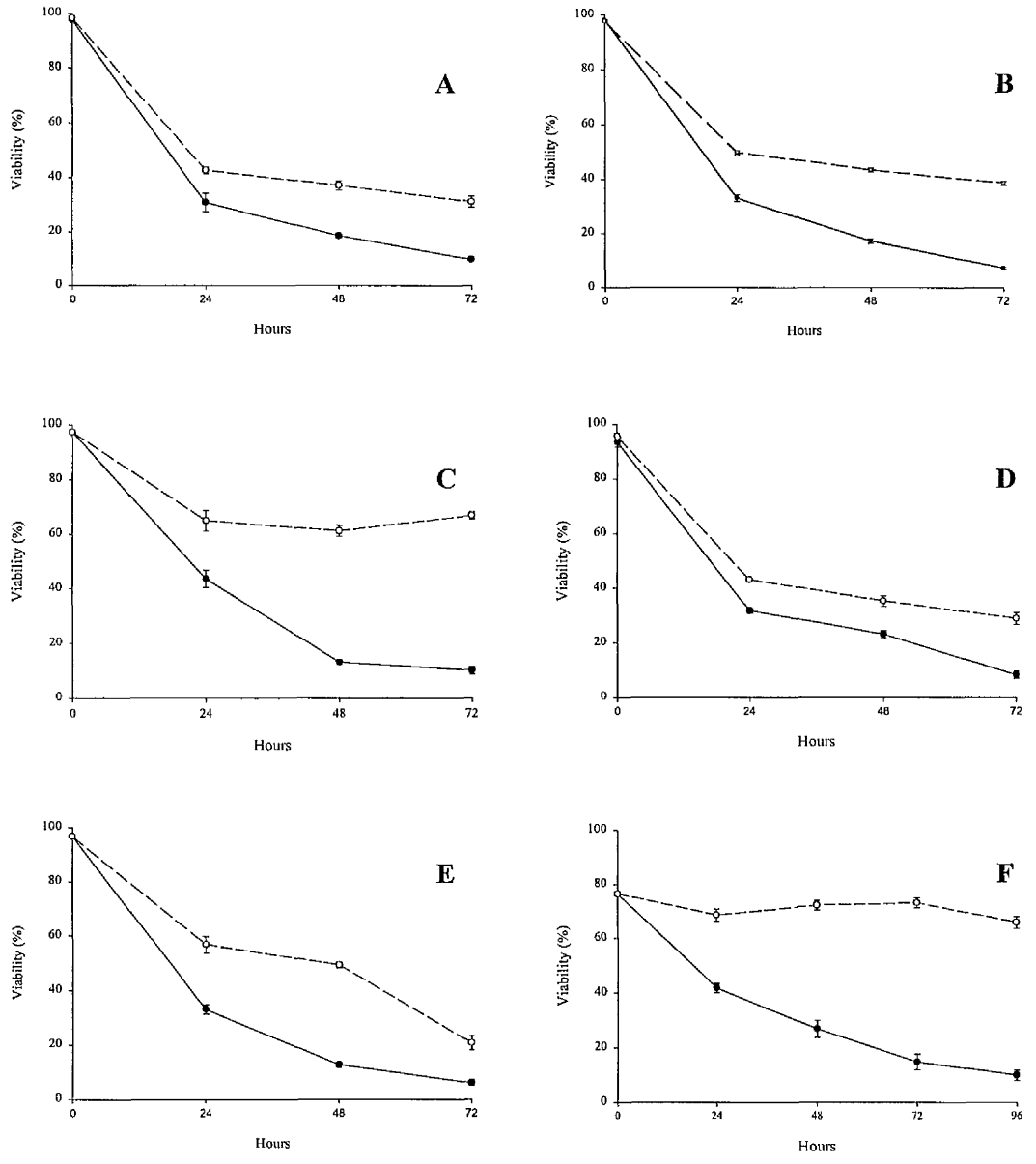


Figure 5.2 ***In Vitro*** **Survival of 4-OHT Treated CD2-*MYC*ER^{TM}Fas^{lpr} T Cell Lymphoma Cells**

A-E, Survival curves of primary tumour cells, and **F**, an established cell line from CD2-*MYC*ER^{TM}/Fas^{lpr} animals. Cells were cultured with (solid line, filled circles) or without (broken line, open circles) 4-OHT, and viability assessed by trypan blue exclusion. Results show live:dead counts performed in quadruplicate as percentage live over total cells, at the time-points indicated. Similar results were obtained for a total of 6 tumours and 3 cell lines.

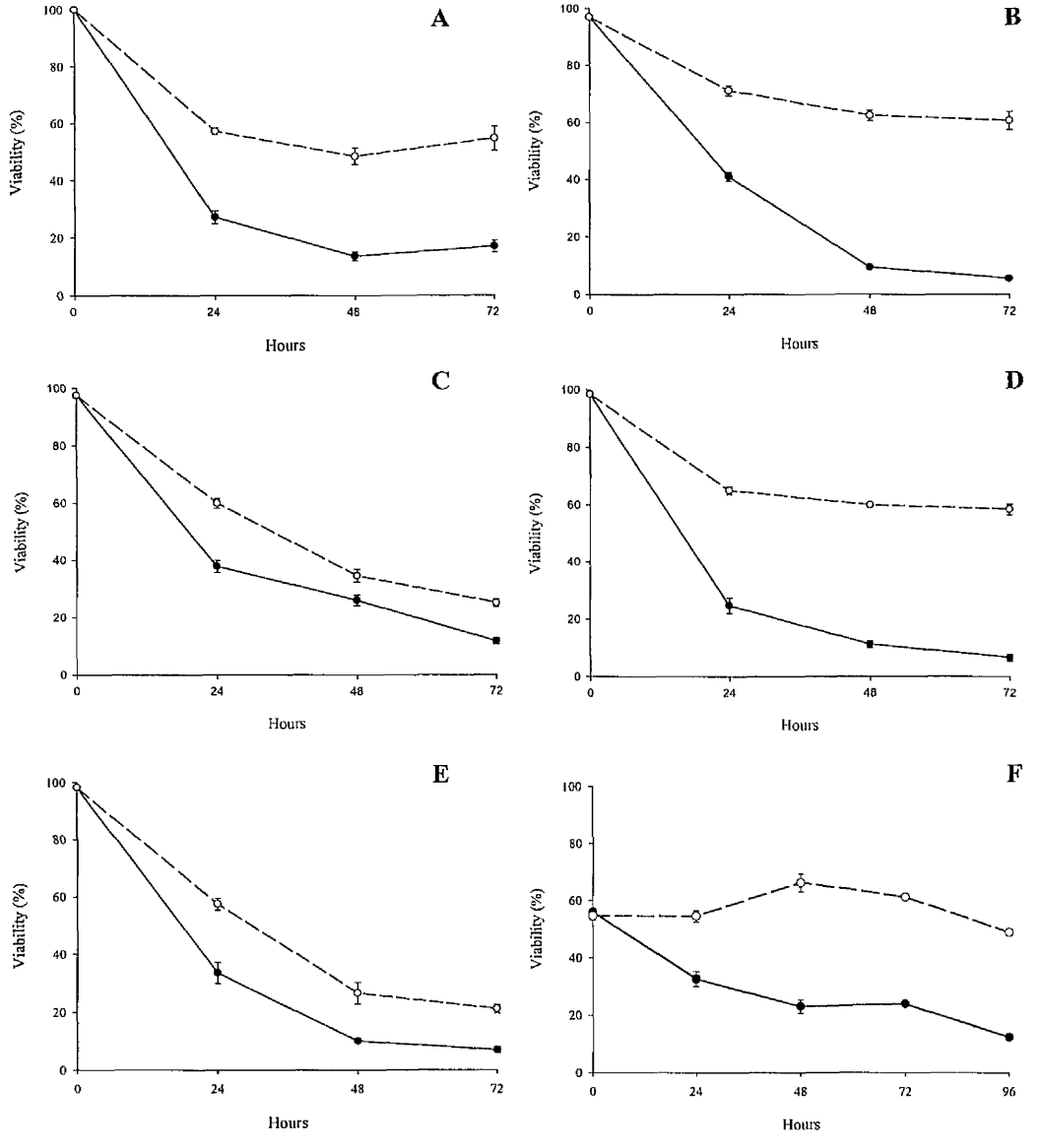


Figure 5.3 *In Vitro* Survival of 4-OHT Treated CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ T Cell Lymphoma Cells

A-E, Survival curves of primary tumour cells, and **F**, an established cell line from CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ animals. Cells were cultured with (solid line, filled circles) or without (broken line, open circles) 4-OHT, and viability assessed by trypan blue exclusion. Results show live:dead counts performed in quadruplicate as percentage live over total cells, at the time-points indicated. Similar results were obtained for a total of 6 tumours and 1 cell line.

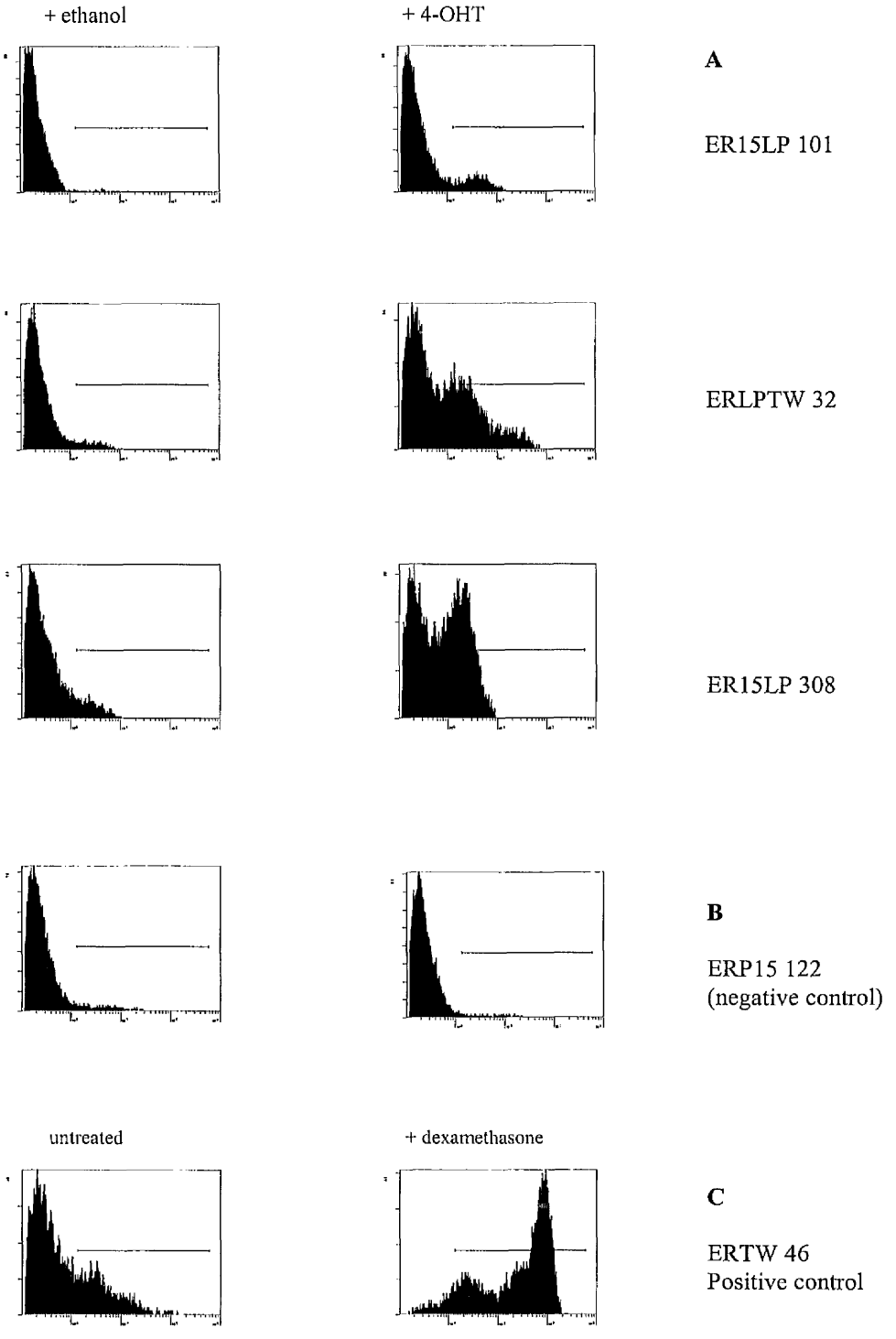


Figure 5.4 Analysis by Annexin V Staining of MYC Induced Apoptosis in CD2-*MYC*ER^{TM} Cell Lines

A, Induction of MYC induced death by 4-OHT in 3 CD2-*MYC*ER^{TM}Fas^{lpr} cell lines. Cell death scored by positive Annexin V staining cells. Cells were stained after 48 hours incubation with 4-OHT. **B,** Control unresponsive cell line (ERP15 122 cells which have lost response to 4-OHT) following 4-OHT treatment. **C,** Cells stained after 48 hours incubation with dexamethasone, as a positive control.

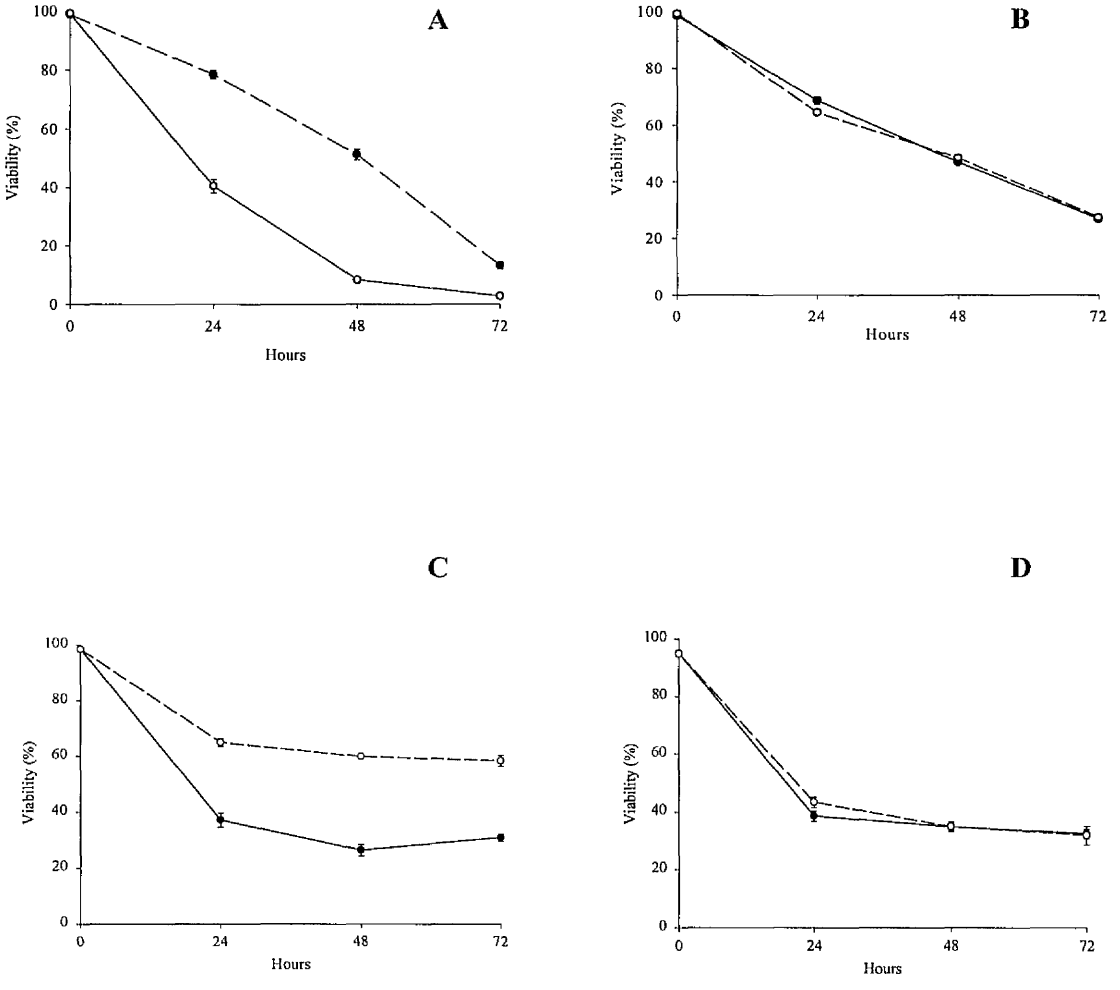


Figure 5.5 Viability of Explanted CD2-*MYC*ER^{TM} lymphoma Cells Following Anti-Fas Treatment

In vitro culture of **A,** control thymocytes, **B,** Fas^{lpr} thymocytes, **C,** CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ lymphoma cells and **D,** CD2-*MYC*ER^{TM}Fas^{lpr} lymphoma cells. Cells were treated with Jo2 antibody (2ng/ml) (solid line, filled circles), or with isotype control (broken line, open circles), and viability was assessed by trypan blue exclusion. Results show live:dead counts expressed as a percentage of live over total, performed in quadruplicate at the indicated time-points. Similar results were obtained for at least six other primary tumours of each genotype.

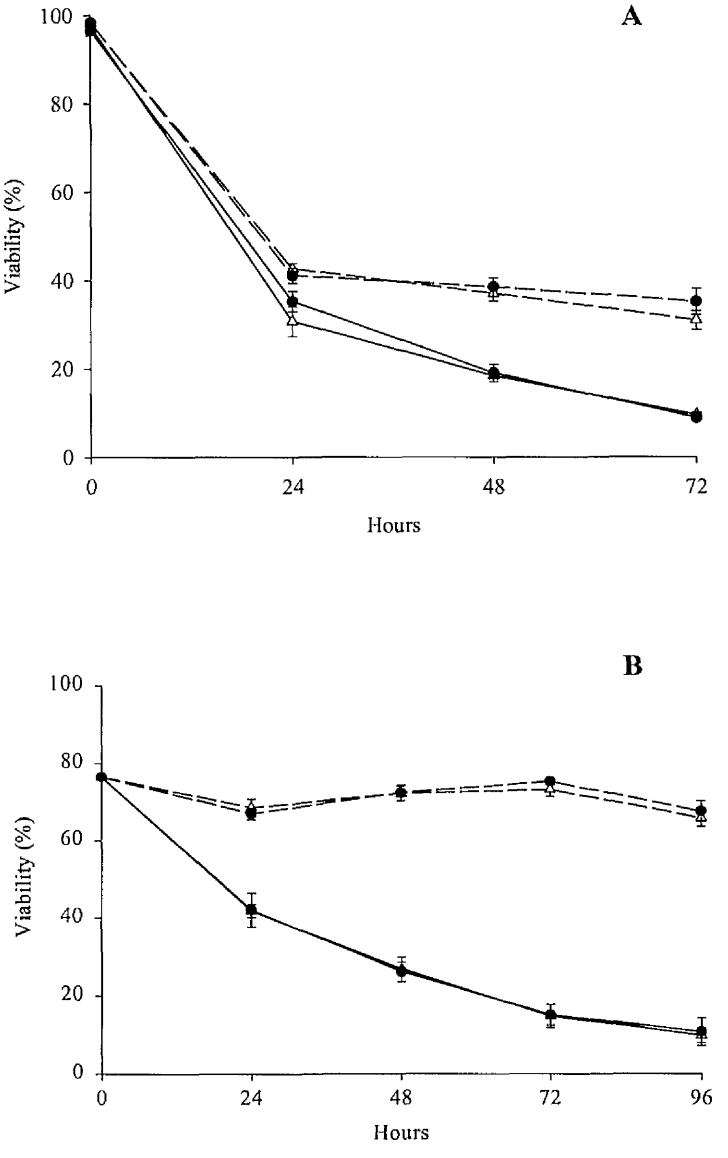


Figure 5.6 Viability of CD2-*MYC*ER^{TM}Fas^{lpr} Lymphoma Cells Following MYC Induction and Anti-Fas Treatment

A, CD2-*MYC*ER^{TM}Fas^{lpr} lymphoma cells and **B,** CD2-*MYC*ER^{TM}Fas^{lpr} lymphoma cell line, incubated in the presence (solid line) or absence (broken line) of 4-OHT, and with Jo2 treatment (filled circles), or isotype control antibody (open triangles). Results show live:dead counts performed in quadruplicate, expressed as percentage live over total, at the indicated time points. Analysis of cell viability is based on trypan blue exclusion.

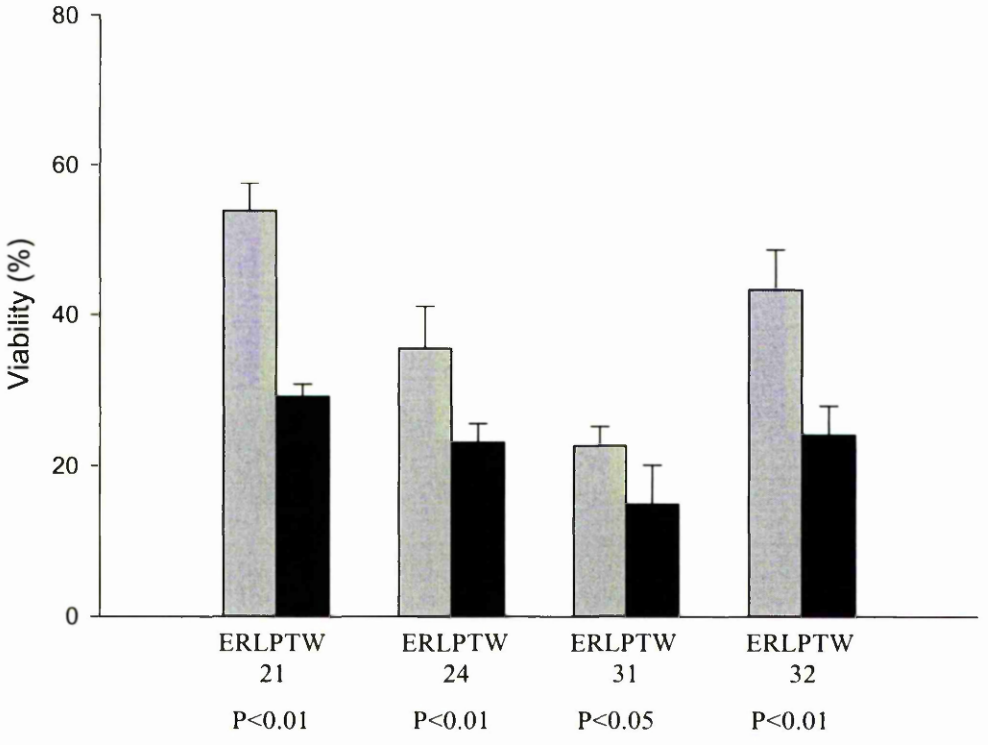


Figure 5.7 Viability of CD2-*MYC*ER^{TM}Fas^{lpr} Lymphoma Cells Explanted from Tamoxifen Treated Animals

A series of lymphoma cells explanted from tamoxifen treated CD2-*MYC*ER^{TM}Fas^{lpr} animals were incubated in the presence (filled bars), or absence (shaded bars) of 4-OHT. Results show live:dead counts expressed as a percentage of live over total, performed in quadruplicate after 72 hours incubation with 4-OHT. Cell viability was assessed by trypan blue exclusion.

5.3 DISCUSSION

5.3.1 The Apoptotic Function of MYC is Not Abrogated During Tumourigenesis

A major area of investigation of the c-*MYC* oncogene is the importance of its apoptotic function in tumourigenesis. It has been suggested that the ability of MYC to induce apoptosis may represent a block in tumour development or progression. Selection may occur for genetic events that help premalignant or malignant cells escape the apoptosis associated with deregulated *MYC*. However, in this study and previously (Blyth *et al.*, 2000), transformed cells from tumours arising in CD2-*MYC*ER^{TM} animals remained highly sensitive to MYC induced apoptosis *in vitro*, indicating that the apoptotic machinery engaged by c-MYC remains intact in these tumours. These results demonstrate that elimination of apoptotic mechanisms associated with *MYC* is not an essential step in the multi-stage process of tumourigenesis. Rather, partial suppression of apoptotic pathways, or upregulation of survival signals may be required in order that MYC induced proliferation outweighs apoptosis during transformation. Nevertheless, apoptosis is still an active process in *MYC* induced tumours. Analysis of tumours arising in CD2-*MYC*ER^{TM} mice showed that elevated MYC activity resulted in increased apoptosis *in vivo* (Blyth *et al.*, 2000), and previous work in B cell lymphomas arising in Eμ-*MYC* transgenic mice showed that areas of extensive *in vivo* apoptosis correspond with increased levels of MYC expression (Prasad *et al.*, 1997) supports this observation.

It is important however that if malignant transformation is to occur, the balance between the growth promoting and apoptotic functions of MYC must favour proliferation. The results in this chapter suggest that in T cells at least, this balance is not achieved by complete inactivation of apoptotic pathways, although certainly apoptotic pathways are likely to be inhibited. One possible way in which overexpressed *MYC* may drive cells into cycle rather than inducing apoptosis is through collaboration with survival signals. Cooperation in tumourigenesis between c-*MYC* and Bcl-2 has already been described (Strasser *et al.*, 1990b). Overexpression of Bcl-2 family members does not block MYC induced apoptosis but

significantly delays commitment to undergo apoptosis (McCarthy *et al.*, 1997; Trudel *et al.*, 1997; Tsuneoka & Mekada, 2000). It is conceivable that in surviving cells where apoptosis is delayed, other genetic lesions will occur which favour proliferation over apoptosis. Ras for instance is analogous with Bcl-2, in that it has been reported to collaborate with *MYC* by increasing cell survival (Kauffmann-Zeh *et al.*, 1997). Ras has been reported to suppress MYC induced apoptosis by activation of the PI3 kinase/Akt survival pathway (Kauffmann-Zeh *et al.*, 1997). Other genes which co-operate with *MYC* during tumourigenesis may have the same properties. Loss of the p53 pathway for example results in significantly accelerated lymphomagenesis in CD2-*MYC* animals (Blyth *et al.*, 1995) however p53 is not required for c-MYC induced apoptosis (Hsu *et al.*, 1995; Blyth *et al.*, 2000). Loss of the p53 pathway may therefore be more important in *MYC* induced tumourigenesis because of the role of p53 in maintaining genomic stability and inhibition of the cell cycle. There may be no requirement for MYC to lose its apoptotic function during tumourigenesis, if the survival of cells undergoing transformation is increased and proliferation occurs unchecked. The mechanisms controlling this are not yet fully understood.

5.3.2 Fas is Not Required for MYC Induced Apoptosis in T Cells

A number of studies have previously shown that expression of FasL in T cells may be regulated by Myc (Wang *et al.*, 1998; Genestier *et al.*, 1999; Brunner *et al.*, 2000). Recently, a binding element for Myc has been identified in the promoter region of FasL, indicating a defined role for Myc in transcription of the FasL gene (Kasibhatla *et al.*, 2000). Previously it had been shown that MYC induced apoptosis in fibroblasts was dependent on the presence of a functional Fas pathway (Hueber *et al.*, 1997). The results of the transgenic studies described in preceding chapters however showed that *MYC* induced T cell lymphomagenesis was not accelerated when the Fas pathway was absent, and there was no oncogenic collaboration between deregulated MYC and loss of Fas *in vivo*. If the Fas pathway were important for MYC to induce apoptosis then it would be expected that loss of this pathway in *MYC* expressing cells would shift the balance towards proliferation rather than death, and facilitate tumour outgrowth. Since lymphoma incidence in CD2-*MYC*ER^{TM} animals was not increased

by loss of Fas, it seemed unlikely that Fas was necessary for MYC induced apoptosis in T cells. This hypothesis was supported by evidence from apoptotic staining of CD2-*MYC* tumours, which revealed no significant difference in the levels of *in vivo* apoptosis in animals homozygous or heterozygous for the Fas^{lpr} mutation (Cameron *et al.*, 2000).

CD2-*MYC*ER^{TM} thymocytes were used to formally investigate the relationship between Fas signalling and MYC induced apoptosis *in vitro*. An apoptotic response was observed in cells in which MYC activity was upregulated, and this response was not blocked by the absence of Fas. It is clear that Fas is not required for MYC induced apoptosis in untransformed T cells. This is in contrast to the findings of Hueber *et al.* (1997), who showed that Fas^{lpr} MEFs were resistant to c-MYC induced apoptosis. That study also showed that MYC induced apoptosis was abrogated in immortalised Swiss 3T3 fibroblasts when the Fas pathway was blocked or absent (Hueber *et al.*, 1997). Investigation of MYC induced apoptosis in explanted tumour cells and cell lines from CD2-*MYC*ER^{TM}Fas^{lpr} animals however, demonstrated a Fas independent mechanism for MYC induced apoptosis in transformed T cells. It may be that there is a fundamental difference between MYC induced apoptosis in T cells and fibroblasts. In all T lymphoma cell cultures tested, upregulation of MYC under conditions of full serum, led to increased cell death. In contrast, MYC induced apoptosis in fibroblasts occurs only in conditions of serum deprivation (Evan *et al.*, 1992), highlighting another difference between the two cell types. It is likely that there is significant variation in the survival signals which are active in thymocytes and fibroblasts. Activation of MYC in fibroblasts may result in less apoptosis than in thymocytes generally, because of higher levels of survival signalling. It may be that cell death in thymocytes is subject to less constraints, because apoptosis of thymocytes is required throughout T cell development, and in down regulation of the immune response. It is also possible that the stimulus for apoptosis affects the pathways recruited by MYC.

Certainly MYC does appear to be able to regulate Fas:FasL interactions. There is strong evidence to suggest that Myc upregulates transcription of FasL in T cells (Wang *et al.*, 1998; Genestier *et al.*, 1999; Brunner *et al.*, 2000; Kasibhatla *et al.*,

2000), and this suggests a role for Fas signalling in MYC induced apoptosis in certain circumstances. There is still no conclusive evidence to directly link *MYC* and the Fas receptor or signalling molecules downstream of Fas. FADD is one of those downstream components of the Fas pathway (Chinnaiyan *et al.*, 1995). Dominant negative FADD is reported to block MYC induced apoptosis in some cell types (Hueber *et al.*, 1997; Juin *et al.*, 1999), however a study using FADD knockout cells concluded that MYC induced apoptosis was independent of the Fas pathway (Yeh *et al.*, 1998). Yet recently, c-MYC activation was reported to act upstream of FADD to induce apoptosis (Rohn *et al.*, 1998). Addition of soluble FasL or expression of a FADD construct induced significant cell death in fibroblasts, but activation of c-MYC in these cells was only able to enhance apoptosis induced by FasL (Rohn *et al.*, 1998).

Further, although MYC is alleged to sensitise cells to apoptosis by inducing cytochrome *c* release, the release of cytochrome *c* does not require Fas signalling (Juin *et al.*, 1999). Fas may simply be more effective at inducing apoptosis once the cytochrome *c* apoptotic cascade has already activated caspase 3. A recent study found that cleavage of the anti-apoptotic factor Bad, by caspase 3, resulted in increased cytotoxicity and acceleration of Fas dependent apoptosis, which would result in positive feedback to maintain the caspase cascade (Condorelli *et al.*, 2001). Other apoptotic signals may be amplified in the same way, making the Fas pathway functionally redundant in some cell types depending on the situation. In addition, the MYC interacting protein Bin-1 has been shown to mediate MYC induced apoptosis through a caspase independent mechanism in a range of human tumour cells (Elliott *et al.*, 2000). These studies confirm that the apoptotic function of MYC is mediated through several different pathways. The importance of Fas and FasL to MYC induced apoptosis will also be context dependent.

5.3.3 The Fas Pathway is Not a Target for Mutation During Tumourigenesis

The results in this chapter indicate that there is no loss of Fas signalling in CD2-*MYC*ER^{TM} induced T lymphoma cells during transformation. Certain studies have however predicted a role for Fas signalling in tumourigenesis (Peng *et al.*, 1996;

Davidson *et al.*, 1998; Newton *et al.*, 2000), and in tumour cell clearance (Schroter *et al.*, 2000). It is possible that some other genetic events occur in these cells which repress apoptotic signalling from Fas and allow proliferation to exceed cell death *in vivo*. For example, overexpression of DcR3, a decoy receptor for FasL which has been detected in several types of tumour (Pitti *et al.*, 1998; Bai *et al.*, 2000; Ohshima *et al.*, 2000), or activation of ERK/MAPK which has recently been shown to override apoptotic signalling from Fas and other death receptors (Tran *et al.*, 2001).

Another possible explanation for the lack of targeting of the Fas pathway in CD2-*MYC*ER^{TM} tumours is that FasL rather than the Fas receptor or any downstream component has been the target of mutagenesis. While there was no loss of heterozygosity at the Fas locus of any of the CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumours tested, and the anti-Fas antibody could induce apoptosis in all of the primary cell cultures tested, loss of FasL in these tumours could not be ruled out. Although MYC induced apoptosis can and does occur independently of Fas in CD2-*MYC*ER^{TM} tumour cells, it is still conceivable that Fas:FasL interactions have some role to play in MYC induced apoptosis, and loss of FasL could occur. The consequences of FasL expression on tumours however, appears to be context dependent. Loss of FasL has been described in tumours before (Ouhtit *et al.*, 2000), however a significant correlation between malignancy and FasL expression has been observed in other studies in which FasL expression on breast and gastric tumour cells has been correlated with metastatic potential, tumour size and malignancy (Mottolese *et al.*, 2000; Liu *et al.*, 2001). This may be due to FasL expression conferring immune privilege on tumour cells, although in some cell types this might be offset by the ability of FasL to induce apoptosis. Induction of FasL expression has been shown in leukaemic T cells following treatment with UV irradiation or cytotoxic drugs, and apoptosis in these cells was reported to be dependent on Fas:FasL interactions (Friesen *et al.*, 1996; Kasibhatla *et al.*, 1998). Another study showed that cytotoxic drug induced apoptosis was Fas independent in T-acute lymphatic leukaemia CEM cells, although an increase in FasL expression was observed following treatment (Villunger *et al.*, 1997). These results were extended by a study showing that apoptosis induced by chemotherapeutic drugs was unaffected in T cells which either lacked Fas or expressed dominant negative FADD (Newton & Strasser, 2000).

Similar findings were generated in B cells (Wieder *et al.*, 2001). Finally, since FasL expression by tumours may well represent a means of immune evasion, it is conceivable that to block Fas mediated apoptosis, Fas or its downstream targets may be more frequent targets for mutagenesis than FasL. In these experiments however, the Fas pathway remained intact in all CD2-*MYC*ER^{TM}Fas$^{lpr/-}$ tumours tested.

The consequences of Fas ligation may be conditional. Two distinct cell types have already been described which use different pathways downstream of Fas. In type I cells, for example most T cells, DISC formation and activation of caspases 8 and 3 is rapid and marked, whereas in type II cells such as B cells and hepatocytes, DISC formation is strongly reduced and caspase activation occurs only after loss of mitochondrial membrane potential (Scaffidi *et al.*, 1998). Overexpression of Bcl-2 blocks caspase activation and apoptosis in type II cells, but has no effect on caspase activation in type I cells (Scaffidi *et al.*, 1998). The functional significance of Fas signalling to a cell may therefore depend on which pathway is activated. Further, recent reports have intimated a role for Fas:FasL interactions in proliferation in T cells (Alderson *et al.*, 1993; Newton *et al.*, 1998; Suzuki & Fink, 1998; Zhang *et al.*, 1998; Zornig *et al.*, 1998; Strasser & Newton, 1999). This aspect of Fas:FasL signalling will be discussed in Chapter 8. Nevertheless, if Fas and its ligand are capable of transducing a proliferative signal, that would have significant bearing on its ability to suppress tumourigenesis through its apoptotic function.

CHAPTER 6

MYC INDUCED APOPTOSIS IN THE COMBINED ABSENCE OF BOTH FAS AND P53 PATHWAYS

6.1 INTRODUCTION

6.1.1 The Role of p53 in *MYC* Induced Apoptosis

Mutations which cause deregulation of c-*MYC* and loss of p53 are among the most common genetic lesions identified in tumours, and are frequently found together in a number of tumour types (Farrell *et al.*, 1991; Gaidano *et al.*, 1991; Smith *et al.*, 1993; Farrugia *et al.*, 1994; Inagaki *et al.*, 1994). Deregulation of c-*MYC* and loss of p53 have been shown to represent synergistic events in T cell lymphomagenesis (Blyth *et al.*, 1995; Elson *et al.*, 1995). The role of p53 in apoptosis induced by many stimuli has been well documented, however its role in c-MYC induced apoptosis is still unclear. Some studies have proposed that p53 is required for c-MYC induced apoptosis (Hermeking & Eick, 1994; Wagner *et al.*, 1994), while others have argued against dependence on p53 (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Amanullah *et al.*, 2000; Blyth *et al.*, 2000). In T cell lymphomas induced by the CD2-*MYC*ER^{TM} transgene in *Trp53* null mice, MYC induced apoptosis can occur, indicating that in T cells at least, MYC induced apoptosis can be p53 independent (Blyth *et al.*, 2000). To date, the potential of MYC induced apoptosis in the absence of functional p53 and Fas pathways has not been determined.

6.1.2 The Relationship Between Fas and p53

Several groups have examined the relationship between Fas and p53 signalling. Because p53 is a transcription factor, the possibility that p53 might upregulate transcription of Fas or FasL as part of its apoptotic function has been explored. An initial study implicated the Fas receptor gene as a transcriptional target gene of p53 in a variety of human cancer cell lines (Owen-Schaub *et al.*, 1995), while a later study

argued that p53 transiently increased surface Fas expression in human vascular smooth muscle cells by promoting transport of Fas from the Golgi complex to the cell surface (Bennett *et al.*, 1998). Later reports however have suggested that Fas and p53 function independently to induce apoptosis. High levels of Fas mediated apoptosis have been found in human leukaemic B and T cells which express mutant p53 (Zhou *et al.*, 1998), and conversely, levels of p53 induced apoptosis in thymocytes from *gld* mice, lacking FasL are apparently normal (O'Connor *et al.*, 2000). Most of the recent evidence indicates that Fas and p53 represent independent apoptotic pathways.

6.1.3 Experimental Aims

In order for deregulation of *MYC* to result in tumourigenesis, additional genetic events must occur before cells become transformed. The ability of MYC to induce apoptosis in addition to proliferation suggests that events that resulted in abrogation of apoptotic function would be strongly selected for. The results presented thus far indicate that MYC induced apoptosis is independent of Fas signalling. Previous studies have also revealed that MYC induced apoptosis can occur in the absence of functional p53 at least in epithelial and T cell systems (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Blyth *et al.*, 2000). What is not clear to date is whether MYC can induce apoptosis when both these death pathways are lost. The aims of these experiments were to investigate whether MYC induced apoptosis was dependent on one or other of these major death pathways, and if not, to explore how apoptosis was mediated in the absence of these signals.

6.2 Results

6.2.1 Crossing of CD2-*MYC*ER^{TM}Fas^{lpr} Animals onto a *Trp53* Null Background

The results described so far show that the Fas pathway is not required for MYC induced apoptosis in T cells. These are reminiscent of previous studies which have

shown that MYC induced apoptosis is not dependent on the presence of functional p53 (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Blyth *et al.*, 2000). To establish whether either the Fas or p53 death signals were indispensable for MYC induced apoptosis when the other was absent, breeding was co-ordinated to generate CD2-*MYC*ER^{TM} animals which were homozygous for the Fas^{lpr} mutation and *Trp53* null. A summary of the breeding is shown in Table 6.1. In the event of one or other of these pathways being essential for MYC induced apoptosis, it might be expected that CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53* null mice would have a very short lifespan, or that the combination of genotypes might even result in embryonic lethality. This has been previously reported in mice which carried both the Eμ-*MYC* and Eμ-*pim-1* oncogenic transgenes, and which developed pre-B cell leukaemia *in utero* (Verbeek *et al.*, 1991). To take account of this possibility, the number of pups in each litter were monitored daily, and any offspring lost during the neonatal period were genotyped. Animals were monitored over a twelve-month period, and sacrificed when clinical signs became apparent. As in previous cohorts, animals developed thymic lymphoma and/or lymphoproliferative disorders associated with the Fas^{lpr} genotype. Additionally, mice null for the *Trp53* gene, but not carrying the CD2-*MYC*ER^{TM} transgene, developed a variety of tumour types of both haemopoietic (including thymic lymphoma) and non-haemopoietic origin.

6.2.2 Failure to Generate CD2-*MYC*ER^{TM}15 Animals on a *Trp53* Null Background

As Table 6.1 shows, the expected proportion of CD2-*MYC*ER^{TM} offspring in the F3 generation that were homozygous for the Fas^{lpr} mutation and null for *Trp53* was 25% (or 6/7 from 26). No animals of this genotype were ever generated however. Although the absence of these mice may suggest embryonic lethality, litter sizes were apparently normal, and an unexpected pattern was also observed with other genotypes. CD2-*MYC*ER^{TM}-/-Fas^{lpr}/*Trp53*+/- animals should have accounted for 25% of the F3 offspring generated, however no mice with this genotype were evident. Further examination of the pattern of genotypes throughout the breeding revealed that all *Trp53* null mice were negative for the CD2-*MYC*ER^{TM} transgene, and all CD2-*MYC*ER^{TM}+/+ animals generated were wild-type for *Trp53*. The results

suggested genetic linkage between the CD2-*MYC*ER^{TM} transgene and the wild-type *Trp53* allele. It would appear that the transgene inserted into or close to the *Trp53* allele during creation of this CD2-*MYC*ER^{TM} line, or that at some stage of the breeding programme, recombination events have resulted in the transgene being linked to the *Trp53* allele. Further attempts by others to generate CD2-*MYC*ER^{TM} mice on a *Trp53* null background reinforced this theory. Only 5 mice of this genotype were generated in a cohort of 207 animals in which 25% were expected to be positive for the CD2-*MYC*ER^{TM} transgene and null for *Trp53* (Blyth *et al.*, unpublished results).

6.2.3 Evidence that MYC May Induce Apoptosis in the Absence of Both Pathways

Although no CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- animals were born, 20 CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*+/- mice were generated. 10 of these developed lymphoproliferative disease with an average latency of 100 (+/- 21.6) days, while 10 developed thymic lymphoma with an average latency of 103 (+/- 12.6) days. This latency was not significantly different from the latency of tumours in untreated CD2-*MYC*ER^{TM}Fas^{lpr} animals. To investigate the status of the remaining wild-type *Trp53* allele in thymic lymphomas in these mice, DNA was prepared from frozen tumour samples and Southern hybridisation analysis performed. The remaining wild-type *Trp53* allele was of significant interest in these mice, because if one or other of the Fas and p53 death pathways is required for MYC induced apoptosis there would be a considerable growth advantage for cells that lose both pathways. It was also of interest because if there was selective advantage for cells to lose *Trp53*, then the transgene might also be lost, depending on how closely linked these two were. For that reason, Southern hybridisation analysis was also carried out to verify the presence of the transgene in those tumours tested. In 7 out of 7 tumours tested however, *Trp53* heterozygosity was maintained at least by this analysis, and not unexpectedly, the transgene remained present and intact in all 7 tumours (Figure 6.1).

This analysis does not account for point mutations that may inactivate *Trp53*. However attempts to establish cell lines from these tumours were without success,

and if p53 function had been lost in these tumours, it would be expected that cell lines could be easily established. It has been observed previously that CD2-*MYC* tumour cells have a poor rate of establishment, but on a *Trp53* null background nearly all CD2-*MYC* tumour cells established as a cell lines (Blyth *et al.*, 1995). Primary cells from one of these tumours were cultured *in vitro*, and despite the high background rate of death, significantly increased cell death was induced following treatment with 4-OHT ($P<0.01$ at 48 hours) indicating that MYC induced apoptosis was not lost during tumourigenesis. Although, complete loss of the wild-type *Trp53* allele in these tumours is unlikely to be selected for, given that the linkage to the transgene may also mean that the transgene would be lost, it might be expected that point mutations would occur to inactivate *Trp53* in these tumours. The failure to establish any cell lines from these tumours, hints that selection for loss of both Fas and *Trp53* may not occur during *MYC* induced lymphomagenesis, suggesting perhaps that MYC may induce apoptosis through other signals as well.

6.2.4 Generation of CD2-*MYC*ER^{TM}Fas^{lpr} Animals on a *Trp53* Null Background (CD2-*MYC*ER^{TM}2 transgene)

Because of the failure to generate CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- animals in the previous breeding programme, a new programme was set up using a different CD2-*MYC*ER^{TM} transgenic line. The new line of CD2-*MYC*ER^{TM} animals were known to have lower transgene copy number (Blyth *et al.*, 2000). It was expected however that in the absence of p53, and on MRL strain, a proportion of these mice would develop thymic lymphoma, since *MYC* induced lymphomagenesis is accelerated on a *Trp53* null background (Blyth *et al.*, 1995), and in MRL strain animals (previously described in Chapter 3). As usual, animals were monitored over a twelve-month period, and sacrificed when clinical signs were present. Transgenic animals homozygous for the Fas^{lpr} mutation developed either thymic lymphoma or lymphoproliferative disease, whereas animals heterozygous for *lpr* developed only thymic lymphoma. On a *Trp53* null background, mice developed a range of tumour types in addition to thymic lymphoma or lymphoproliferative disease.

6.2.5 Lymphomagenesis in CD2-*MYC*ER^{TM}2Fas^{lpr}/*Trp53*-/- Mice

CD2-*MYC*ER^{TM}2 mice homozygous for the *lpr* mutation and null for *Trp53* were generated in expected numbers (Table 6.2A), indicating that these mice were viable and the combination of these genetic lesions was not lethal prenatally. There were no obvious developmental abnormalities in these mice, however it was noted that in Fas^{lpr}/*Trp53*-/- mice, independent of whether they carried the transgene, there was a general trend of accelerated lymphadenopathy. These results extend earlier findings that the onset of lymphoproliferative disease in Fas^{lpr} animals is significantly accelerated by the *Trp53* null genotype (Johnston *et al.*, unpublished results). At post-mortem, the pathological signs of lymphadenopathy and splenomegaly appeared more severe in these mice, suggesting that lymphadenopathy may be accelerated in these animals but other clinical symptoms associated with autoimmunity, such as pruritis, may not be affected by the *Trp53* genotype. Acceleration of lymphoproliferation was also previously reported in *pim-1* transgenic mice on an Fas^{lpr} background (Moroy *et al.*, 1993).

The number of mice of each genotype, and the incidence of thymic lymphoma, lymphadenopathy or other tumour type is shown in Table 6.2B. The results of this experiment were inconclusive since the ongoing removal of mice from the cohort due to development of lymphadenopathy caused the incidence of thymic lymphomas in these mice to be very low. Additionally, only mice null for *Trp53* ever developed thymic lymphoma over the course of this experiment. Without induction, transgene expression was not high enough to induce spontaneous lymphoma, at least in this small cohort. Therefore it was not possible to detect significant differences in tumour incidence and latency between CD2-*MYC*ER^{TM}Fas^{lpr} mice null or heterozygous for *Trp53*, although trends can be observed from this cohort.

Table 6.1 CD2-*MYC*ER^{TM}15 x Fas^{lpr} x *Trp53* Null Cross

***MYC*ER^{TM}+/- Fas^{lpr} *Trp53*+/+**	**x**	***MYC*ER^{TM}-/- Fas^{lpr} *Trp53*-/-**	
F1 Offspring		**Expected**	**Actual**
*MYC*ER^{TM}+/- Fas^{lpr}	*Trp53*+/-	½	4/9
*MYC*ER^{TM}-/- Fas^{lpr}	*Trp53*+/-	½	5/9
***MYC*ER^{TM}+/- Fas^{lpr} *Trp53*+/-**	**x**	***MYC*ER^{TM}+/- Fas^{lpr} *Trp53*+/-**	
F2 Offspring			
*MYC*ER^{TM}+/+ Fas^{lpr}	*Trp53*+/+	$^{1}/_{16}$	2/11
*MYC*ER^{TM}+/+ Fas^{lpr}	*Trp53*+/-	$^{2}/_{16}$	0
*MYC*ER^{TM}+/+ Fas^{lpr}	*Trp53*-/-	$^{1}/_{16}$	0
*MYC*ER^{TM}+/- Fas^{lpr}	*Trp53*+/+	$^{2}/_{16}$	0
*MYC*ER^{TM}+/- Fas^{lpr}	*Trp53*+/-	$^{4}/_{16}$	4/11
*MYC*ER^{TM}+/- Fas^{lpr}	*Trp53*-/-	$^{2}/_{16}$	0
*MYC*ER^{TM}-/- Fas^{lpr}	*Trp53*+/+	$^{1}/_{16}$	0
*MYC*ER^{TM}-/- Fas^{lpr}	*Trp53*+/-	$^{2}/_{16}$	0
*MYC*ER^{TM}-/- Fas^{lpr}	*Trp53*-/-	$^{1}/_{16}$	5/11
***MYC*ER^{TM}+/- Fas^{lpr} *Trp53*+/-**	**x**	***MYC*ER^{TM}-/- Fas^{lpr} *Trp53*-/-**	
F3 Offspring			
*MYC*ER^{TM}+/- Fas^{lpr}	*Trp53*+/-	¼	12/26
*MYC*ER^{TM}+/- Fas^{lpr}	*Trp53*-/-	¼	0
*MYC*ER^{TM}-/- Fas^{lpr}	*Trp53*+/-	¼	0
*MYC*ER^{TM}-/- Fas^{lpr}	*Trp53*-/-	¼	14/26

Table 6.2A CD2-*MYC*ER^{TM}2 x Fas^{lpr} x *Trp53* Null Cross

***MYC*ER^{TM}2+/-**	**Fas$^{+/+}$**	***Trp53*+/+**	**x**	***MYC*ER^{TM}2-/-**	**Fas^{lpr}**	***Trp53*-/-**

F1 Offspring			**Expected**	**Actual**
*MYC*ER^{TM}2+/-	Fas$^{lpr/-}$	*Trp53*+/-	½	6/8
*MYC*ER^{TM}2-/-	Fas$^{lpr/-}$	*Trp53*+/-	½	2/8

***MYC*ER^{TM}2+/-**	**Fas$^{lpr/-}$**	***Trp53*+/-**	**x**	***MYC*ER^{TM}2-/-**	**Fas^{lpr}**	***Trp53*-/-**

F2 Offspring

*MYC*ER^{TM}2+/-	Fas$^{lpr/-}$	*Trp53*+/-	1/8	9/66
*MYC*ER^{TM}2-/-	Fas$^{lpr/-}$	*Trp53*+/-	1/8	4/66
*MYC*ER^{TM}2+/-	Fas^{lpr}	*Trp53*+/-	1/8	8/66
*MYC*ER^{TM}2-/-	Fas^{lpr}	*Trp53*+/-	1/8	8/66
*MYC*ER^{TM}2+/-	Fas$^{lpr/-}$	*Trp53*-/-	1/8	8/66
*MYC*ER^{TM}2-/-	Fas$^{lpr/-}$	*Trp53*-/-	1/8	11/66
*MYC*ER^{TM}2+/-	Fas^{lpr}	*Trp53*-/-	1/8	8/66
*MYC*ER^{TM}2-/-	Fas^{lpr}	*Trp53*-/-	1/8	10/66

***MYC*ER^{TM}2+/-**	**Fas^{lpr}**	***Trp53*-/-**	**x**	***MYC*ER^{TM}2-/-**	**Fas^{lpr}**	***Trp53*-/-**

F3 Offspring

*MYC*ER^{TM}2+/-	Fas^{lpr}	*Trp53*-/-	½	8/18
*MYC*ER^{TM}2-/-	Fas^{lpr}	*Trp53*-/-	½	10/18

Table 6.2B Incidence of Lymphoma in CD2-*MYC*ER^{TM}2Fas^{lpr}/*Trp53*-/- Mice

genotype	total animals	cause of death			latency range of thymic lymphoma
		T.L	*lpr*	tumour/ lymphoma*	
CD2-*MYC*ER^{TM}2Fas$^{lpr/-}$ *Trp53*+/-	16	0	1	2	
CD2-*MYC*ER^{TM}2Fas^{lpr} *Trp53*+/-	10	0	9	0	
CD2-*MYC*ER^{TM}2Fas$^{lpr/-}$ *Trp53*-/-	11	5	0	4	75-209 days
CD2-*MYC*ER^{TM}2Fas^{lpr} *Trp53*-/-	16	1	11	4	164 days
Fas$^{lpr/-}$ *Trp53*+/-	5	0	0	1	
Fas^{lpr} *Trp53*+/-	13	0	13	0	
Fas$^{lpr/-}$ *Trp53*-/-	13	3	0	6	106-220 days
Fas^{lpr} *Trp53*-/-	20	2	16	1	84-129 days

* other than thymic lymphoma

Lymphomagenesis did occur in mice homozygous and heterozygous for the Fas^{lpr} mutation, allowing comparison of the kinetics of lymphomagenesis between these two groups. In CD2-*MYC*ER^{TM}Fas$^{lpr/-}$/*Trp53*-/- mice, the incidence of thymic lymphoma was 45% (5 from 11) compared to 6% (1 from 16) in the CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- group. These results are somewhat misleading however, since many of the mice in the latter group had to be sacrificed due to development of lymphadenopathy. The latency of tumour development was not accelerated in CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- mice compared to CD2-*MYC*ER^{TM}Fas$^{lpr/-}$/*Trp53*-/- animals. The average latency of tumours in the *lpr* heterozygous group was 132 days, and the only CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- tumour to develop had a latency of 164 days. Although results cannot be based on one tumour, it is reasonable to suggest that latency was not increased in this group. The average lifespan of CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- mice sacrificed due to development of lymphadenopathy was 139 days. If lymphomagenesis was accelerated in these mice, then it might be expected that a greater proportion of the cohort could develop thymic lymphoma with a reduced latency, before developing lymphoproliferative disease.

6.2.6 Loss of p53 in CD2-*MYC*ER^{TM}Fas^{lpr} Cell Lines

The ultimate goal of the previous experiments was to generate CD2-*MYC*ER^{TM} cell lines which lacked both Fas and p53 apoptotic pathways by genotype. These efforts were hindered for a number of reasons. Using these cell lines it would have been possible to investigate whether MYC induced apoptosis could still occur in the absence of these two pathways. We have noticed however, that establishment of explanted tumour cells in long term culture is correlated with loss of p53 function. For the purpose of exploring MYC induced apoptosis in the absence of both Fas and p53 pathways, CD2-*MYC*ER^{TM}15Fas^{lpr} and CD2-*MYC*ER^{TM}15/*Trp53*-/- cell lines were studied since there may be selective pressure for cell lines to lose death pathways in order to survive *in vitro* (Cheng & Haas, 1990; Mazars *et al.*, 1992).

A number of cell lines were tested for their response to γ-irradiation with the aim of assessing the integrity of their p53 pathway. Although this assay is not conclusive, a significant increase in the level of apoptosis in an exposed cell line indicates the presence of a functional p53 pathway, and conversely, unaltered levels of apoptosis strongly suggest loss of functional p53 (Lowe *et al.*, 1993). Figure 6.2 shows the panel of cell lines that were irradiated. Following irradiation, irradiated and untreated cells were stained with Annexin V and the percentage of positive staining apoptotic cells analysed by flow cytometry (Figures 6.2 & 6.3).

Out of three CD2-*MYC*ER^{TM}Fas^{lpr} cell lines tested in this way, both ERLPTW 32 and ER15LP 101 showed no increase in cell death following irradiation, strongly suggesting that functional p53 had been lost in these lines. The third CD2-*MYC*ER^{TM}Fas^{lpr} line, ER15LP 308 retained its response to irradiation, implying that p53 was still functional in these cells. Significantly increased cell death was also seen in ER15LP 52, a Fas^{lpr} heterozygous cell line which had shown an apoptotic response to irradiation previously, and which was included as a positive control for functional p53. A CD2-*MYC*ER^{TM}/*Trp53*-/- cell line, ERP15 122, was also included as a negative control, and as expected there was no increase in cell death following irradiation of this cell line, confirming the absence of p53. Representative examples of the flow cytometric analysis of these cell lines are shown in Figure 6.3, together

with dexamethasone treated cells which act as a positive control for staining of apoptotic cells.

To confirm that wild-type p53 function was lost in these cells, immunoblotting analysis was carried out. Protein extracts were prepared from cell lines before and after irradiation, and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nylon membranes by Western transfer and detected by incubation with antibodies, followed by electro-chemical luminescence. Figure 6.4A shows detection of p53 protein in these irradiated cell lines. Detection of actin is shown to control for any inconsistency of gel loading (Figure 6.4B).

Since p53 promotes the transcription of its own negative regulator, MDM2, mutations which cause p53 function to be lost actually result in increased cellular levels of the mutant protein, since negative regulation no longer occurs. The anti-p53 antibody used in these experiments is designed to detect both normal and mutant p53, so if p53 function has been lost by mutation in any of these cell lines, high levels of expression may be observed by this method. If the protein is not functional then no difference should be observed between p53 levels in irradiated and unirradiated cells, since the mutant p53 should no longer be able to initiate negative feedback on p53 expression. The results acquired parallel those obtained from studies of apoptosis in these cell lines following irradiation. ERLPTW 32 cells which were not induced to die by irradiation show high expression of p53 by Western analysis, before and after irradiation, while in ER15LP 101 cells p53 protein was not detected in either irradiated or unirradiated cells. ER15LP 308 cells however, which showed increased death following irradiation, expressed low levels of p53 before irradiation, which increased following irradiation suggesting the presence of functional p53 which is activated in response to DNA damage. The data available make it reasonable to conclude that p53 function has been abrogated in the two CD2-*MYC*ER^{TM}Fas^{lpr} cell lines, ERLPTW 32 and ER15LP 101.

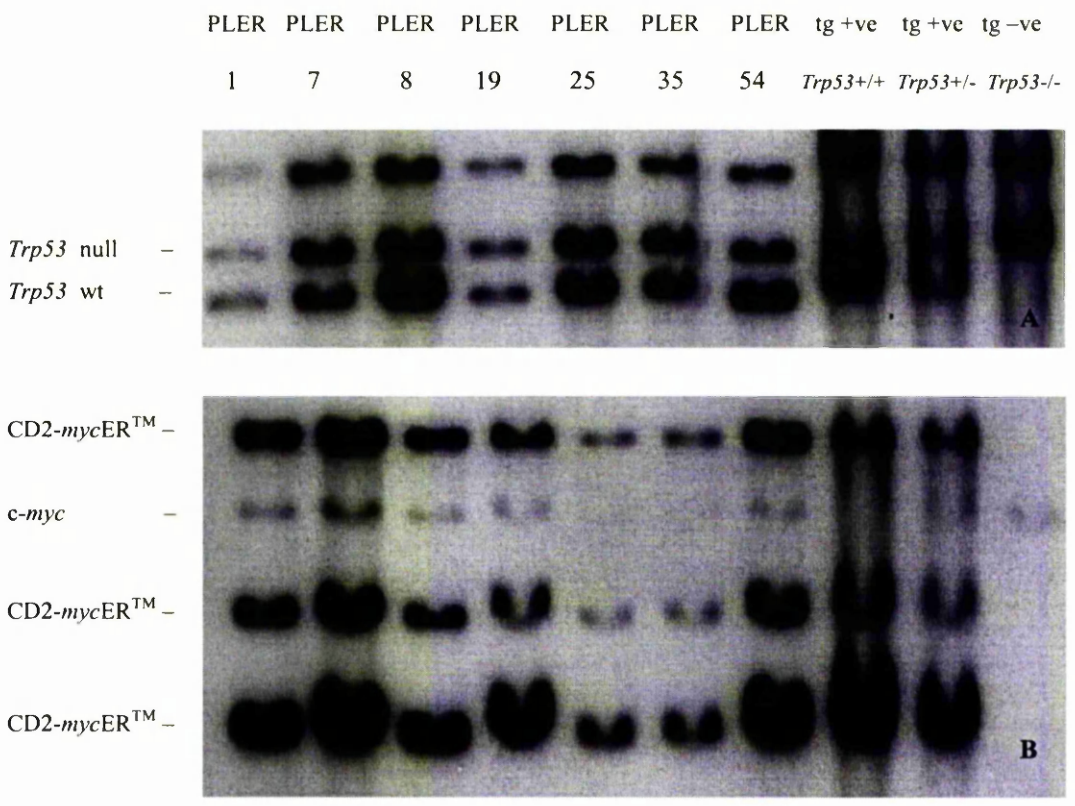


Figure 6.1 Analysis of *Trp53* and Transgene Status in CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*+/- Tumours

A, Southern hybridisation analysis was carried out to investigate the status of the remaining *Trp53* wild-type allele in tumours from CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*+/- mice The figure shows analysis of 7 tumours, and *Trp53* controls as indicated. **B,** Southern hybridisation analysis was also used to identify presence of the CD2-*MYC*ER^{TM} transgene in these tumours. Again 7 tumours are shown, and controls for the transgene as indicated.

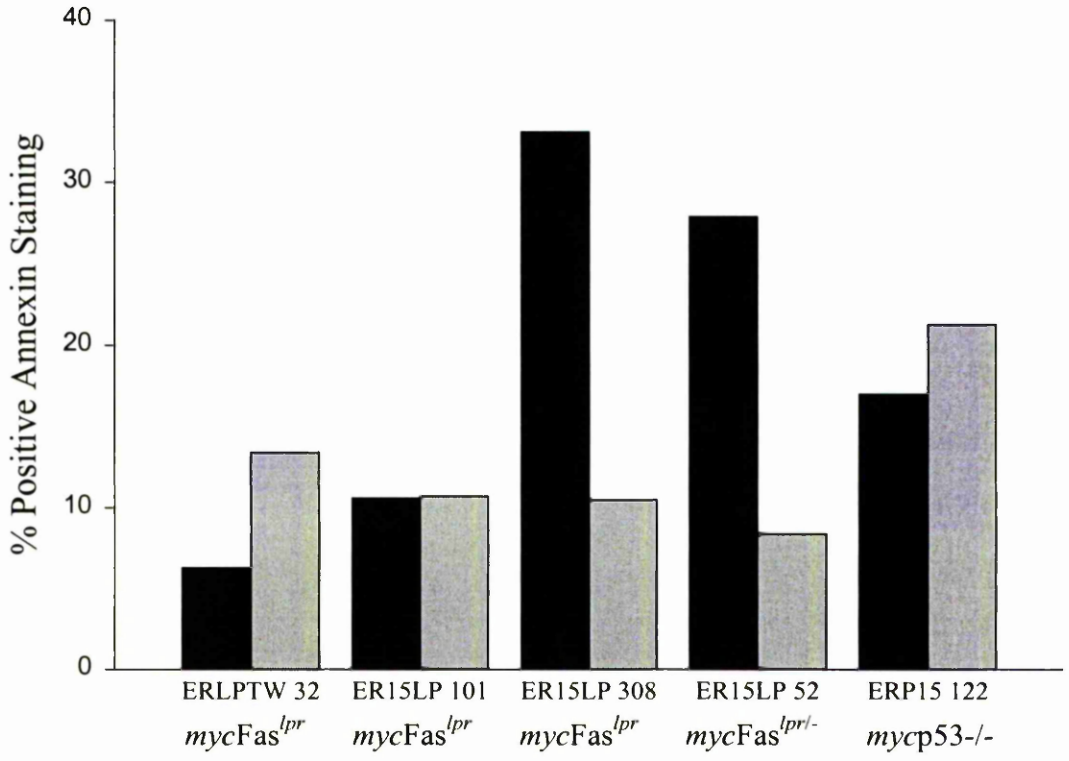


Figure 6.2 Response to Irradiation in CD2-*MYC*ER™ Cell Lines

CD2-*MYC*ER™ cell lines were stained with Annexin V antibody before and after exposure to γ-radiation. Cells were exposed to 5 Grays γ-irradiation from a Cobalt source. Positive staining cells were scored as apoptotic. Figure shows five cell lines tested before irradiation (grey bars) and after (black bars), with genotypes indicated. (*MYC* = CD2-*MYC*ER™15). Figure 6.3 shows flow cytometry profiles.

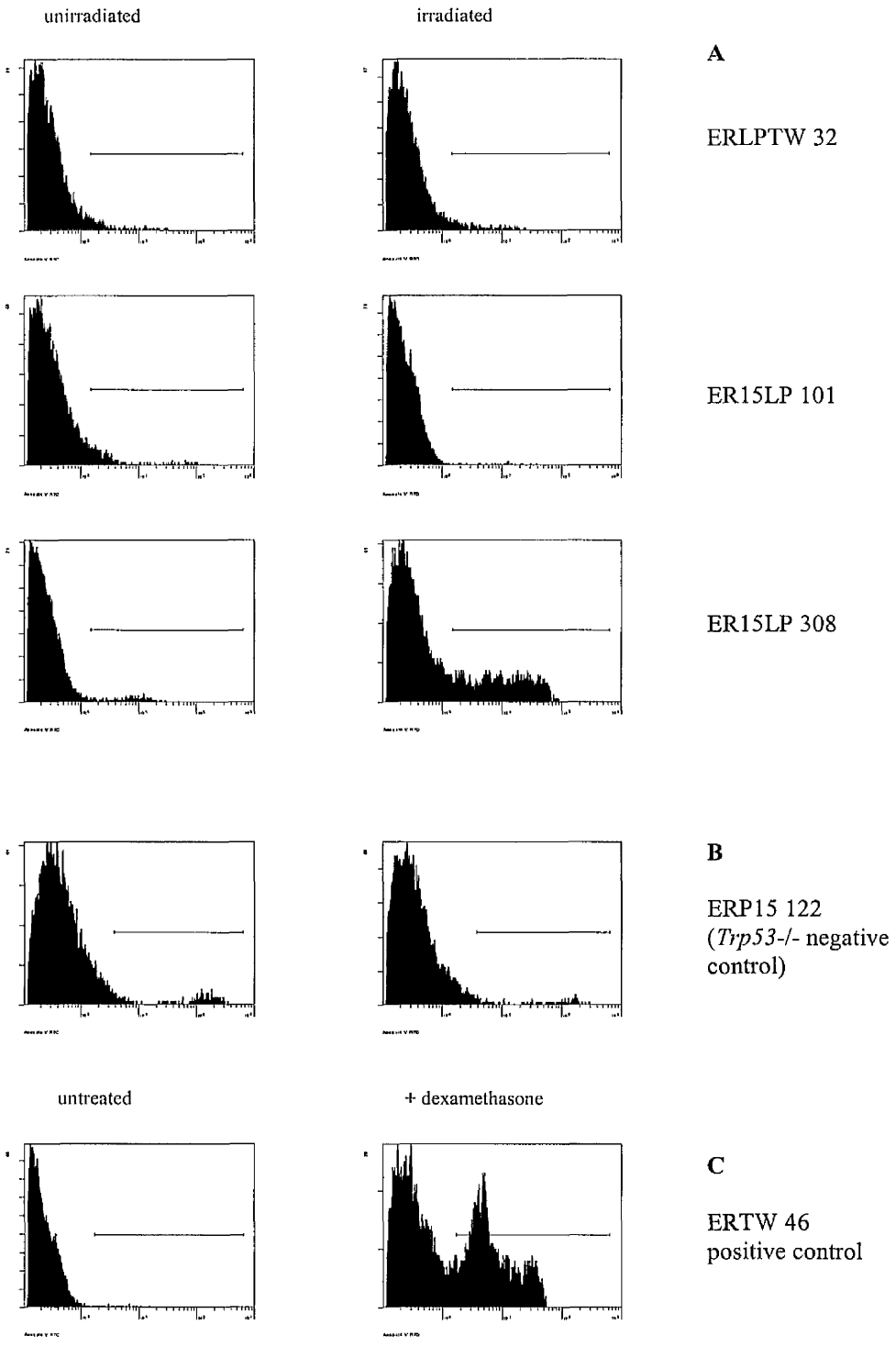


Figure 6.3 Analysis by Annexin V Staining and Flow Cytometry of Apoptosis in Irradiated CD2-*MYC*ER^{TM} Cell Lines

A, Profile of induction of apoptosis before and after γ-irradiation in CD2-*MYC*ER^{TM}Fas^{lpr} cell lines. Cell death scored by positive Annexin V staining cells. **B,** Control *Trp53*-/- cell line before and after irradiation. **C,** Positive control for Annexin staining. CD2-*MYC*ER^{TM}15 tumour cell line (ERTW 46) following 24-hour incubation with dexamethasone.

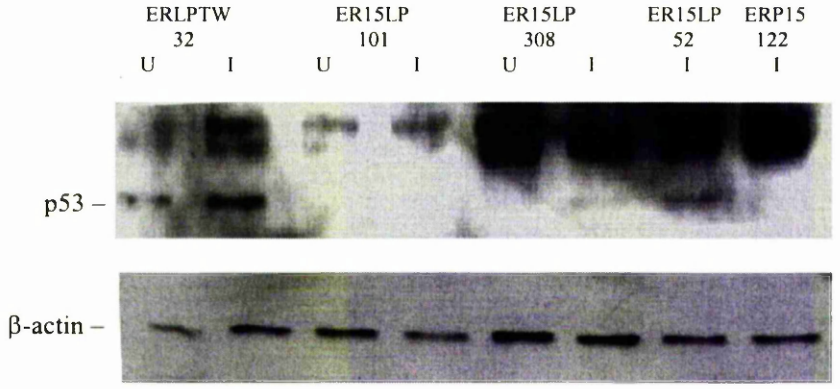


Figure 6.4 Detection of p53 in CD2-*MYC*ER^{TM}15Fas^{lpr} Cell Lines by Immunoblotting

A, Detection of p53 protein in the cell lines indicated, before and after γ-irradiation. Antibody against normal and mutant p53 was used for detection. **B,** Detection of actin protein in the cell lines indicated (U = γ-unirradiated, I = γ-irradiated). Cells were exposed to 5 Grays γ-irradiation from a Cobalt source.

6.2.7 MYC Induced Apoptosis in CD2-*MYC*ER^{TM}Fas^{lpr} Cell Lines in which Functional p53 is Lost

For practical purposes these cell lines (ERLPTW 32 and ER15LP 101) were taken to represent CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- cells, and were used to investigate MYC induced apoptosis in the absence of functional Fas and p53 pathways. Both cell lines were cultured with and without 4-OHT *in vitro*, and cell viability assessed after 48 hours, by trypan blue exclusion (Figure 6.5). Induction of MYC activity elicited a significant increase in cell death in both these cell lines indicating that the apoptotic function of MYC was not blocked. These data argue strongly, that MYC is able to induce apoptosis in the absence of both Fas and p53.

6.2.8 Fas Induced Apoptosis in CD2-*MYC*ER^{TM}/*Trp53*-/- Cell Lines in which MYC's Apoptotic Function is Lost

Indirect evidence to suggest that MYC may use other pathways to mediate its apoptotic function was generated from a CD2-*MYC*ER^{TM} cell line null for *Trp53*. It was noted that after prolonged time in culture, a small number of CD2-*MYC*ER^{TM} cell lines lost their apoptotic response to 4-OHT treatment. This was not unexpected since there is constant selective pressure *in vitro* for cells to lose apoptotic signals. In one such cell line, ERP15 122, which was *Trp53* null by genotype, MYC's apoptotic function was lost over a period of time in culture, and subsequently, a second sub-clone of this cell line also became resistant to MYC induced apoptosis in culture (Figure 6.6A). If the Fas pathway was important in mediating MYC induced apoptosis, it might be expected that in cell lines in which the apoptotic function of MYC was lost this may be due to loss of Fas. Both of these 'new' 4-OHT resistant cell lines were cultured in the presence of Jo2 antibody or with isotype control antibody. Viability was assessed 48 hours after addition of antibody (Figure 6.6B). In both lines, significantly increased death was seen in response to Jo2, indicating that the Fas pathway was still intact in these cells. Despite selective pressure to block apoptosis in these cells, and the absence of p53, the Fas pathway was not a target for loss. These findings support the results of earlier *in vitro* experiments which have shown that MYC does not depend on Fas and p53 alone to induce apoptosis. The

results suggest that MYC may induce apoptosis by a Fas and p53 independent mechanism, and it may be this pathway which is lost in cell lines which no longer undergo MYC induced apoptosis.

6.2.9 Protection of a CD2-*MYC*ER^{TM}Fas^{lpr} Cell Line from MYC Induced Apoptosis by α-FasL Antibody

Further studies were performed *in vitro* on CD2-*MYC*ER^{TM}Fas^{lpr} cell lines lacking p53 function to determine what pathway *MYC* may be activating or sensitising these cells to, in the absence of Fas and p53. Cells were cultured *in vitro* in the presence of 4-OHT, and the effects of different apoptotic inhibitors were tested. In the first instance ER15LP 101 cells, which are CD2-*MYC*ER^{TM}Fas^{lpr} cells lacking functional p53, were cultured with 4-OHT in the presence of inhibitors of caspase 8 and 3. Both of these caspases are involved in apoptotic pathways, however caspase 8 activity has only been reported in death induced by TNF-R family members, while caspase 3 activity is more widespread and occurs downstream of the so-called 'initiator' caspases like caspase 8. Cells were also incubated with the anti-FasL antibody, MFL-3, primarily to test whether FasL has any function in these cells, despite the absence of Fas, either through activation of unknown death receptors, or by reverse signalling in the cells which express it. Cells were cultured for 72 hours, and the viability of those cells assessed by trypan blue exclusion.

In this cell line, caspase 8 inhibitor did not allow significantly increased survival following MYC induction, while caspase 3 inhibitor offered slight but significant protection from MYC induced apoptosis compared to control untreated cells, $P<0.05$ (Figure 6.7). The graph shows however that the level of background death was also slightly reduced in cells treated with caspase 3 inhibitor ($P<0.05$) compared with untreated cells, indicating that the protective effects of this inhibitor may not be specific to MYC induced apoptosis. Further, there was no significant difference between the protection against MYC induced apoptosis offered by inhibition of caspase 3 or of caspase 8. Intriguingly however, although increased MYC activity could still induce considerable apoptosis, cells treated with the anti-FasL antibody,

MFL-3 showed a small but significant level of protection compared to cells treated with isotype-matched control antibody ($P<0.01$).

Because of the leaky nature of the *lpr* mutation, it was necessary to exclude the possibility that the effect of the anti-FasL antibody on this cell line was merely due to very low levels of surface Fas, although the anti-Fas antibody, Jo2 had previously been shown to have no effect on this cell line (see Figure 5.6A). Cells were incubated in the presence or absence of anti-FasL antibody again, and MYC activity induced by addition of 4-OHT. On this occasion however, Jo2 was included at high concentration (10ng/ml) to replace blocked FasL, and activate any Fas receptor on the surface of these cells. Figure 6.8 shows that Jo2 neither induced death, nor blocked the protection afforded by the anti-FasL antibody on this cell line. There was significantly increased survival in cells treated with either anti-FasL alone ($P<0.05$), or with anti-FasL and excess Jo2 ($P<0.01$), compared with isotype control antibody treated cells. These data suggest that the effect of the anti-FasL antibody is independent of Fas.

To confirm that the anti-FasL antibody was in fact mediating its effect directly through FasL, cells were treated with cyclosporin A, and their viability assessed following MYC upregulation. Cyclosporin A (CsA) has been reported to inhibit transcription of FasL but not Fas, while having no effect on Fas signalling (Brunner *et al.*, 1996). When treated with CsA, these cells were protected from MYC induced apoptosis at a level comparable to that seen with anti-FasL antibody, $P<0.01$ (Figure 6.9). This finding implies that the protection afforded by anti-FasL is due to a direct effect on FasL, which is Fas independent. It was not possible to say whether this is due to a block in an apoptotic interaction between FasL and an unknown receptor, or whether anti-FasL antibody is having a direct effect on FasL, since reverse signalling through FasL has been reported previously (Suzuki & Fink, 1998; Suzuki *et al.*, 2000).

In order to investigate further the apoptotic pathway affected by anti-FasL, cells were incubated with or without caspase inhibitors in the presence or absence of anti-FasL. Viability was assessed following MYC upregulation. Figure 6.10 shows that anti-

FasL had a significant ($P<0.01$) protective effect against MYC induced apoptosis, as seen in previous experiments. When the cells were treated with anti-FasL along with caspase 8 inhibitor, the level of apoptosis induced by MYC was significantly reduced still further ($P<0.05$) suggesting that whichever pathway anti-FasL inhibits, it is not dependent on caspase 8, although the levels to which caspase inhibitors and anti-FasL inhibit apoptosis may affect the results. In contrast however, inhibiting caspase 3 in the presence of anti-FasL had no additive effect, indicating that these pathways do overlap (Figure 6.10). The data suggest that anti-FasL is inhibiting an apoptotic signal rather than stimulating proliferation through FasL, and that caspase 3 is involved in this pathway.

The consequences of incubation with anti-FasL antibody have been tested in 5 other cell lines, including CD2-*MYC*ER^{TM}Fas^{lpr} lines, however this protective effect in response to MYC upregulation has not been seen in any other cell line tested so far. A representative cell line is shown in Figure 6.11A. One explanation for this may be that in other cell lines this is a death pathway that is masked by other more fundamental pathways, and is only revealed in this cell line because of the lack of functional Fas and p53 signalling. Anti-FasL treatment had no protective effect against MYC induced apoptosis in ERLPTW 32 cells however, although they also lack functional Fas and p53 (Figure 6.11B), suggesting that this may be an over-simplification. Analysis of other death receptors in this cell line may be necessary to elucidate the mechanism by which anti-FasL protects against MYC induced apoptosis.

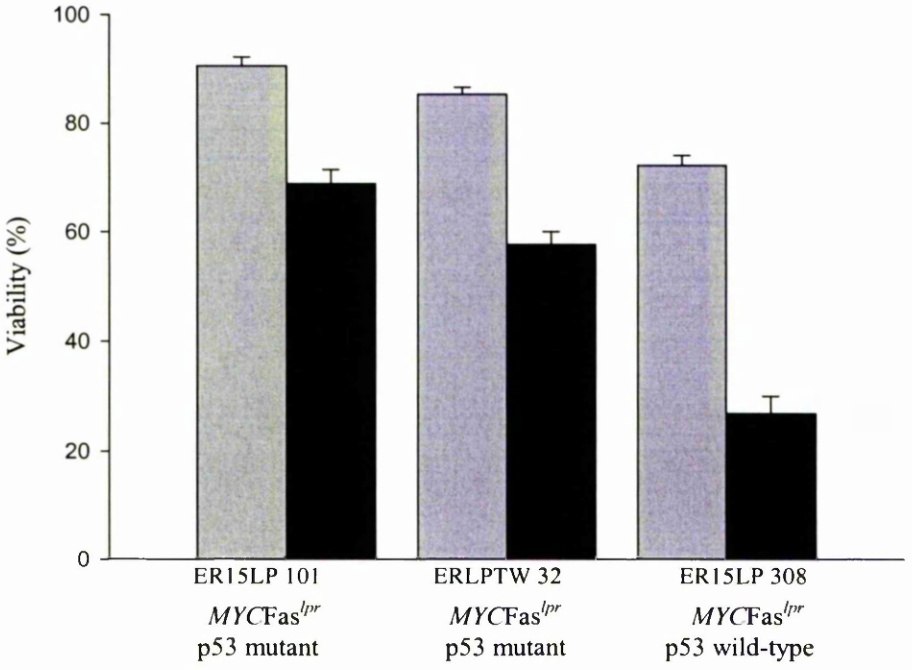


Figure 6.5 Viability of CD2-*MYC*ER^{TM}15Fas^{lpr} Cell Lines Following 4-OHT Treatment

CD2-*MYC*ER^{TM} cell lines were incubated with (filled bars) or without (shaded bars) the addition of 4-OHT and viability was assessed after 48 hours by trypan blue exclusion. Results show the average of live:dead counts performed in quadruplicate expressed as a percentage of live over total. The cell line genotype and p53 status are indicated.

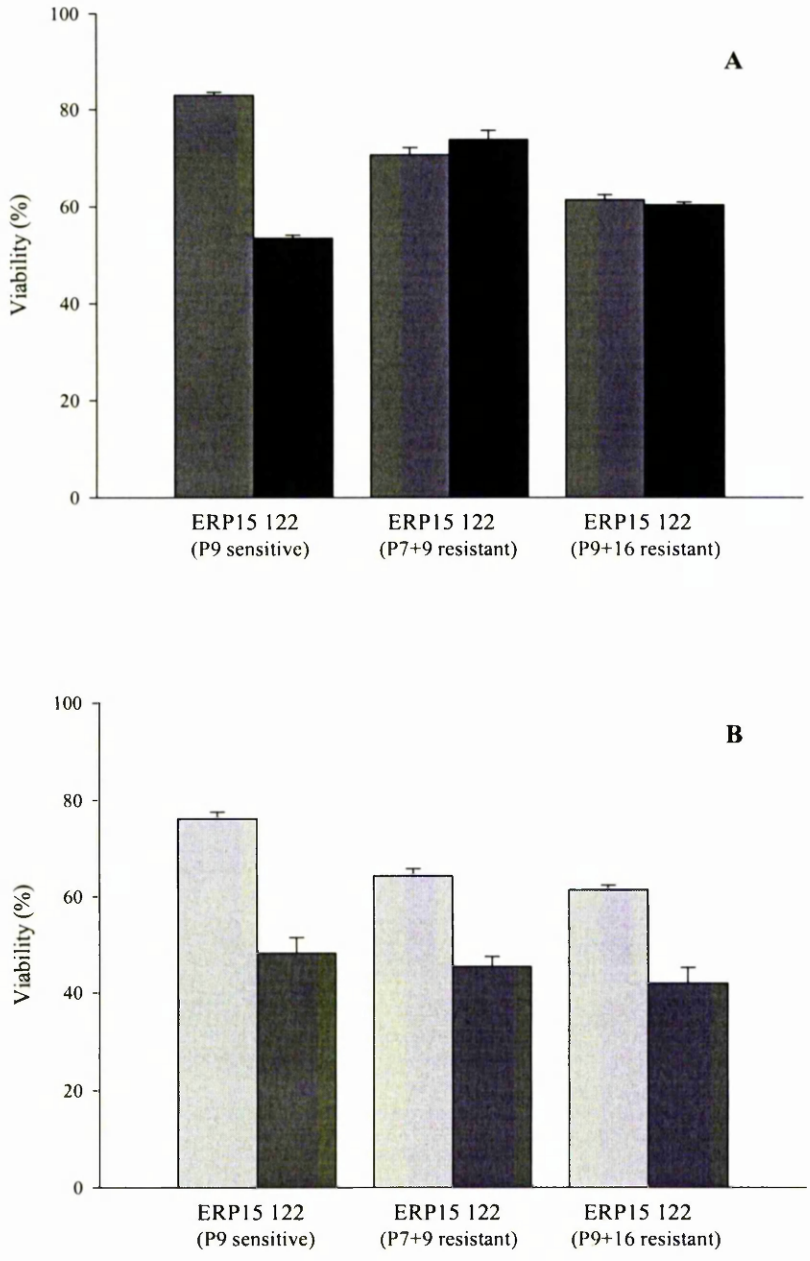


Figure 6.6 Viability of a 4-OHT Resistant CD2-*MYC*ER^{TM}/*Trp53*-/- Cell Line Following Anti-Fas Treatment

A, A CD2-*MYC*ER^{TM}/*Trp53*-/- cell line (ERP15 122) before and after resistance to 4-OHT was incubated with (black bars) or without (dark-grey bars) the addition of 4-OHT, or **B,** with Jo2 (mid-grey bars) or isotype control antibody (light grey bars). Viability was assessed after 48 hours by trypan blue exclusion. Results show the average of live:dead counts performed in quadruplicate, expressed as a percentage of live over total.

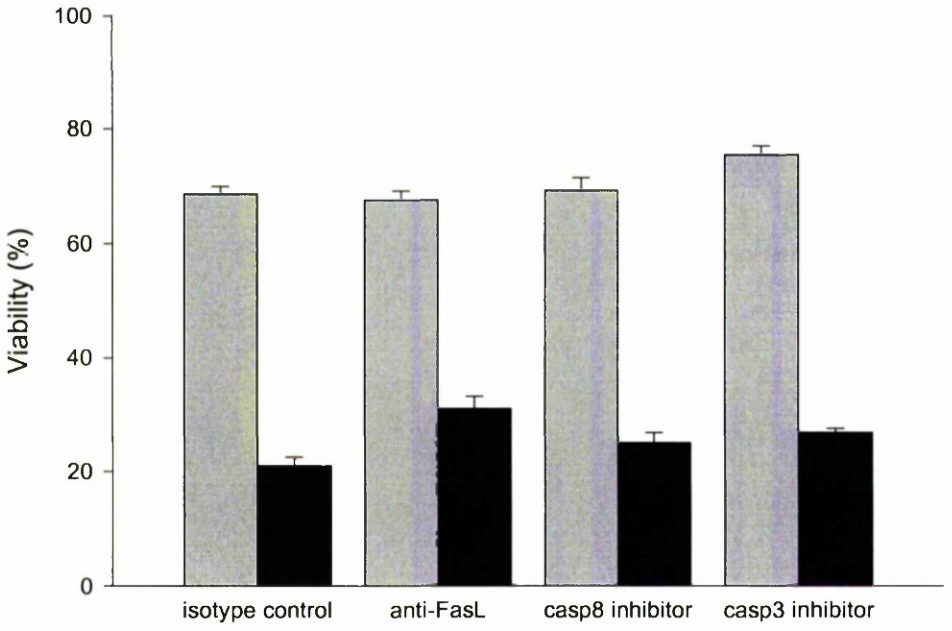


Figure 6.7 Viability of 4-OHT Treated CD2-*MYC*ER^{TM}Fas^{lpr} Cells Following Treatment with Anti-FasL Antibody and Caspase Inhibitors

ER15LP 101 cells were cultured in the presence (filled bars) or absence (shaded bars) of 4-OHT and treated with anti-FasL antibody or inhibitors of caspases 8 and 3. Results shown represent live:dead counts performed in quadruplicate, expressed as a percentage of live over total. Viability was assessed after 72 hours by trypan blue exclusion. Significant protection against *MYC* induced apoptosis was observed with anti-FasL treatment ($P<0.01$), and caspase 3 inhibitor ($P<0.05$).

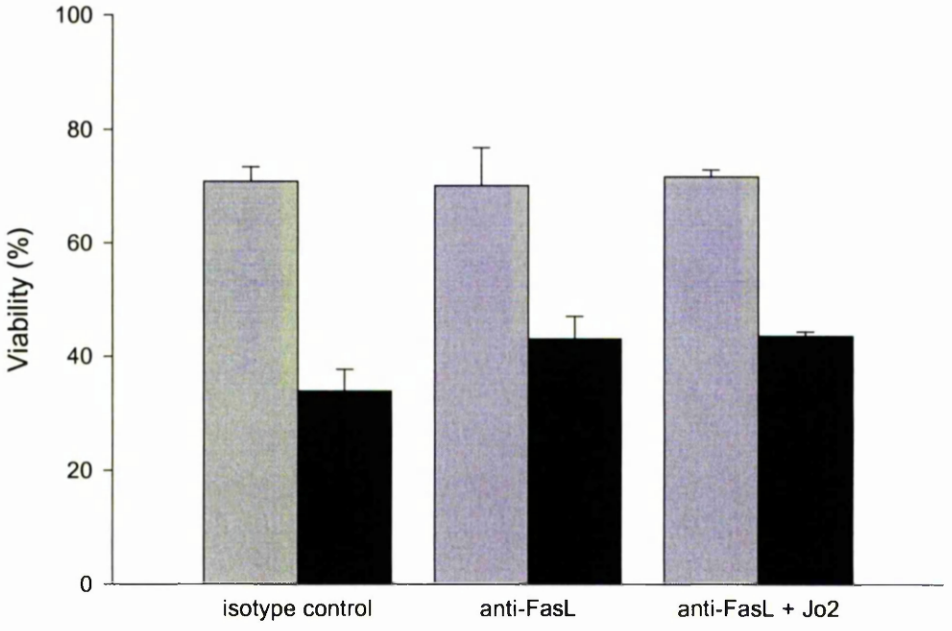


Figure 6.8 Viability of 4-OHT Treated ER15LP 101 Cells Following Incubation with Anti-FasL and Anti-Fas Antibodies

ER15LP 101 cells were incubated in the presence (filled bars) or absence (shaded bars) of 4-OHT, and treated with anti-FasL +/- Jo2 antibody, or with isotype control antibody as indicated. Results show live:dead counts performed in quadruplicate, expressed as a percentage of live over total. Viability was assessed by trypan blue exclusion after 72 hours. Significant protection against *MYC* induced apoptosis was observed with anti-FasL treatment, with ($P<0.01$), or without addition of Jo2 ($P<0.05$).

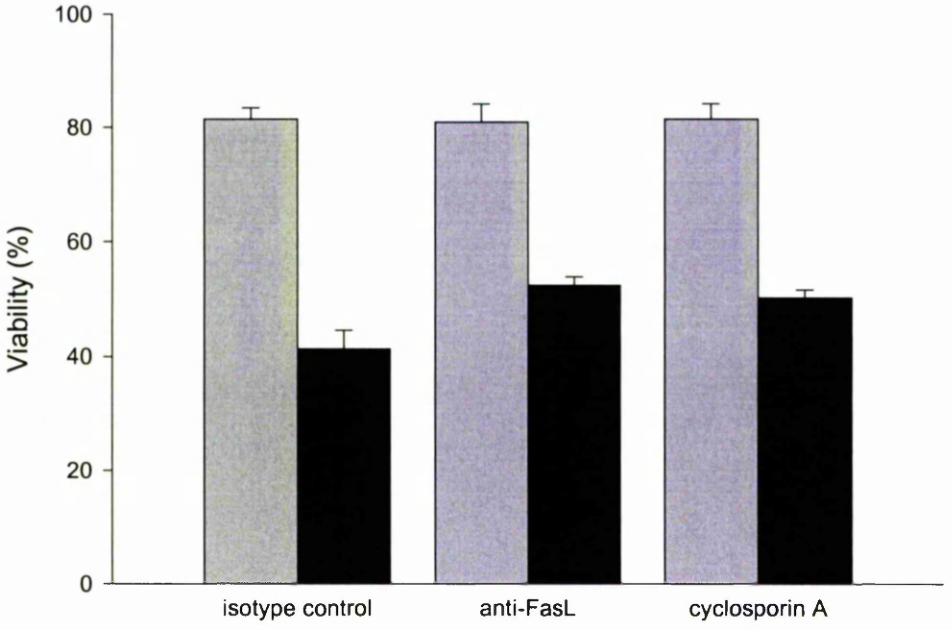


Figure 6.9 Viability of 4-OHT Treated CD2-*MYC*ER^{TM}Fas^{lpr} Cells Following Incubation with Cyclosporin A

ER15LP 101 cells were incubated in the presence (filled bars) or absence (shaded bars) of 4-OHT, and treated with anti-FasL, cyclosporin A or isotype control as indicated. Results of live:dead counts performed in quadruplicate are shown, expressed as a percentage of live over total. Viability was assessed after 72 hours culture, by trypan blue exclusion. Significant protection against *MYC* induced apoptosis was observed with anti-FasL treatment and cyclosporin A ($P<0.01$).

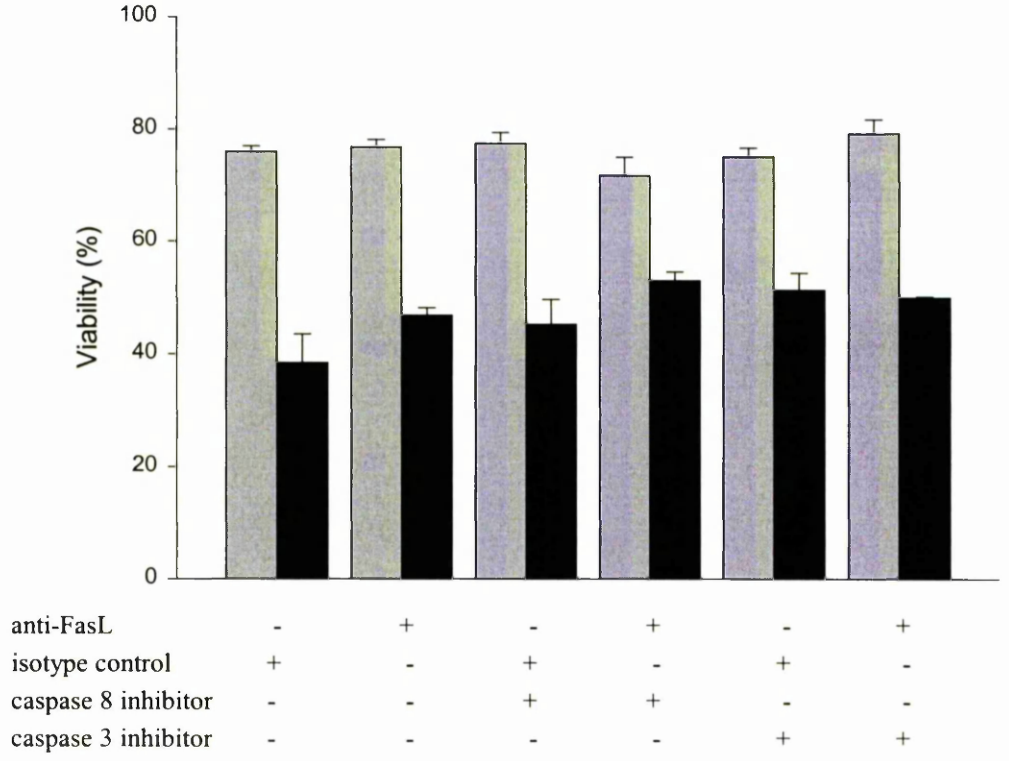


Figure 6.10 Viability of CD2-*MYC*ER^{TM}Fas^{lpr} Cells Following Treatment with Anti-FasL and Caspase Inhibitors

ER15LP 101 cells were cultured in the presence (filled bars) or absence (shaded bars) of 4-OHT, and treated with anti-FasL or isotype control with or without inhibitors of caspase 8 and 3 as indicated. Results shown represent live:dead counts performed in quadruplicate after 72 hours expressed as percentage live over total. Viability was assessed by trypan blue exclusion. Significant protection against *MYC* induced apoptosis was observed with anti-FasL treatment and caspase 3 inhibitor, ($P<0.01$), and addition of caspase 8 inhibitor significantly increased anti-FasL mediated protection from *MYC* induced apoptosis ($P<0.05$).

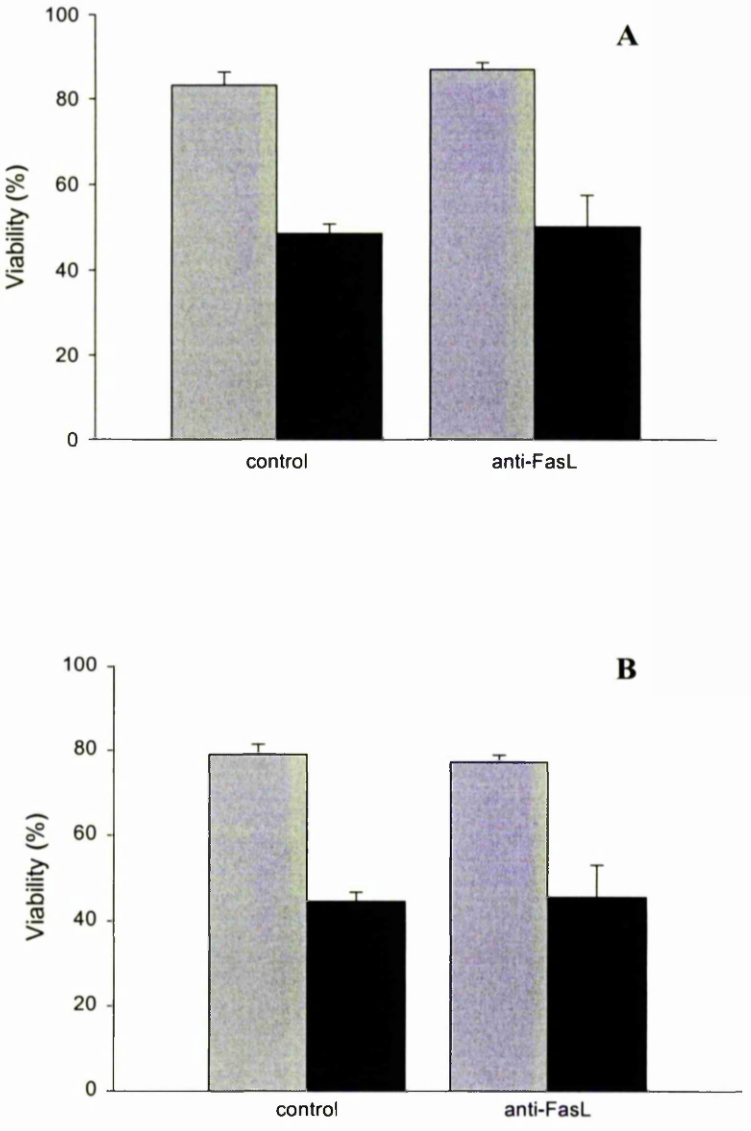


Figure 6.11 Viability of CD2-*MYC*ER^{TM}Cells Following Treatment with 4-OHT and Anti-FasL

A, ERP15 92 (CD2-*MYC*ER^{TM}15/*Trp53*-/-) cells and **B,** ERLPTW 32 (CD2-*MYC*ER^{TM}15Fas^{lpr}) cells were treated with anti-FasL in the presence (black bars) or absence (grey bars) of 4-OHT. Results shown represent averages of live:dead counts performed in quadruplicate expressed as a percentage of live over total. Viability was assessed after 72 hours culture by trypan blue exclusion.

6.3 DISCUSSION

6.3.1 MYC Induced Apoptosis in the Absence of Both Fas and p53 Signalling

It has previously been reported elsewhere that MYC induced apoptosis requires functional Fas or p53 pathways, in certain cell types (Wang *et al.*, 1993a; Hermeking & Eick, 1994; Hueber *et al.*, 1997). Certainly strong evidence exists to indicate that MYC is able to utilise both of these pathways to induce apoptosis. Other evidence suggests however, that neither pathway is essential for MYC induced apoptosis. MYC induced apoptosis has been demonstrated on a *Trp53* null background, indicating that p53 is dispensable for MYC induced apoptosis (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Blyth *et al.*, 2000). Similarly, the data in Chapter 5 suggest that MYC induced apoptosis occurs in the absence of Fas. The finding in this chapter that MYC induced apoptosis can occur in the absence of both pathways suggests the presence of additional mechanisms which mediate the apoptotic function of MYC.

In tumours arising in CD2-*MYC*ER^{TM}Fas^{lpr} animals heterozygous for *Trp53*, it appears that the remaining wild-type *Trp53* allele was not a target for mutagenesis during lymphoma development or progression, since no loss of heterozygosity was observed at the *Trp53* locus in tumours arising in these mice. In these circumstances however, this is perhaps not unexpected, since the transgene and the wild-type *Trp53* allele are linked, and thus to lose *Trp53*, tumours may also have to lose the transgene. Felsher and Bishop (1999) have previously shown that tumours driven by upregulation of a MYC transgene regress following inactivation of the transgene. Nevertheless it might be expected that there could be selection for transformed cells that had developed a mechanism to lose the wild-type p53 allele without loss of the transgene, however this did not occur. Further, the failure of cells explanted from these tumours to establish in culture suggests that perhaps p53 function may be maintained in these cells. Sequence analysis of the tumour DNA however would be required to test exhaustively the integrity of the remaining wild-type p53 allele.

The possibility of functional overlap between the Fas and p53 pathways has been suggested since p53 has been reported to upregulate surface Fas expression both by transcriptional activation (Owen-Schaub *et al.*, 1995), and by transport of Fas from cytoplasmic stores to the cell surface (Bennett *et al.*, 1998). Further, protection from Fas mediated apoptosis in tumour cell lines in which p53 was inactivated has been reported (Maecker *et al.*, 2000). However, later studies in human cancer cells showed that while p53 may initiate death partially through Fas signalling, it is also able to mediate apoptosis through alternative signalling pathways (Fukazawa *et al.*, 1999; Hara *et al.*, 2000).

Comparison of lymphomagenesis in CD2-*MYC*ER^{TM} transgenic animals either heterozygous or null for either Fas or *Trp53* was essential to investigate the importance of these two apoptotic effectors. Although useful data generated *in vitro* from the CD2-*MYC*ER2^{TM} model was limited, the survival of mice in this cohort was very informative. The incidence of thymic lymphoma was not increased in untreated CD2-*MYC*ER^{TM}Fas^{lpr}/*Trp53*-/- mice compared to the same mice heterozygous for the Fas^{lpr} mutation. In addition, the latency of thymic lymphoma was not significantly altered by the status of the Fas gene in CD2-*MYC*ER^{TM} animals null for *Trp53*. Although the number of thymic lymphomas in this cohort is small, the results imply that even when p53 is absent, loss of Fas does not accelerate *MYC* induced lymphomagenesis.

Perhaps the most convincing results to suggest that MYC induced apoptosis can occur independently of Fas and p53 are those generated in tumour cell lines from CD2-*MYC*ER^{TM}Fas^{lpr} mice. Although these mice were genotypically wild-type for *Trp53*, loss of functional p53 has been demonstrated in two CD2-*MYC*ER^{TM}Fas^{lpr} cell lines. Loss of p53 was not surprising since the selective advantage for cell lines to lose p53 function in culture is high (Cheng & Haas, 1990; Mazars *et al.*, 1992). Crucially, MYC upregulation in these cell lines still resulted in significantly increased apoptosis. This finding suggests that MYC induced apoptosis can occur in the absence of both Fas and p53 pathways, and implies that additional mechanisms exist by which MYC can mediate its apoptotic function.

If the ability of MYC to induce cell death *in vitro* represents a survival disadvantage, it might be selected against. Indeed this does occur, however it is not accompanied by loss of the Fas pathway. In a CD2-*MYC*ER^{TM} cell line, null for *Trp53*, the ability of MYC to induce apoptosis was lost over a period of time in culture, but the Fas apoptotic pathway remained intact. The suggestion is that selection has occurred against some other apoptotic mechanism that may mediate MYC induced apoptosis. This hypothesis is supported by studies in another CD2-*MYC*ER^{TM} cell line which is heterozygous for the *lpr* mutation and has retained functional p53, but which became resistant to MYC induced apoptosis. It seems likely that some unknown apoptotic pathway has been targeted for mutation in these cells, either during transformation or *in vitro* culture, because some genetic event during transformation has shifted the balance in these cells to favour proliferation.

The results presented here are perhaps not surprising given the recent assertion that MYC induces apoptosis by sensitising cells to signalling through death pathways by inducing cytochrome *c* release from the mitochondria (Juin *et al.*, 1999). Indeed neither Fas nor p53 signalling were required for MYC mediated cytochrome *c* release however cytochrome *c* release was required for MYC induced apoptosis (Juin *et al.*, 1999). If MYC does act initially through the mitochondrial apoptotic pathway to simply sensitise cells to apoptotic signals, then many other pathways in addition to Fas and p53 could be influenced by deregulated *MYC* expression. The reliance on any particular apoptotic pathway is likely to vary between cell type.

If the ability of MYC to induce cytochrome *c* release does indeed sensitise cells to multiple death pathways, it is reasonable to speculate that in order for cells to be driven to proliferate during the process of tumourigenesis, complementary genetic lesions are more likely to upregulate survival signals rather than block death pathways. Presumably activation of a survival signal upstream of cytochrome *c* release would be more efficient than abrogation of several apoptotic pathways activated by MYC. Members of the Bcl-2 family are prime candidates for the role of allowing growth and survival of cells transformed by deregulation of *MYC*. Bcl-2 is able to prevent apoptosis induced by a variety of stimuli (Merry & Korsmeyer, 1997), and a number of studies have shown that Bcl-2 can inhibit MYC induced apoptosis

(Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993). Further, c-*myc* and Bcl-2 have been shown to act synergistically to induce lymphomagenesis *in vivo* (Strasser *et al.*, 1990b), and in CD2-*MYC*ER^{TM} cell lines, Bcl-2 has been shown to block MYC induced apoptosis (Blyth *et al.*, 2000). The theory that Bcl-2 may block the ability of MYC to sensitise cells to apoptosis is supported by reports suggesting that the prevention of apoptosis by Bcl-2 occurs by blocking cytochrome *c* release (Kluck *et al.*, 1997; Yang *et al.*, 1997a; Brustugun *et al.*, 1998; Shimizu *et al.*, 1999). Bcl-2 has been implicated in protection from p53 mediated apoptosis in lymphoma cells both *in vitro* and *in vivo* (Wang *et al.*, 1993b; Marin *et al.*, 1994). The role of Bcl-2 family members in Fas mediated apoptosis has been the subject of more controversy. While reports have been made of Bcl-2 family members' ability to prevent Fas mediated cell death (Itoh *et al.*, 1993; Rodriguez *et al.*, 1996; Schneider *et al.*, 1997b; Peter *et al.*, 1997), conflicting results from other groups have shown that Fas induced apoptosis is not blocked by Bcl-2 family members (Memon *et al.*, 1995; Strasser *et al.*, 1995; Huang *et al.*, 1999). Recent studies however have described amplification of Fas mediated apoptosis by caspase 8 and 3 mediated cleavage of the pro-apoptotic Bcl-2 family member Bid, which in turn promotes cytochrome *c* release from the mitochondria and further caspase activation (Li *et al.*, 1998; Bossy-Wetzel & Green, 1999). If Bcl-2 becomes deregulated in tumours in which Fas:FasL interactions play a considerable role in mediating cell death, then it is possible to speculate that even though Fas signalling is likely to induce some death, this will not be amplified and proliferation of cells will outweigh loss by apoptosis. Other apoptotic pathways may be subject to the same regulation.

Although apoptosis may be inhibited in tumour cells *in vivo* in order for proliferation to outweigh death, MYC induced apoptosis was clearly observed in CD2-*MYC*ER^{TM}Fas^{lpr} tumour cells lacking functional p53 *in vitro*. A possible explanation for this is that levels of apoptosis are increased *in vitro* due to the lack of signals which activate survival pathways *in vivo*. This will be discussed further in Chapter 7. It is clear however that apoptosis can still be induced by MYC in these cells, indicating the presence of an intact death pathway independent of Fas or p53.

The data presented here is mirrored by a study of v-Jun induced apoptosis in fibroblasts. v-Jun is similar to c-*MYC* in that it is an oncogene able to promote cell-cycle progression and apoptosis in serum deprivation (Bossy-Wetzel *et al.*, 1997). The study showed that v-Jun induced apoptosis and cytochrome *c* release from the mitochondria occur simultaneously. Release of cytochrome *c* could be blocked by overexpression of Bcl-2, but not by inhibition of the Fas or p53 pathways (MacLaren *et al.*, 2000).

6.3.2 Possible Mechanisms for MYC Induced Apoptosis in the Absence of Fas and p53 Signalling

As discussed in Chapter 1, Fas belongs to the tumour necrosis factor receptor (TNF-R) superfamily, many of whose members also possess the ability to induce apoptosis of the cell in which they are expressed. Receptors are stimulated by members of the TNF family, the most closely related members of which, in terms of homology are FasL and TRAIL (for TNF-Related Apoptosis Inducing Ligand). The ability of TRAIL to inhibit tumour growth *in vivo* has already been demonstrated (Walczak *et al.*, 1999). In contrast with FasL, TRAIL has two putative death receptors, DR4 and DR5 (or Killer), binding of which leads to the activation of apoptosis through a caspase cascade (Ogasawara *et al.*, 1993; Mariani *et al.*, 1997; Griffith & Lynch, 1998). TRAIL does activate a pathway distinct from that initiated by the Fas receptor however, since cells resistant to FasL mediated apoptosis may still show sensitivity to TRAIL (Mariani *et al.*, 1997), and TRAIL induced apoptosis is independent of FADD (Marsters *et al.*, 1996a). Further, FasL and TRAIL have been detected together on the surface of many tumour cell lines (Mariani & Krammer, 1998), suggesting that there is no functional redundancy between these two death pathways.

It is possible that TRAIL plays some role in MYC induced apoptosis independent of the Fas pathway. In fact, the TRAIL death pathway may be particularly important in Bcl-2 overexpressing tumour cells, since it has been reported that although TRAIL initiates a death signal that involves mitochondrial apoptogenic activity to some degree, Bcl-2 is unable to block TRAIL induced apoptosis in transformed T cells (Keogh *et al.*, 2000). The signals which regulate expression and activity of TRAIL

and its receptors are unknown as yet. It is possible to speculate however that if MYC can sensitise cells to death by cytochrome *c* release, then it may also sensitise cells to the effects of the TRAIL apoptotic pathway. If cells overexpressed Bcl-2, then while most apoptosis induced by MYC would be inhibited (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992), the TRAIL death pathway could become particularly significant.

Certainly there are other death signals through which MYC might mediate apoptosis. Apoptosis induced by engagement of other TNF family members has also been described, but less is known of these pathways. Apoptosis through two novel death receptors of the TNF family, THANK and DR6 has recently been described (Pan *et al.*, 1998; Mukhopadhyay *et al.*, 1999). Further investigation of these signals will obviously be required, however it is feasible that apoptosis induced by MYC could activate or amplify any of these pathways.

So far, the research on *MYC* and loss of p53 in tumourigenesis has focused on the apoptosis induced by the p53 protein. The collaboration between deregulation of *MYC* and loss of p53 does not appear to be due to a dependence on p53 for MYC induced apoptosis however, since MYC induced apoptosis can occur in the absence of p53 (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Amanullah *et al.*, 2000; Blyth *et al.*, 2000). It may be that the loss of other tumour suppressor functions of p53 are responsible for the acceleration of tumourigenesis in *Trp53* deficient *MYC* transgenic mice. Another possibility is that p53 interacting proteins may have some ability to regulate apoptosis in cells lacking p53. A recent study in fact showed that transfection of MDM2, the protein responsible for degradation of p53, into p53 deficient human thyroid carcinoma cells can induce apoptosis (Dilla *et al.*, 2000).

There is also a possibility that members of a recently described family of p53 related genes have an apoptotic function independent of p53. The two other members of this family identified so far, p63 and p73 share substantial sequence homology with p53 and when overexpressed can transcriptionally activate p53 responsive target genes and induce apoptosis (Jost *et al.*, 1997; Osada *et al.*, 1998; Yang *et al.*, 1998). There are fundamental differences between family members however, for instance p73 is not induced by DNA damage (Kaghad *et al.*, 1997), and while both p63 and p73 have

important roles during development, mice deficient in either of these proteins show no elevation in spontaneous tumourigenesis (Mills *et al.*, 1999; Yang *et al.*, 1999; Yang *et al.*, 2000). Nevertheless, studies of the mechanisms which regulate these two genes, and of the functions unique to each protein are still ongoing. Either of these proteins may have the potential to mediate MYC induced apoptosis in the absence of functional p53. There may be many more as yet undiscovered apoptotic pathways which could be targets for regulation by *MYC*.

6.3.3 Engagement of FasL Protects from MYC Induced Apoptosis

During investigation of MYC induced apoptosis in CD2-*MYC*ER^{TM} cell lines in the absence of Fas and p53, increased survival in response to MYC upregulation was observed when anti-FasL antibody was included in culture. Although this effect was modest, and only observed in one cell line, it was significant and reproducible. There are a number of possible explanations for this result, and it is possible that whatever the pathway engaged or blocked by anti-FasL, its effect is only uncovered in this cell line because of the lack of death induced by either Fas or p53.

There are two possible distinct mechanisms by which anti-FasL antibody may act in lymphoma cells *in vitro*. Firstly, the antibody may block interactions between FasL and a death receptor. This death receptor is unlikely to be the Fas receptor since the anti-Fas antibody, Jo2 had no effect on MYC induced apoptosis in the same cell line. Thus if anti-FasL protects cells from MYC induced apoptosis by an antagonistic mechanism, then these results suggest the existence of a novel death receptor for FasL, or at least an additional function of a previously described death receptor.

It is possible that the death receptors preference for their 'own' ligand may not be as specific as previously thought. For example, a novel TNF related death ligand (TRDL-1) has been reported to be able to signal through the Fas receptor (Kelly *et al.*, 2000). If, as these results suggest, Fas induced apoptosis can be triggered independently of its ligand, then it is not inconceivable that upregulation of FasL has effects that are distinct from its role in stimulating its receptor. It also appears from recent studies that a domain exists in members of the TNF-R superfamily that allows

receptor trimerisation and activation which is ligand independent (Chan *et al.*, 2000; Siegel *et al.*, 2000). In fact induction of Fas ligand independent, Fas dependent apoptosis was observed in human colon cancer cell lines in response to treatment with camptothecin, a chemotherapeutic agent (Shao *et al.*, 2001).

Another interesting feature of the apoptotic members of the TNF-R superfamily, is the recent discovery of autoamplification of death receptor induced apoptosis by enhanced transcription of death ligands on receptor ligation (Herr *et al.*, 2000). Ligation of the Fas receptor for example induced expression not only of FasL, but also of TRAIL and TNF-α. Similarly, enhanced transcription of all three death inducing ligands was observed following ligation of either TRAIL or TNF (Herr *et al.*, 2000). Given the homology and this level of 'cross-talk' between receptors of the TNF-R family, it is possible that FasL may interact with related death receptors other than Fas, and similarly other death inducing ligands may act on different receptors. Already TRAIL has been reported to interact with two death receptors (Pan *et al.*, 1997a; 1997b; Walczak *et al.*, 1997), and two decoy receptors (Degli-Esposti *et al.*, 1997a; 1997b; Marsters *et al.*, 1997b), and only recently a decoy receptor for FasL was identified in human tumours (Pitti *et al.*, 1998; Ohshima *et al.*, 2000). The presence of further receptors for FasL cannot be ruled out, and future investigation into the other TNF-R family death receptors in ER15LP 101 cells would be valuable.

The other mechanism by which anti-FasL may protect from MYC induced apoptosis, or more accurately, may increase survival in response to MYC upregulation, is by initiating a proliferative or survival signal through FasL. Since cyclosporin A treatment, which inhibits FasL expression, had a protective effect on ER15LP 101 cells however, it seems improbable that FasL delivers a proliferative signal at least in this cell line. Nevertheless, FasL has recently been implicated in proliferation of $CD8^+$ T cells (Suzuki & Fink, 1998). This study described a role for FasL as a signalling receptor. Proliferation assays using either Fas^{lpr}, Fas^{gld} or wild-type CTL cell lines demonstrated that CTLs lacking FasL had a diminished capacity for proliferation following sup-optimal activation. This was not as a result of lacking Fas:FasL interactions, since no such proliferative defect was observed in *lpr* cell lines (Suzuki & Fink, 1998). A proliferative signal mediated by reverse signalling through

FasL could explain the increased survival of this CD2-*MYC*ER^{TM}Fas^{lpr} cell line in response to MYC upregulation when anti-FasL is present, although the results of treatment with cyclosporin A make this unlikely. Presumably this effect would only be evident when FasL becomes upregulated following MYC induction, in the absence of Fas receptor. In that case the reverse signal through FasL would to some extent counter-balance the apoptotic signal. The possibility of further involvement of the Fas:FasL system in the capacity of T cells to proliferate will be discussed in greater detail in Chapter 8.

CHAPTER 7

PROTECTION FROM *MYC* INDUCED APOPTOSIS MEDIATED BY CELL CONTACT

7.1 INTRODUCTION

7.1.1 Detachment Induced Apoptosis ("Anoikis")

Apoptosis can occur not only as a result of cellular damage or as a mechanism to maintain homeostasis. In many cell types loss of attachment to, or contact with the extracellular matrix (ECM) induces apoptosis. This has been variously called detachment-induced apoptosis, anchorage-related apoptosis or "anoikis" (Greek for homelessness). Anoikis was first observed in epithelial and endothelial cells that were experimentally dissociated from the extracellular matrix (Meredith *et al.*, 1993; Frisch & Francis, 1994). Apoptosis was induced in cells by disruption of the interactions between these cells and the extracellular matrix, and in epithelial cells, this death was blocked by overexpression of Bcl-2 (Frisch & Francis, 1994). Among the signals implicated in contact mediated protection from apoptosis are hyaluronic acid (HA), a principal component of the extracellular matrix, and its receptor the CD44 cell surface antigen (Ayroldi *et al.*, 1995). A great deal of research has also focused on various members of the integrin family (reviewed by Frisch & Ruoslahti, 1997; Giancotti & Ruoslahti, 1999), and a number of pathways initiated by PI3 kinase activation (Krasilnikov, 1999). These are discussed in more detail below.

In normal tissues detachment-induced apoptosis should prevent the establishment of cells in inappropriate locations, that have lost contact with the matrix. It may also play a role in the involution of tissues such as the mammary gland (Boudreau *et al.*, 1995). In transformed cells apoptosis may be regulated by the level of integrins and other matrix adhesion molecules and receptors, and their associated signalling molecules. Changes in cell:cell contact or cell:matrix contact in neoplastic cells might be important because of the possibility that they may allow contact-

independent growth and/or metastasis. Recent reports have suggested that in order for transformed cells to survive *in vivo* and *in vitro*, they must overcome anchorage dependence by constitutively activating certain survival signals, or by suppressing apoptotic signals (Frisch & Francis, 1994; Sethi *et al.*, 1999). Tumour cells which are able to upregulate certain integrins for example may be able to evade apoptosis (reviewed by Frisch & Ruoslahti, 1997).

Focal adhesion kinase (FAK) appears to play a major role as a mediator of protection from detachment induced apoptosis. Upon integrin mediated cell-matrix attachment, FAK becomes autophosphorylated and thus activated to initiate a survival signalling cascade (or cascades, Schlaepfer *et al.*, 1994). This role of FAK in anoikis has been clearly established. Blocking the expression of FAK was shown to induce apoptosis in human tumour cell lines (Xu *et al.*, 1996). Further, cells in which FAK is constitutively activated are protected significantly from anoikis, and conversely inactivation of FAK in the same cells causes apoptosis (Frisch *et al.*, 1996b). Cells carrying constitutively active FAK exhibited anchorage independent growth, and formed tumours in nude mice (Frisch *et al.*, 1996b), emphasising the importance of this phenomenon in tumourigenesis.

The signalling cascades downstream of FAK activation are complicated and still not fully understood. One candidate for mediating protection from apoptosis is phosphotidylinositidol 3-kinase (PI3K), which binds to FAK and also activates the survival factor protein kinase B/Akt (Chen & Guan, 1994; Khwaja *et al.*, 1997; King *et al.*, 1997). Akt has already been shown to have an anti-apoptotic function in another system. MYC overexpression in fibroblasts cultured in conditions of low serum induces apoptosis which is blocked by Akt (Kauffmann-Zeh *et al.*, 1997; Rohn *et al.*, 1998). Significantly, in the same cells which were shown to be protected from anoikis by constitutive expression of FAK, introduction of either activated PI3K or Akt mediated protection from anoikis (Khwaja *et al.*, 1997). Akt protects from apoptosis at least in part by phosphorylating and thus inactivating Bad and caspase 9 (Datta *et al.*, 1997), which are involved in apoptotic signalling, however the downstream pathways from Akt itself are not yet fully defined (reviewed by Coffer *et al.*, 1998, Datta *et al.*, 1999).

The PI3K/Akt pathway is not the only survival pathway involved in protection of detached cells. Alternative routes have been proposed, and it now seems likely that anoikis is controlled in an integrin-specific manner. Under disparate circumstances, attachment to the ECM through a particular integrin may be necessary for survival. Binding of the α5β1 integrin to fibronectin for example, induces expression of Bcl-2, which subsequently protects from environmental stresses (Zhang *et al.*, 1995). Furthermore, in cells lacking α5β1, overexpression of Bcl-2 blocked apoptosis (Zhang *et al.*, 1995). On endothelial cells, the αvβ3 integrin promotes survival by suppression of the p53 response and activation of NF-κB and Bcl-2 (Stromblad *et al.*, 1996). In turn Bcl-2 may suppress the activation of caspases and of the MEKK/JNK pathway which induces apoptosis in response to stress. There is some evidence to support the involvement of this pathway since JNK activity was recently found to be rapidly induced in detached epithelial cells (Frisch *et al.*, 1996a). In the future, comparison of the expression and activity of various integrin types and other adhesion molecules in a variety of cancer types may aid our understanding of both anoikis and metastasis.

Several studies have demonstrated a role for integrin signalling in co-stimulation and activation of T cells. In particular, engagement of β1 family integrins on the surface of activated T cells has been shown to increase proliferation of these cells, at the same time FAK activation has been observed (Matsuyama *et al.*, 1989; Dang *et al.*, 1990; Finkelstein *et al.*, 1997). More recently, signalling through integrins has been implicated in modulation of T cell apoptosis, either by providing a survival signal or by inducing a death signal. Co-stimulation of integrin α4β1 with T cell receptor engagement blocks dexamethasaone induced apoptosis in human thymocytes (Zaitseva *et al.*, 1998), while α5β1 integrin has been reported to mediate protection of $CD8^+$ T cells against TGF-β induced apoptosis (Rich *et al.*, 1996). Since AICD in T cells is dependent on Fas:FasL interactions, it is unsurprising that several studies have shown involvement of Fas pathway components in integrin mediated protection from apoptosis. In Jurkat T cells engagement of α2β1 integrin by collagen type I or with agonistic antibodies was shown to inhibit AICD with a concomitant reduction in FasL mRNA expression (Aoudjit & Vuori, 2000). Protection was specific to

apoptosis induced by TCR stimulation. Fas-mediated and cycloheximide-mediated apoptosis were not affected by engagement of $\alpha 2\beta 1$. Moreover, inhibition of FasL expression and of AICD required FAK, since a dominant negative form of FAK blocked the effects of integrin ligation when overexpressed (Aoudjit & Vuori, 2000).

Downstream targets of Fas have also been implicated in detachment induced apoptosis recently (Rytomaa *et al.*, 1999). Anoikis was blocked in several untransformed epithelial cell lines by expression of DN-FADD, although blocking ligation of Fas itself did not affect anoikis, suggesting activation of the Fas pathway at least in this system is not ligand dependent. Caspase 8 was strongly activated following loss of contact of cells with the matrix., and appeared to be the initiating event leading to apoptosis. Overexpression of Bcl-2 protected cells from apoptosis following detachment, and also inhibited the activation of caspase 8, suggesting the possible existence of a positive feedback loop (Rytomaa *et al.*, 1999). There may be many other pathways responsible for both induction of apoptosis in response to detachment, or for survival mediated by cell contact or attachment. The dependence on cell contact in *MYC* induced tumours has not been examined so far, but may be significant given the need for tumour cells to overcome MYC induced apoptosis

7.1.2 Experimental Aims

Throughout the course of working on the CD2-*MYC*ER^{TM} model, a number of observations led to the formulation of a hypothesis that cell contact could protect tumour cells against MYC induced apoptosis. Firstly, it was suspected that the likelihood of CD2-*MYC*ER^{TM} tumours establishing as cell lines appeared to be enhanced when adherent cells were present in the initial culture, although no formal data were collected. Secondly, it was apparent that in some established CD2*MYC*ER^{TM} tumour cell lines, cell contact in culture is maximised, since cells often appear to gather in clusters in culture, or become 'clumpy'. Further, the appearance of this phenotype coincided with the development of resistance to MYC induced apoptosis in a small number of cell lines (Blyth and Morton, unpublished observations). Since some CD2-*MYC*ER^{TM} cell lines appeared to retain at least a partial dependence on cell interactions in order to survive *in vitro*, these lines may

provide an understanding of the mechanism behind cell contact mediated protection from apoptosis, and may lead to a better idea of which apoptotic pathways are triggered when these survival signals are lost.

The initial aim of the following experiments was to investigate the observation that CD2-*MYC*ER^{TM} cell lines may be protected from MYC induced apoptosis by cell contact in culture. To generate evidence to support these observations, it was necessary to assess the level of protection offered by cell contact, and to investigate whether overall cell survival was increased, or if MYC induced apoptosis specifically was inhibited when adherent cells were present in culture. Finally, a possible mechanism responsible for providing contact mediated protection was examined.

7.2 RESULTS

7.2.1 Survival of CD2-*MYC*ER^{TM} Cell Lines is Increased when Cultured with Adherent Cells

During efforts to establish CD2-*MYC*ER^{TM} thymic lymphoma cell lines, it was noted that the chances of long-term survival of a cell line appeared to be increased when adherent cells were included in culture. Normally, the preparation of lymphoma cell lines included a Ficoll® density gradient to achieve pure lymphocyte cell suspensions, however occasionally a few cells of different origin, possibly thymic stromal cells, escaped this selection process. The impression that inclusion of these cells might enhance long-term survival of thymic lymphoma cells in culture was purely speculative: no data was collected on the successful establishment of cell lines with and without significant contamination of adherent cells, and this may vary between tumour cells of different genotype. Neither do we know whether the number of adherent cells that escape the screening process during preparation of suspension cell cultures is random, or is due to increased dependence of the tumour on those cells *in vivo*. However the observation did lead us to consider whether cell contact may be important for the survival of these tumour cells.

Once cell lines became well established on a supporting layer of adherent cells, it became possible to establish separate cultures purely of transformed lymphocytes. To assess whether a supporting layer of adherent cells had a general protective effect on tumour cells, or whether protection was specific against MYC induced apoptosis, two such CD2-*MYC*ER^{TM} cell lines grown with and without the support of adherent cells were studied. The genotype of these and other cell lines discussed in this chapter is given in Table 7.1. Corresponding cell lines remaining in culture with adherent cells, or approximately 6 weeks following separation, were incubated in the presence of 4-OHT, and their viability monitored at 24 hour intervals over a 96 hour period. Viability was assessed by trypan blue exclusion.

Figure 7.1 shows the survival curves of these two cell lines, ER15LP 52 and ER15LP 101. Treatment with 4-OHT resulted in significantly increased cell death in the cell lines tested at every time point from 48 hours ($P<0.01$), consistent with previous results in CD2-*MYC*ER^{TM} cell lines. In the same two cell lines cultured with the support of adherent cells however, significant although not complete protection from MYC induced apoptosis was observed at every time point from 48 hours ($P<0.01$). In cultures of ER15LP 101 cells, adherent cells also had a general protective effect against background death (Figure 7.1B). The results suggest that the survival of lymphoma cell lines cultured with supporting adherent cells was increased at least in part, as a result of partial protection from MYC induced apoptosis.

Table 7.1 Genotypes of CD2-*MYC*ER^{TM} Cell Lines

Cell Line	**Mouse Genotype**	**Events *In Vitro***
ER15LP 52	CD2-*MYC*ER™Fas$^{lpr/-}$	p53 retained
ER15LP 101	CD2-*MYC*ER™Fas^{lpr}	functional p53 loss
ER15LP 308	CD2-*MYC*ER™Fas^{lpr}	p53 retained
ERLPTW 32	†CD2-*MYC*ER™Fas^{lpr}	functional p53 loss
ERP15 122 sensitive	CD2-*MYC*ER™/*Trp53*-/-	
ERP15 122 resistant	CD2-*MYC*ER™/*Trp53*-/-	loss of MIA*
ERTW 46	†CD2-*MYC*ER™	functional p53 loss

† these cell lines were generated from animals administered tamoxifen as described in Chapter 3.

*MYC induced apoptosis

7.2.2 Mediation of Protection is a General Feature of Fibroblasts

Although the protection conferred on cell lines by incubation with adherent cells was potentially very interesting, it was necessary to confirm that this was a general feature of fibroblasts, and not some effect provided solely by these uncharacterised adherent cells isolated from tumours. Mouse embryo fibroblasts (MEFs) or NIH/3T3 fibroblasts were added to 24 well plates, and allowed to adhere over a sixteen hour period. ER15LP 52 cells were added to plates with or without fibroblasts, and incubated in the presence or absence of 4-OHT. The viability of lymphoma cells was assessed by trypan blue exclusion after 48 hours in culture. Results are shown in Figure 7.2. Significant levels of apoptosis in lymphoma cells incubated alone, were induced following treatment with 4-OHT ($P<0.01$). MYC induced apoptosis was reduced however when the same cell lines were cultured in the presence of MEFs or NIH/3T3 cells. Both MEFs and NIH/3T3 cells offered significant protection against MYC induced apoptosis ($P<0.01$). In fact NIH/3T3 fibroblasts offered complete protection from MYC induced apoptosis in this experiment, while 4-OHT could still induce significant apoptosis in ER15LP 52 cells on supporting MEFs ($P<0.05$). Induction of MYC induced apoptosis in the control cells in Figure 7.2A was also significantly higher than in control cells in Figure 7.2B ($P<0.01$). Further, significant protection of ER15LP 52 cells from background death, was observed when cultured on MEFs ($P<0.01$). The reasons for the differences between the two experiments, in death induction following 4-OHT treatment, and in rate of background death, is likely to be due to differences in the viability of the cell line at the 0 hour time-point. Although all cell concentrations in short-term cultures are initially the same, the confluence of the cultures from which the cells are taken can affect their kinetics in short-term cultures. This is not unexpected, as it is possible that survival factors may become limiting in culture, and affect the viability of the cells in these cultures. The kinetics of cell growth may also be affected by the level of contact with the other cells in culture. It is clear from the results however, that protection from MYC induced apoptosis in this CD2-*MYC*ER^{TM} cell line is mediated by cell contact provided by a fibroblast layer, and is not specific to the cell type of the supporting layer. Further experiments later in this chapter (shown in Figure 7.5) show that fibroblast mediated protection occurs in 3 other cell lines.

7.2.3 Protection Is Mediated by Cell Contact

Initial observations on cell lines in culture suggested that protection from MYC induced apoptosis was dependent on cell contact. Firstly, explanted lymphoma cells in culture adhered to available adherent cells if any were present following cell preparation. Figure 7.3A shows an example of a cell line which established on a supporting fibroblast layer. In addition, in some cell lines cell contact is clearly maximised, as the cells adhere to each other and become 'clumpy'. Figure 7.3B shows an example of a clumpy cell line. It was shown that after serial passaging, some CD2-*MYC*ER^{TM} cell lines can specifically lose susceptibility to MYC induced apoptosis. On 5 out of 7 occasions, this altered response to MYC upregulation coincided with an apparent change in the appearance of the culture, due to cells becoming clumpy (Blyth and Morton, unpublished observations). The loss of MYC induced apoptosis in one such cell line, ERP15 122, is shown in Figure 6.6A. The hypothesis arising from these observations was that cell contact appeared to be one mechanism for escaping MYC induced apoptosis. Nonetheless it was important to establish whether direct cell contact was responsible for the increased survival of cells cultured with fibroblasts, since survival may be mediated by a soluble growth factor or cytokine released by the fibroblasts.

To investigate which of these theories was accurate, MEFs were cultured as before in a 24 well plate, and when the cells reached near confluence, the medium was harvested. This medium was then filtered to remove any suspended fibroblasts. ER15LP 52 cells which were known to be protected from MYC induced apoptosis by culture with fibroblasts were grown in unconditioned or conditioned medium, in the presence or absence of 4-OHT. Viability of cells was assessed after 48 hours incubation, by trypan blue exclusion. Although activation of MYC resulted in significant induction of cell death in both conditioned ($P<0.01$), and unconditioned medium ($P<0.01$), there was no significant difference in the levels of apoptosis between the two growth conditions (Figure 7.4A). These data suggest that protection from MYC induced apoptosis is not mediated by soluble growth factors given off by the fibroblasts.

Although conditioned medium offered no protection from MYC induced apoptosis in the previous experiment, it was not yet possible to rule out protection mediated by soluble factors. In order to investigate if the continued production of putative anti-apoptotic factors was protecting the cells from MYC induced apoptosis, a similar experiment was performed using a co-culture system. This system allowed both lymphoma cells and fibroblasts to be cultured in the same wells of a 24 well plate, but separated by a membrane insert that prevented cell contact but allowed diffusion of soluble factors. ER15LP 52 cells were incubated alone, in direct contact with a supporting layer of MEFs, or separated from MEFs by membrane inserts. The viability of the suspension cells was assessed by trypan blue exclusion 48 hours following 4-OHT treatment to induce MYC upregulation. Again significant levels of apoptosis were induced following 4-OHT treatment of controls (where no MEFs were present, $P<0.01$), however a layer of supporting MEFs offered almost complete protection from MYC induced apoptosis (Figure 7.4B). Treatment with 4-OHT did not result in significant induction of death in the ER15LP 52 cells when cultured on MEFs. This protective effect was abolished when contact with the MEFs was blocked. In these cultures significant induction of death occurred following MYC upregulation ($P<0.01$), and there was no significant difference between induction of death in these cultures, and in ER15LP 52 cells cultured alone. The results confirm that protection is indeed mediated by cell contact, and not by soluble factors released by the fibroblasts.

7.2.4 Cell Contact Mediated Protection is Dependent on PI3 Kinase

Recent studies have suggested that MYC induced apoptosis might be suppressed by signalling through the survival kinase Akt, which is itself activated by PI3 kinase (Kauffmann-Zeh *et al.*, 1997; Rohn *et al.*, 1998). It was therefore of interest to investigate whether the PI3 kinase pathway might protect CD2-*MYC*ER^{TM} cell lines against apoptosis following MYC upregulation. Further, a number of reports have highlighted a protective effect of cell contact on tumour cells (Xu *et al.*, 1996; Sethi *et al.*, 1999), and one report of particular relevance to these results suggested that Integrin Linked Kinase (ILK), a molecule that can mediate the effects of cell

adhesion through its interaction with integrin subunits, may act as an effector for PI3 kinase activation of Akt (Delcommenne *et al.*, 1998).

Experiments were set up to assess whether the protection from MYC induced apoptosis by contact with fibroblasts seen in some CD2-*MYC*ER^{TM} cell lines was dependent on PI3 kinase signalling. Initially, 3 CD2-*MYC*ER^{TM} cell lines; ER15LP 101; ERP15 122; and ERTW 46, which were known to be protected from MYC induced apoptosis by cell contact, were cultured with and without NIH/3T3 cells in the presence or absence of the PI3 kinase specific inhibitor, LY294002 (Vlahos *et al.*, 1994). MYC activity was upregulated by addition of 4-OHT, and cell viability was assessed after 72 hours by trypan blue exclusion. As observed previously, 4-OHT treatment resulted in significantly increased cell death in each cell line ($P<0.01$ for each line), and contact with fibroblasts offered significant protection from MYC induced apoptosis in the cell lines tested (ERTW 46 and ER15LP 101, $P<0.01$, ERP15 122, $P<0.05$). However in all these cell lines (3 from 3), fibroblast mediated protection was significantly reduced, indeed completely abrogated, when LY294002 was present in culture ($P<0.01$, Figure 7.5). Contact with fibroblasts offered no significant protection against MYC induced apoptosis in these lines, when LY294002 was included in culture. It is reasonable to suggest therefore, that inhibition of the PI3 kinase pathway blocks protection conferred by cell contact, at least in these cell lines, and suggest that the protective effect of contact is mediated through the PI3 kinase pathway.

Not all CD2-*MYC*ER^{TM} cell lines demonstrated the same effect with LY294002. In some cell lines unprotected by cell contact, LY294002 had no significant effect, however in two further cell lines, ER15LP 52 and ER15LP 308, it was found that LY294002 was lethal to the cells. The results of these experiments are shown in Figure 7.6. This effect was independent of the ability of fibroblasts to confer protection on the cells, since one of these cell lines was protected against MYC induced apoptosis by cell contact ($P<0.05$, Figure 7.6A), and the other was not (Figure 7.6B). The data suggest that in these cell lines PI3 kinase activates some pathway that is essential for survival of the cells. The reason for this effect in some cell lines but not others is not clear, however, the two cell lines in which LY294002

initiated rapid cell death share one property. Both of these cell lines were shown to have functional p53, by response to irradiation, whereas the cell lines in which inhibition of PI3 kinase is not lethal, but rather blocks contact mediated protection, have lost p53 function by the same criteria. It may be that cells in which p53 is still functionally active rely more heavily on certain survival signals which may be mediated by the PI3 kinase pathway. Since PI3 kinase has multiple functions, it is also possible that PI3 kinase directly blocks p53 induced apoptosis, and hence in cells with functional p53, inhibition of PI3 kinase activity would result in rapid cell death. In cells lacking p53, PI3 kinase may simply mediate certain survival signals, such as those engaged during cell contact.

7.2.5 Levels of Phosphorylated Akt in CD2-*MYC*ER^{TM} Cell Lines Protected by Cell Contact

One of the pathways activated by PI3 kinase, the Akt survival pathway (King *et al.*, 1997), has previously been shown to play a role in protection from anoikis (Khwaja *et al.*, 1997). To investigate whether PI3 kinase was acting through the Akt pathway in the cell lines in which inhibition of PI3 kinase activity blocked cell contact mediated protection, levels of phosphorylated (active) Akt were determined by immunoblotting. CD2-*MYC*ER^{TM} cells which were protected against *MYC* induced apoptosis by cell contact, and in which protection was abolished by PI3 kinase inhibitor were studied. Protein extracts were prepared from these cells 18 hours following incubation in the presence or absence of 4-OHT, with or without LY294002, and cultured with or without supporting fibroblasts. Levels of phosphorylated Akt and β-actin were detected by immunoblotting (see Chapter 2). Figure 7.7A shows the results obtained in ER15LP 101 cells. It might be expected that if PI3 kinase were acting through Akt, then phosphorylated Akt levels would be increased in 4-OHT treated cells on supporting fibroblasts, compared to 4-OHT treated cells cultured alone. It would also be expected that addition of LY294002 would result in a decrease in the levels of phosphorylated Akt. Any differences in the levels of phosphorylated Akt in ER15LP 101 cells following these treatments did not appear to be of great magnitude however. Results from one other cell line protected from MYC induced apoptosis by cell contact, showed similarly low levels

of phosphorylated Akt. These early results although not conclusive would indicate that Akt does not play a major role in the protection of these cells from MYC induced apoptosis.

7.2.6 Increased Levels of Phosphorylated Akt in CD2-*MYC*ER^{TM} Cell Lines in which PI3 Kinase Inhibition is Lethal

It was also necessary to determine the levels of phosphorylated Akt in the two cell lines, ER15LP 52 and ER15LP 308, in which PI3 kinase inhibition was lethal. Both of these cell lines express functional p53, and a recent report has suggested that the PI3 kinase/Akt pathway promotes translocation of MDM2 to the nucleus and subsequent degradation of p53 (Mayo & Donner, 2001). If this was the case, then it might be expected that when PI3 kinase is inhibited in these cells there would be an increase in active p53, which would explain the rapid induction of cell death. Levels of phosphorylated Akt in untreated ER15LP 52 and ER15LP 308 were compared to control cell lines studied, in which LY294002 was not toxic (ER15LP 101 and ERP15 122), by immunoblotting. Detection of β-actin was also carried out to control for differences in loading. The results presented in Figure 7.7B show that levels of phosphorylated Akt in these two untreated cell lines in which inhibition of PI3 kinase was lethal, are markedly increased compared to the two control cell lines in which PI3 kinase inhibitor had no effect except to abrogate contact mediated protection from 4-OHT. The data suggest that in cell lines in which functional p53 is retained, constitutive activation of the PI3 kinase/Akt survival pathway may be critical for survival, at least *in vitro*.

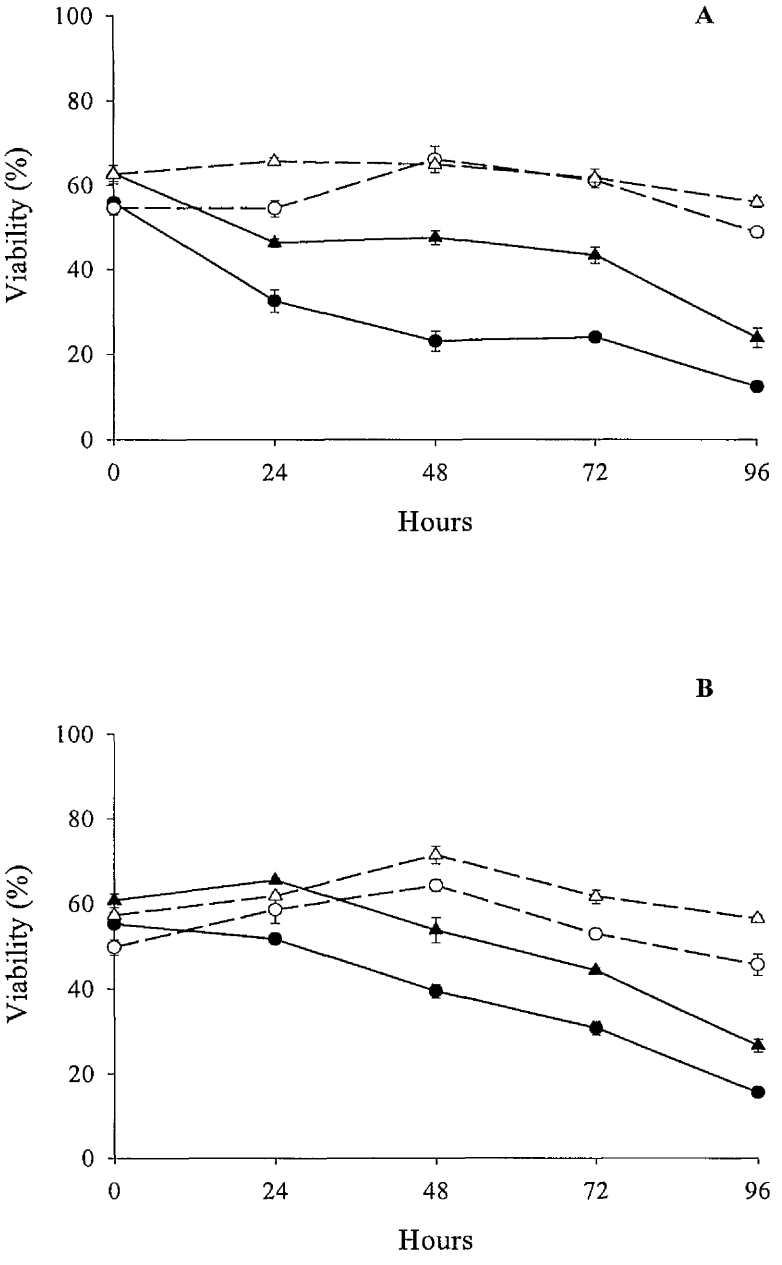


Figure 7.1 Viability of Explanted CD2-*MYC*ER^{TM} Cell Lines Growing With or Without Supporting Adherent Cells

CD2-*MYC*ER^{TM} cell lines, **A**, ER15LP 52 and **B**, ER15LP 101, were grown with supporting adherent cells (triangles) or separated from those adherent cells (circles) were incubated in the presence (solid lines, filled symbols) or absence (broken lines, open symbols) of 4-OHT. Viability was assessed at the indicated time points by trypan blue exclusion. Results shown are based on live:dead counts performed in quadruplicate and expressed as percentage live over total.

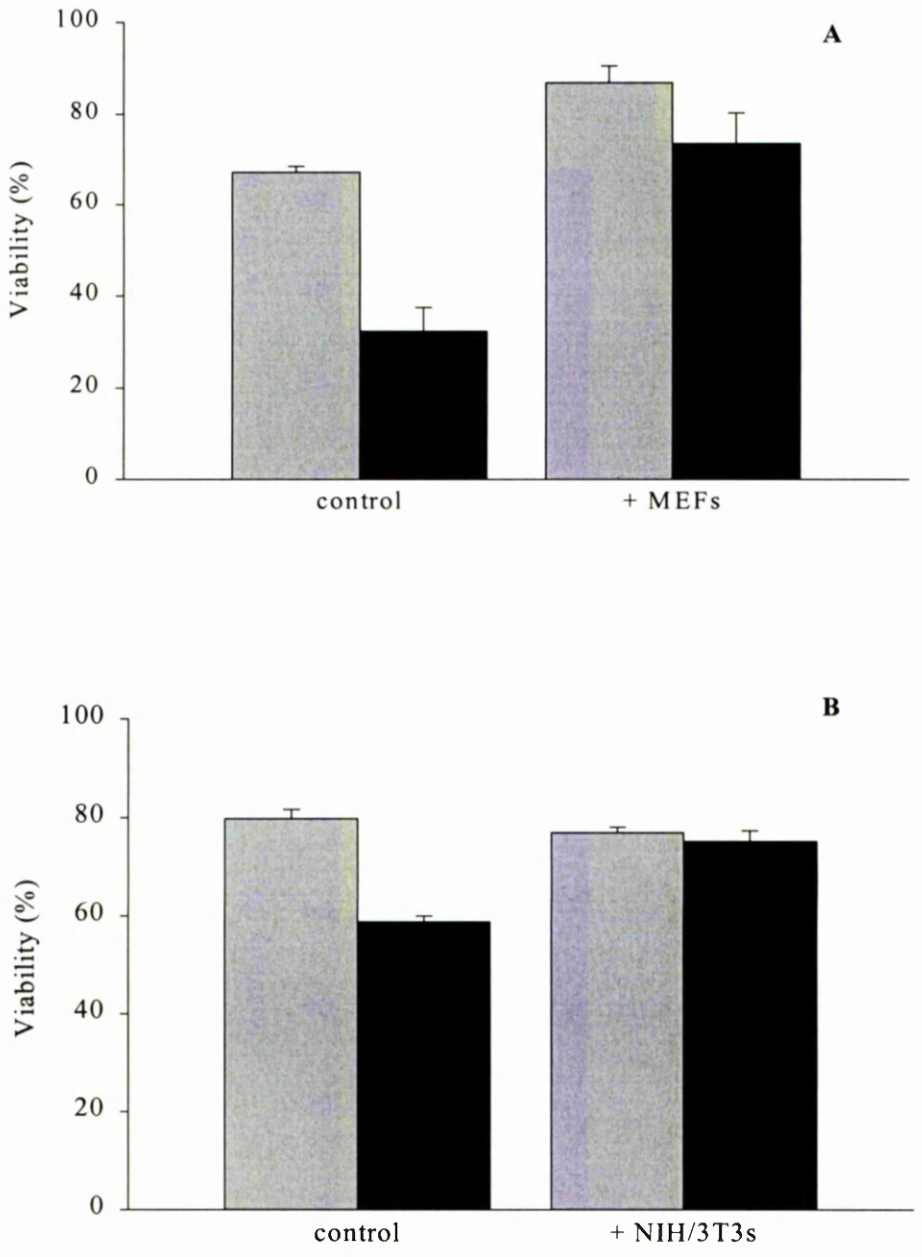


Figure 7.2 Viability of an Explanted CD2-*MYC*ER^{TM} Cell Line (ER15LP 52) on MEFs or NIH/3T3 Cells

A, ER15LP 52 cells were cultured with or without a supporting layer of MEFs, or **B,** with or without supporting NIH/3T3 cells. Cells were incubated in the presence (black bars) or absence (grey bars) of 4-OHT. Representative results are shown here. Viability of CD2-*MYC*ER^{TM} (ER15LP 52) cells was assessed following 48 hours in culture, by trypan blue exclusion. Results shown are based on live:dead counts performed in quadruplicate, expressed as a percentage of live over total.

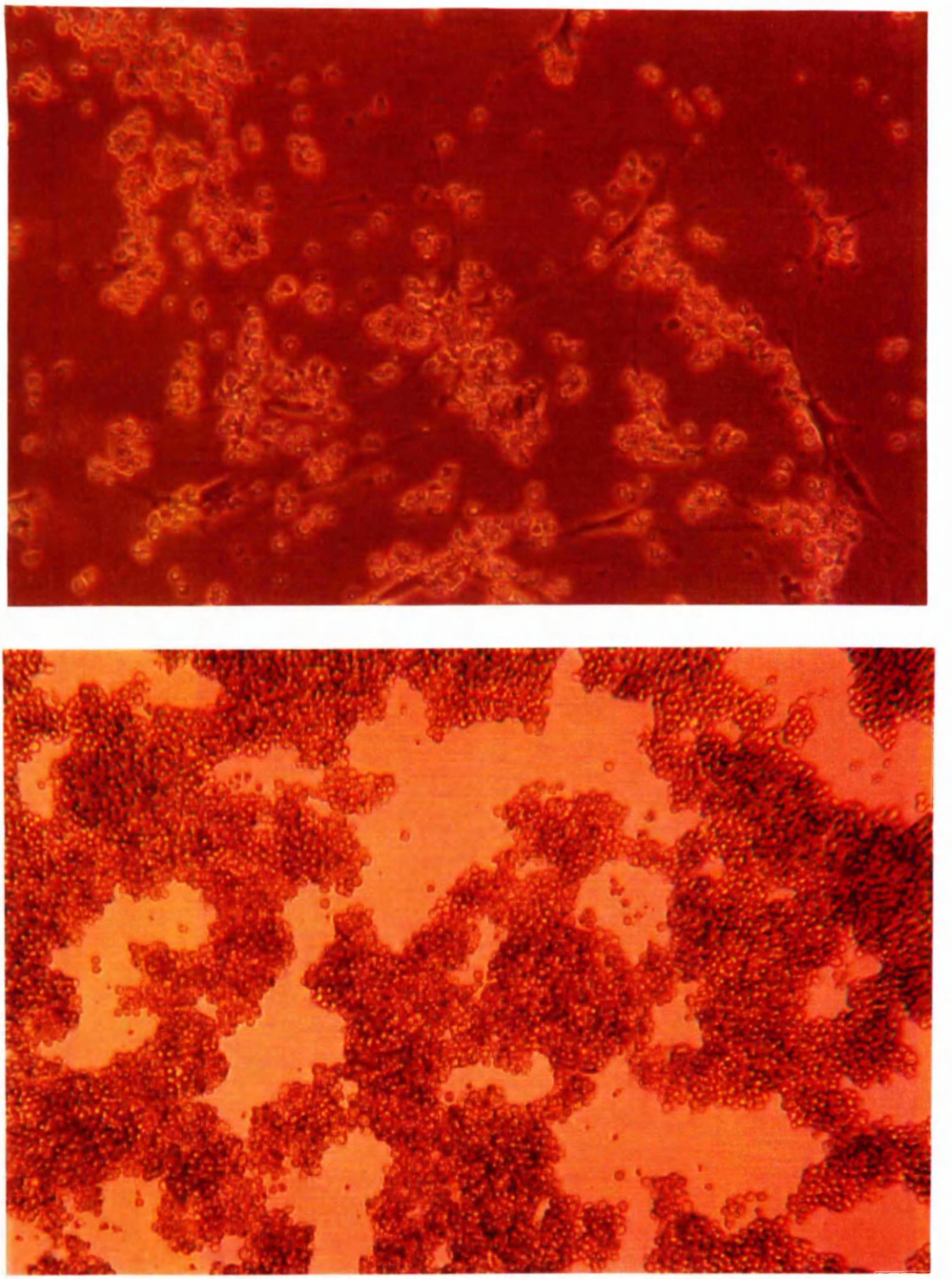


Figure 7.3 Photomicrographs of CD2-*MYC*ER^{TM} Cell Lines

A, Photomicrographs show a CD2-*MYC*ER^{TM} cell line, ER15LP 101, with supporting adherent cells carried through from initial suspension cell preparation. **B**, CD2-*MYC*ER^{TM} cell line, ERTW 46, with 'clumpy' phenotype. Cells shown are untreated cells in unconditioned medium.

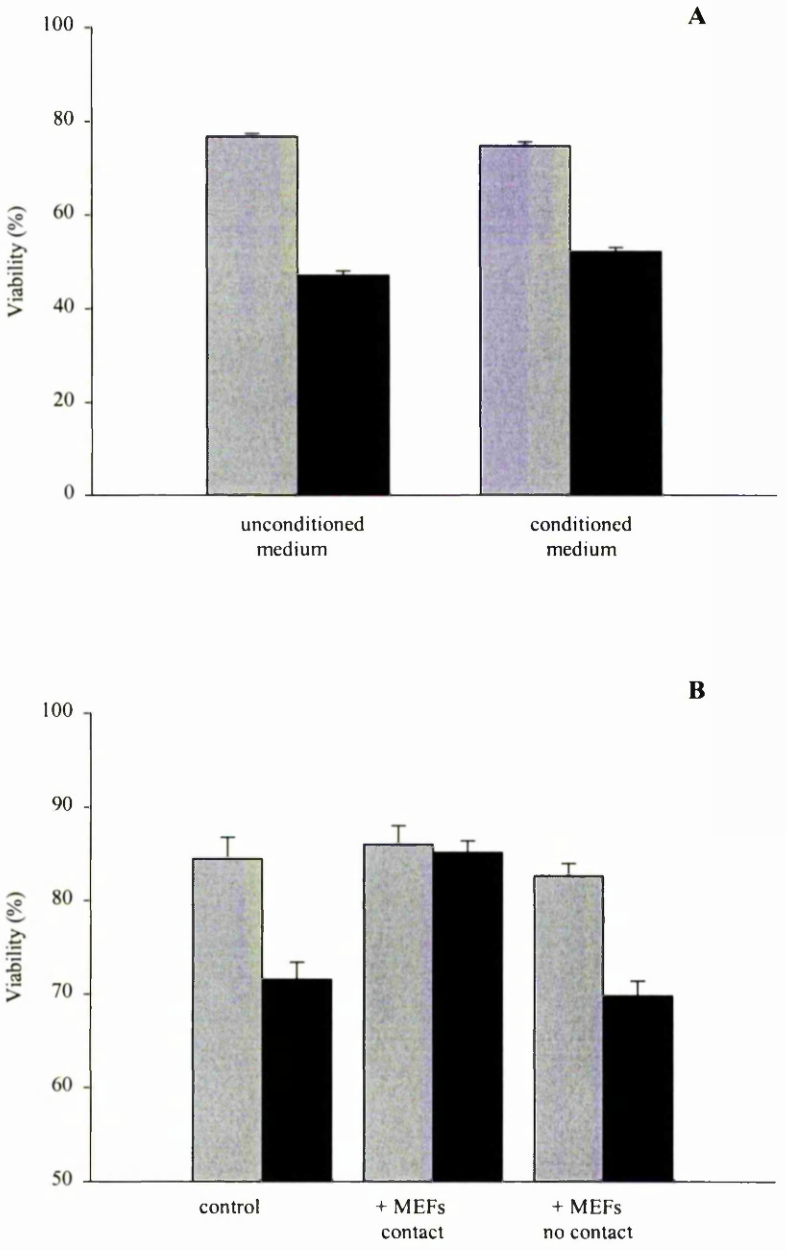


Figure 7.4 Viability of CD2-*MYC*ER^{TM} (ER15LP 52) Cells in Conditioned Medium or in Contact with Fibroblasts

A, ER15LP 52 cells were cultured in unconditioned or conditioned medium (described in section 7.2.3), as indicated, and treated with (solid bars) or without (shaded bars) 4-OHT. **B,** ER15LP 52 cells were incubated with or without MEFs, in contact or not as indicated, using the co-culture system described in section 7.2.3. Cells were treated with (solid bars) or without (shaded bars) 4-OHT. Viability was assessed following 48 hours in culture. Results shown are based on live:dead counts performed in quadruplicate, expressed as a percentage live over total.

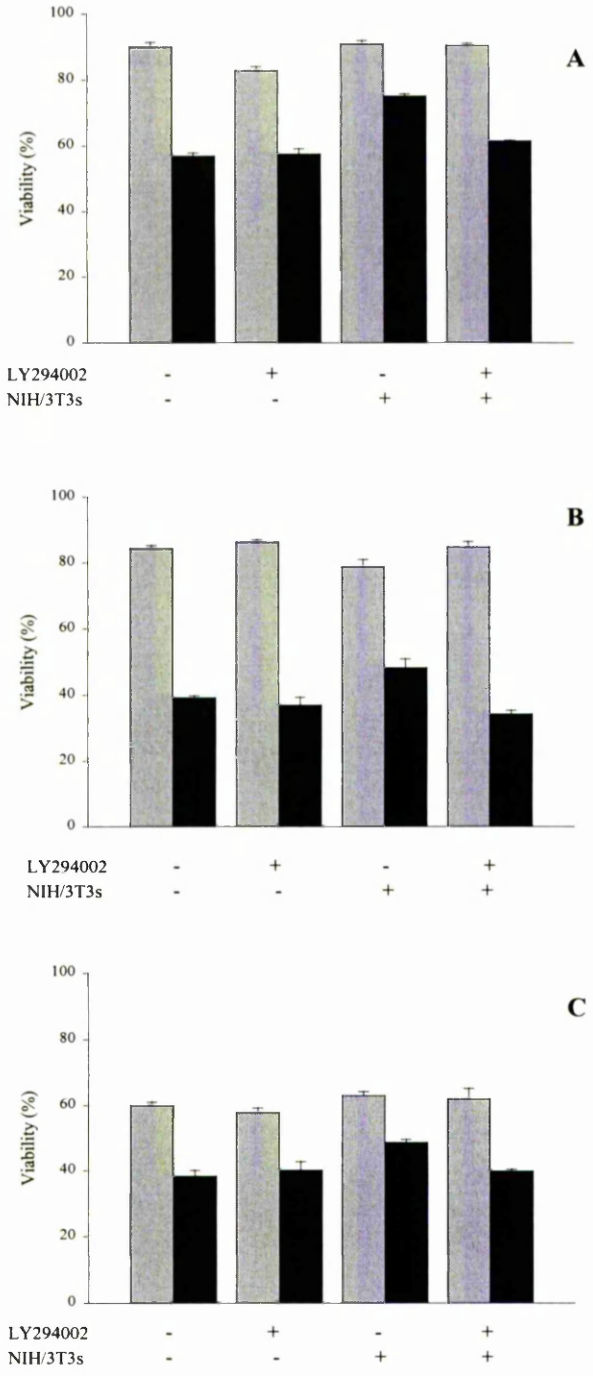


Figure 7.5 Viability of Explanted CD2-*MYC*ER^{TM} Cell Lines Growing With or Without Supporting Adherent Cells Following Inhibition of PI3 Kinase

CD2-*MYC*ER^{TM} cells, **A**, ERTW 46, **B**, ERP15 122, and **C**, ER15LP 101 were grown with or without supporting fibroblasts. Cells were treated with (black bars) or without (grey bars) 4-OHT, in the presence or absence of PI3 kinase inhibitor (LY294002, 10μM) as indicated. Viability was assessed after 72 hours in culture by trypan blue exclusion. Results shown are based on live:dead counts performed in quadruplicate, expressed as percentage live over total. Cell contact offered significant protection against *MYC* induced apoptosis in ERTW 46, ER15LP 101 ($P<0.01$) and ERP15 122 ($P<0.05$) cell lines.

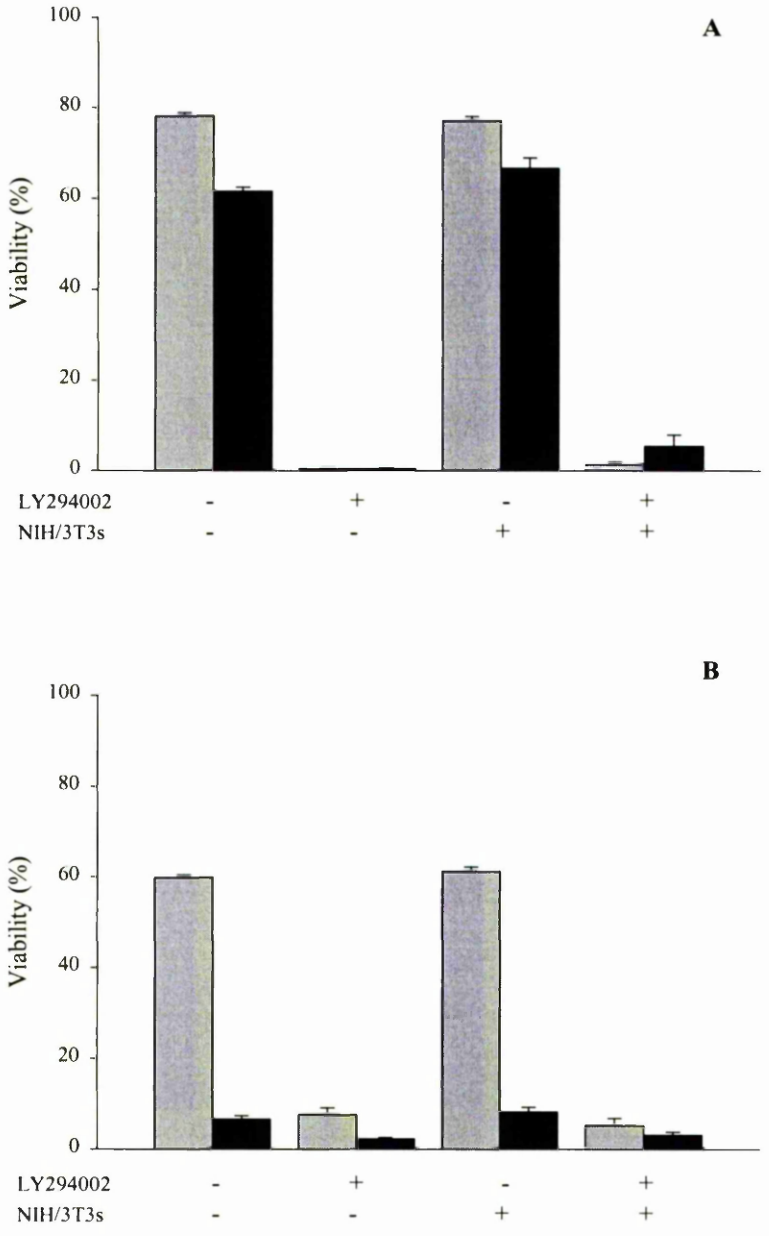


Figure 7.6 Viability of Explanted CD2-*MYC*ER^{TM} Cell Lines Growing With or Without Supporting Adherent Cells Following Inhibition of PI3 Kinase

A, ER15LP 52 (cell contact protected) and **B,** ER15LP 308 (unprotected) cell lines were grown with or without supporting fibroblasts. Cells were treated with (filled bars) or without (shaded bars) 4-OHT, in the presence or absence of PI3 kinase inhibitor (LY294002, 10μM) as indicated. Viability was assessed after 72 hours in culture by trypan blue exclusion. Results shown are based on live:dead counts performed in quadruplicate, expressed as percentage live over total.

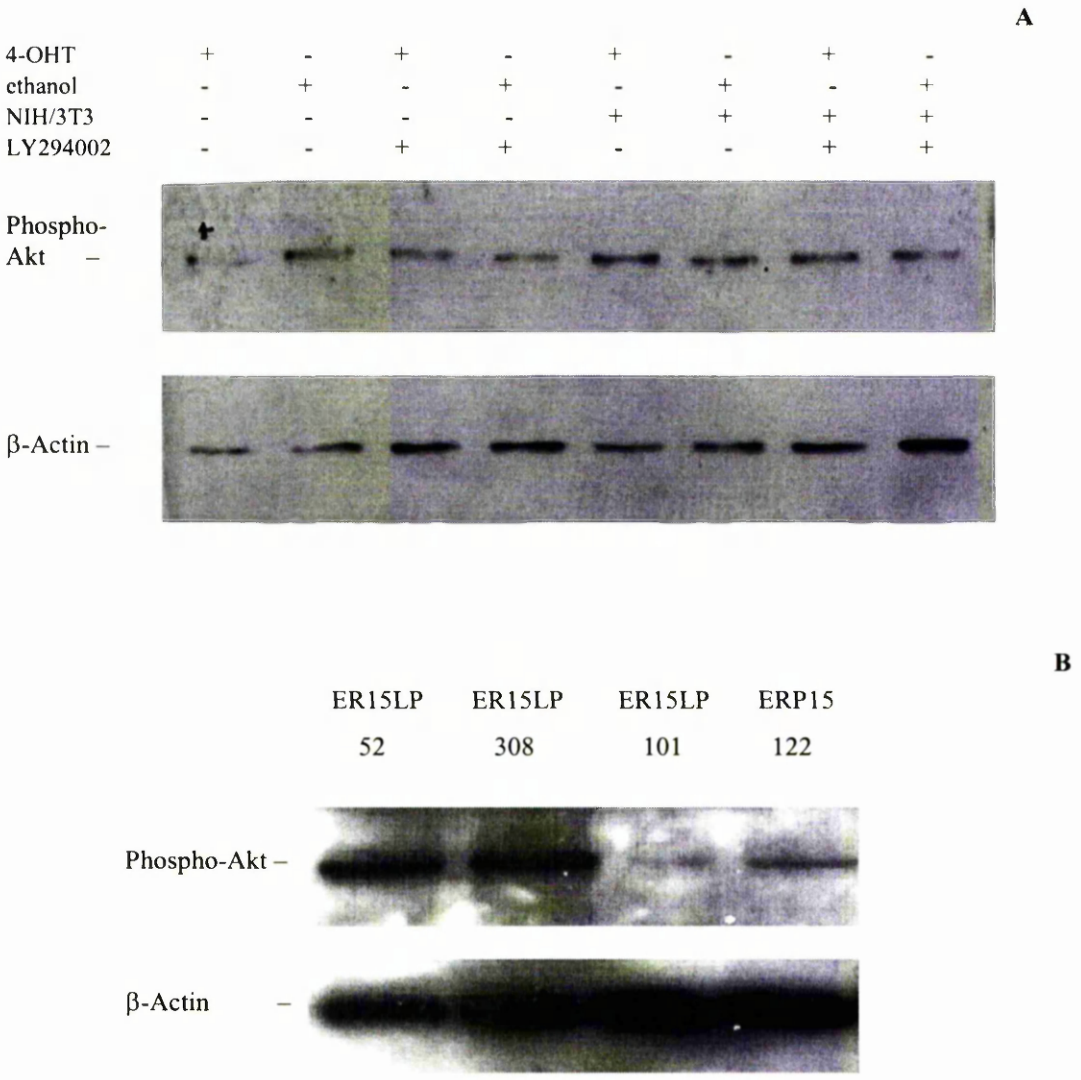


Figure 7.7 Detection of Phosphorylated Akt in CD2-*MYC*ER^{TM} Cell Lines by Immunoblotting

A, Levels of phosphorylated Akt were examined in ER15LP 101 cells, (which are protected from MYC induced apoptosis by cell contact), 18 hours following treatment with 4-OHT or ethanol, with or without PI3 kinase inhibitor, in the presence or absence of NIH/3T3 cells, as indicated. Detection of β-actin is also shown. **B,** Levels of phosphorylated Akt in untreated CD2-*MYC*ER^{TM} cell lines were detected by immunoblotting. Differences in loading were controlled for by detection of β-actin.

7.3 DISCUSSION

7.3.1 Cell Contact Increases Survival of Transformed T Cells *In Vitro*

During *in vitro* studies of transformed CD2-*MYC*ER^{TM} cells, a number of observations led to speculation that cell contact could protect T lymphoma cells against MYC induced apoptosis. Namely, that the likelihood of a cell line establishing when cultured on supporting adherent cells appeared to be increased, and that there was some correlation between resistance to MYC induced apoptosis, and clumpiness of a cell line. It is possible that this phenomenon is particularly observed in CD2-*MYC*ER^{TM} cells lines, because of the reported ability of *MYC* to sensitise cells to a number of death pathways (Juin *et al.*, 1999). Perhaps in MYC overexpressing cells, additional mechanisms are required to protect those cells from background death, enhanced by deregulation of MYC. A number of experiments were carried out to test this hypothesis. CD2-*MYC*ER^{TM} cell lines that had been established with a supporting layer of undefined adherent cells had increased survival in response to MYC upregulation when the adherent cells were present. This protective effect was shown to be a general feature of fibroblasts that was mediated by cell contact and not by soluble survival factors released by the fibroblasts, as demonstrated in the co-culture system.

These results are supported by data from other studies which also suggest that cell contact may protect T cells from apoptosis induced by certain stimuli. During early thymocyte development, before positive and negative selection occur, interaction with fibroblasts in the thymic stroma is essential for the development of certain stages of T cell precursors, and this dependency is mediated by contact through integrins and other adhesion molecules (Anderson *et al.*, 1997b). Experiments have also been described in which co-culture of mature activated T lymphocytes with fibroblasts prolonged the survival of activated T lymphocytes in the absence of mitogenic signals (Scott *et al.*, 1990; Gombert *et al.*, 1996).

The results described in this chapter are reminiscent of the phenomenon of anoikis which was first described as apoptosis induced by the disruption of interactions

between the extracellular matrix and normal epithelial cells (Frisch & Francis, 1994). The same study however showed that anoikis was abrogated when epithelial cells were transformed with a number of oncogenes and suggested that anchorage independence could accompany transformation (Frisch & Francis, 1994). If complete anchorage independence occurred in all tumours however, cell contact might not be expected to enhance survival of transformed cells. Nevertheless protection from apoptosis by cell contact has been observed in tumour cells in other systems, as well as in CD2-*MYC*ER^{TM} cell lines. A study of small cell lung cancer (SCLC) showed that extracellular matrix surrounds these tumours at both primary and secondary sites. Investigation of chemotherapy induced apoptosis in these cells *in vivo* demonstrated an integrin mediated protection from apoptosis, and the authors also reported enhanced survival of these cells *in vitro* when cultured on a feeder layer of fibroblasts (Sethi *et al.*, 1999). It is likely that the way in which tumours evade anoikis will affect the tumour cells ability to be rescued from apoptosis by cell contact. For example some tumour cells may evade anoikis by specific upregulation of adhesion molecules or their downstream targets, while other tumours may have upregulated more general survival pathways, and may yet be subject to protection from apoptosis by cell contact, while still able to survive detachment.

7.3.2 Mechanisms by which Cell Contact Protects Against MYC Induced Apoptosis

Many apoptotic pathways have been implicated in detachment induced apoptosis, and consequently inhibition of those pathways should confer protection on the cells. From the experiments carried out on CD2-*MYC*ER^{TM} cell lines however, it appears that PI3 kinase plays a major role in the protection/survival signal stimulated by cell contact. This conclusion is dependent on the specificity of LY294002 as an inhibitor of PI3 kinase. The use of LY294002 as a recognised specific inhibitor of PI3 kinase is widespread throughout the literature however, and only one case of non-specificity has been reported, expressly inhibition of nitric oxide production in murine astrocytes *in vitro* (Jung *et al.*, 1999). PI3 kinase itself is involved in many different signalling pathways but its anti-apoptotic function is reported to be mediated by the survival kinase Akt (or Protein Kinase B, Franke *et al.*, 1995). Akt itself is able to enhance

survival of cells in which it is activated, by inhibiting a number of apoptotic signals (reviewed by Coffer *et al.*, 1998; Datta *et al.*, 1999; Kandel & Hay, 1999) including caspase 9 activity (Cardone *et al.*, 1998), the pro-apoptotic Bcl-2 family member Bad (Datta *et al.*, 1997; del Peso *et al.*, 1997), and glycogen synthase kinase 3 (GSK-3, Pap & Cooper, 1998). NF-κB activity has also been reported to be induced by activated Akt (Kane *et al.*, 1999), and subsequently shown to suppress TNF induced apoptosis (Burow *et al.*, 2000).

Signalling through the PI3 kinase/Akt pathway has been previously reported to protect fibroblasts from MYC induced apoptosis (Kauffmann-Zeh *et al.*, 1997) and it seems likely that the protection conferred on CD2-*MYC*ER™ cells by cell contact is mediated through the same pathway. There are a number of ways in which cell contact might allow increased survival of T lymphoma cells in response to MYC upregulation. The most likely of these are the pathways controlled by members of the integrin family, which are reported to be of particular importance in anchorage dependence in tumour cells (reviewed by Giancotti & Ruoslahti, *et al.*, 1999). In turn, integrin signalling may be transduced by two major signalling kinases; focal adhesion kinase (FAK) which has been implicated in integrin signalling, and inhibition of which induces loss of attachment and apoptosis in a variety of human tumour cell lines (Xu *et al.*, 1996), and integrin linked kinase (ILK), overexpression of which allows anchorage independent growth and survival of epithelial cells (Radeva *et al.*, 1997). Both of have been shown to activate the PI3 kinase/Akt survival pathway under certain circumstances (Delcommenne *et al.*, 1998; Almeida *et al.*, 2000). In CD2-*MYC*ER™ cell lines however, examination of the levels of phosphorylated Akt could not conclusively show that this pathway was responsible for contact mediated protection. Early results suggest that, at least in this system, the Akt pathway does not play a major role in cell contact mediated protection from MYC induced apoptosis.

7.3.3 Mechanism of Protection May Vary Between Individual Tumours

It may be that the mechanism of protection depends on the genetic lesions which have arisen during tumourigenesis. Both TNF-R family members and p53 have been

reported to be influenced by integrin signalling. Ilic *et al.* (1998) studied the dependence of endothelial cells and fibroblasts on survival signals transmitted through integrins and focal adhesion kinase (FAK), and showed that these were transduced by FAK through a p53 dependent pathway. Further, when p53 was inactivated in these cells, they became anchorage independent and no longer relied on signalling through FAK for survival (Ilic *et al.*, 1998). Activation of Akt in these cells however did not prevent apoptosis induced by the inhibition of FAK, suggesting that loss of FAK/p53 mediated apoptosis does not operate through the PI3K/Akt survival pathway. A recent study in carcinoma cells did suggest that p53 and Akt had overlapping roles in an integrin survival pathway. Specifically, the $\alpha 6\beta 4$ integrin was reported to activate the Akt survival signal in p53 deficient carcinoma cells, but in carcinoma cells that expressed wild-type p53, integrin $\alpha 6\beta 4$ stimulation inhibited this survival pathway by inducing p53 dependent cleavage of Akt (Bachelder *et al.*, 1999). This data might partly explain why cell death was induced so rapidly by inhibition of PI3 kinase in those CD2-*MYC*ER™ cell lines which retained functional p53. It is therefore possible that functional p53 may reduce the survival of CD2-*MYC*ER™ cells *in vitro*, by cleaving Akt and partially inhibiting the PI3 kinase/Akt survival pathway. If PI3 kinase is inhibited in these cells, then cell death may be rapid once no more Akt can be activated by PI3 kinase. It is also possible that in MYC overexpressing cells in which p53 function is retained, survival pathways mediated by PI3 kinase are required to suppress p53 induced apoptosis, and so death is rapidly induced when these survival pathways are inhibited. This hypothesis is supported by a recent study which showed that PI3 kinase could signal through Akt to promote translocation of MDM2 to the nucleus where it would effect ubiquitination and degradation of p53 (Mayo & Donner, 2001). Detection of phosphorylated Akt in protein extracts from ER15LP 52 and ER15LP 308 cells without treatment confirmed that levels of activated Akt were clearly higher than in the untreated cell lines which lacked functional p53 and in which PI3 kinase inhibition was not lethal. Since three cell lines lacking functional p53 exhibited the ability to be protected from MYC induced apoptosis by cell contact, the results do clearly suggest that in these cells at least, anoikis can be p53 independent.

During efforts to establish CD2-*MYC*ER™ cell lines homozygous for the Fas^{lpr} mutation it was observed that these lines were susceptible to detachment induced death, and that survival of these lines was increased by cell contact, implying that Fas:FasL interactions were not involved in anoikis. Fas signalling has also been described as a target for inhibition by cell contact however, although presumably not in cells in which this pathway is lost. Engagement of another member of the integrin family, $\alpha 2\beta 1$, has been reported to protect T cells from activation induced cell death by inhibiting FasL expression via activation of FAK (Aoudjit & Vuori, 2000). The involvement of FADD in promoting anoikis has been described in epithelial cells and in MDCK cells, since dominant negative FADD was reported to block anoikis in these cells (Frisch, 1999; Rytomaa *et al.*, 1999), and more recently, matrix attachment was reported to regulate expression levels of Fas and FasL in endothelial cells, and detachment was shown to induce Fas:FasL interaction, FADD recruitment and activation of caspase 8 (Aoudjit & Vuori, 2001). This study also showed that although anoikis could be blocked in these cells by activation of the PI3 kinase/Akt pathway, this survival effect was not mediated through regulation of expression of Fas or its ligand (Aoudjit & Vuori, 2001). Other studies have demonstrated a link between the Fas pathway and PI3 kinase. Ras activation of the PI3 kinase/Akt survival pathway for example has been reported to mediate survival by downregulation of Fas expression in fibroblasts and epithelial cells (Peli *et al.*, 1999).

There may be many other death and survival pathways regulated by cell contact. Recent research in mammary carcinoma cells showed that TRAIL may be involved in anoikis, since expression of TRAIL and susceptibility to TRAIL induced apoptosis were suppressed in anchored cells compared to unattached cells (Goldberg *et al.*, 2001). The tumour suppressor protein PTEN has also been linked with both anoikis and the PI3/Akt survival pathway. PTEN was shown to induce anoikis in breast cancer cells (Lu *et al.*, 1999), and a number of studies have reported that Akt is negatively regulated by PTEN (Haas-Kogan *et al.*, 1998; Stambolic *et al.*, 1998; Dahia *et al.*, 1999; Tamura *et al.*, 1999).

In the CD2-*MYC*ER™ cell lines that were tested for response to cell contact mediated protection and inhibition of PI3 kinase, a variety of results were generated. In the

cell lines which were protected by contact with fibroblasts, inhibition of PI3 kinase resulted in loss of that protection, suggesting that at least in *MYC* induced T cell tumours, contact mediated protection from MYC induced apoptosis was dependent on signalling through PI3 kinase. It is not clear yet how that signalling progressed in these cells, and whether the survival signals were the same in each case. It is apparent however that some cell lines are more sensitive to inhibition of PI3 kinase activity than others, and that may be dependent on which apoptotic pathways are functional, and on the extent that upregulation of survival signalling contributed to transformation in those cells. For instance, cell lines in which p53 function was still detected were highly sensitive to PI3 kinase inhibition, suggesting that viability of these cells may be highly dependent on overexpression of survival signalling pathways. It is also clear that some cell lines may have become anchorage independent either *in vivo*, or during establishment *in vitro*, since a basal layer of fibroblasts confers no protection from MYC induced apoptosis on these cells. These cells may have lost anchorage dependent apoptotic pathways during tumourigenesis, or have deregulated expression of survival signals downstream of PI3 kinase. In tumours which remain anchorage dependent, cell contact *in vivo* must confer a considerable growth advantage. The results suggest that the potential of cell death and survival pathways differs between individual tumours, and emphasise that tumourigenesis is a multi-step process. Even in tumours of the same cell type, induced by the same *MYC* transgene, there may be many different genetic lesions selected for mutagenesis during the progression towards malignancy.

CHAPTER 8

ACTIVATION INDUCED PROLIFERATION IS DIMINISHED IN T CELLS FROM FAS^{lpr} MICE

8.1 INTRODUCTION

8.1.1 T Cell Receptor Activation and the Role of Fas Signalling

Activation induced cell death has been described in detail in Chapter 1. In order for homeostasis to be maintained within the T cell compartment, antigenic activation through the T cell receptor (TCR) must first result in proliferation and expansion of the T cell population, and also in deletion of previously activated cells to prevent accumulation of these cells in the organs of the lymphoid system (reviewed by Kabelitz *et al.*, 1993). A number of groups have demonstrated that activation induced death of T cells is dependent on Fas:FasL signalling (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). It seems likely therefore that the apoptotic response to TCR activation may be defective in thymocytes from Fas^{lpr} mice compared with cells from control mice. What is less clear however is whether the non-apoptotic response of T cells to activation is altered by the loss of Fas signalling, and whether thymocytes from Fas^{lpr} mice are otherwise normal in their response to activation.

8.1.2 Anti-CD3 Antibodies Simulate T Cell Receptor Activation *In Vitro*

The TCR complex consists of a heterodimer of alpha and beta transmembrane polypeptide chains which each have constant and variable domains, associated with the invariant CD3 complex. The TCR allows recognition of specific antigen, while the CD3 complex signals to the cell that antigen binding has occurred. Engagement of the CD3 complex on immature mouse thymocytes, with antibodies to CD3 was shown to result in death of the treated cells through an endogenous apoptotic pathway (Smith *et al.*, 1989). These findings suggested that crosslinking of CD3 on

T cells with anti-CD3 antibodies mimicked the effect of antigenic activation of the TCR complex. Antibodies against CD3 are now widely accepted to reproduce the effects of TCR activation.

8.1.3 Experimental Aims

The experiments described in this chapter were carried out to investigate the consequences of TCR activation in thymocytes explanted from healthy control and Fas^{lpr} mice. Since activation can lead to both proliferation and cell death, it may have consequences for the development and progression of T cell lymphoma. It was therefore necessary to examine whether activation induced cell death was defective in Fas^{lpr} thymocytes in this system. It might be expected that lack of activation induced cell death would result in increased incidence or reduced latency of T cell lymphomas in Fas^{lpr} animals. The results presented in Chapters 3 and 4 show that this was not the case, at least in this model. Therefore it was also important to examine the non-apoptotic response in Fas^{lpr} thymocytes, compared to those from control mice, since studies have suggested that cell death may not be the only consequence of T cell activation (Malissen & Schmitt-Verhulst, 1993). If there was a defect in the proliferative response to activation in Fas^{lpr} T cells, then this could affect lymphoma development in these animals. Finally, it was also of interest to assess whether there was any correlation between cell surface markers and response to activation since response to activation may also vary between cells of different developmental stage and cell surface phenotype.

8.2 RESULTS

8.2.1 T Cell Activation Results in Cell Death and Proliferation

The Fas signalling pathway has been widely reported as an important mediator of activation induced cell death in T cells (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). Activation induced cell death can be studied *in vitro* using plate bound anti-CD3 antibodies which cross-link the CD3 co-receptor,

and represent activation through the TCR, or antigen induced activation. This method was used to study defects in activation induced cell death in Fas^{lpr} cells.

To investigate differences in the level of activation induced cell death in Fas^{lpr} T cells it was first necessary to investigate the effects of CD3 crosslinking in normal healthy T cells. Healthy control MRL strain mice were sacrificed at 3 - 8 weeks of age, and their thymii removed. Single cell suspensions of thymocytes were prepared, and these were incubated on anti-CD3 antibody, or isotype-matched control antibody coated 96-well plates. The preparation of these plates is described in section 2.5. Cell viability was assessed at 24 hour intervals over a 144 hour period, by the trypan blue exclusion method. During the initial 72 hours in culture, cell viability was assessed every 12 hours, to observe at what time-point death induction by CD3 crosslinking was most significant. Figure 8.1A shows pooled results of 3 identical experiments.

Under these conditions of T cell activation, control thymocytes undergo significantly accelerated cell death for 48 hours following CD3 crosslinking, compared to those incubated with isotype-matched control antibody ($P<0.01$ at every time point between 12 hours and 48 hours). The most significant induction of death in anti-CD3 treated cells, compared with the background death of isotype matched control treated thymocytes occurred at 36 hours. Induction of cell death in these cells appears to be maximal at around 48 hours in culture following crosslinking. The figure shows that after 48 hours, the percentage of viable T cells starts to increase, and continues to increase until at least 144 hours following CD3 stimulation. In contrast, thymocytes treated with isotype matched control antibody continue to lose viability. There was significantly increased viability in the anti-CD3 treated thymocytes, compared to the isotype-matched control treated thymocytes at every time point from 60 hours until 144 hours after CD3 crosslinking ($P<0.01$).

From studying thymocyte viability alone, it was not possible to say whether the increased viability observed in anti-CD3 treated thymocytes after 60 hours in culture was due to proliferation of live cells, or loss of dead cells from the culture. From studying the total live cell numbers however, it appears that the increased survival is

as a result of induction of proliferation in these cells. Figure 8.1B shows the pooled results of three identical experiments. For the first 60 hours following CD3 crosslinking, the number of live cells in culture decreases, after which time, the total number of live cells starts to increase and after 144 hours exceed the number of live T cells originally set up in culture. This is in contrast to the isotype-matched control antibody treated cultures, in which the number of live cells continues to diminish over the experimental period. These results indicate that the increased viability of anti-CD3 stimulated thymocytes after the initial induction of death is due to proliferation.

8.2.2 Loss of Fas Signalling Results in Loss of Activation Induced Cell Death and Proliferation

Studies have shown that crosslinking of CD3 induces apoptosis in T cells by upregulation of Fas and FasL expression (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). It follows then, that in Fas^{lpr} thymocytes, where the Fas pathway is abrogated, CD3 crosslinking should fail to stimulate apoptosis. Thymocytes from age, strain and sex-matched 3-6 week old control and Fas^{lpr} mice were incubated with plate-bound anti-CD3 antibody and their viability determined over a period of 144 hours.

As seen in previous experiments, treatment of control thymocytes with plate-bound anti-CD3 antibody resulted in enhanced cell death in the first 48 hours in culture, and then increased cell survival and total live cell numbers from 72 hours following treatment. Representative survival curves for (A) control MRL and (C) Fas^{lpr} thymocytes, and curves for total live cell number for (B) MRL and (D) Fas^{lpr} thymocytes, are shown in Figure 8.2. The results were reproducible in 6 similar experiments. As the literature suggests, treatment with anti-CD3 antibody failed to induce apoptosis in Fas^{lpr} thymocytes (Russell *et al.*, 1993). These findings suggest that Fas signalling is required for activation induced cell death of T cells. This result is not surprising given the reported simultaneous upregulation of Fas and FasL and induction of cell death in response to anti-CD3 stimulation (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). Interestingly however, the

proliferation induced by CD3 crosslinking in control thymocytes was also blocked in Fas^{lpr} thymocytes, as the total number of live cells steadily declined. Although Fas^{lpr} thymocytes were not susceptible to activation induced cell death, and despite the fact that Fas^{lpr} T cells accumulate *in vivo* (Cohen & Eisenberg, 1991), there was no detectable increase in viability or number of live Fas^{lpr} cells following CD3 crosslinking. Further, isotype-matched control antibody treated Fas^{lpr} thymocytes did appear to have diminished survival *in vitro* compared to control MRL thymocytes. This was in line with observations made in other experiments during the course of this study, in which the background death of explanted Fas^{lpr} cells appeared to be higher compared to control cells.

8.2.3 Enhanced Survival of Normal T Cells in Response to CD3 Crosslinking is Due to Increased Proliferation

The most likely explanation for the increase in cell viability and live cell number of control MRL thymocytes following treatment with anti-CD3, was proliferation, thus indicating a proliferative defect in Fas^{lpr} thymocytes. To confirm this hypothesis, proliferation assays were carried out on these cells. Thymocytes were explanted from age-matched 3-6 week-old control C57/CBA and MRL strain mice, which is the control strain for Fas^{lpr} mice, from Fas^{lpr} mice, and from Bcl-2 transgenic mice (generously donated by Prof. S.J. Korsmeyer). Fas^{lpr} mice have been described in detail previously. The Bcl-2 transgenic mice harbour a human Bcl-2 transgene on the lck promoter, which targets expression to the T cell lineage (Linette *et al.*, 1995). Bcl-2 transgenic thymocytes were included to determine whether activation would induce a different response in these cells, since they overexpress a survival factor, in contrast to Fas^{lpr} cells which lack a death pathway. Thymocytes were incubated in 96 well plates coated with either anti-CD3 antibody or with isotype-matched control antibody. Following 72 hours in culture, a $[^3H]$ thymidine incorporation proliferation assay was carried out. This assay depends on the uptake of thymidine into dividing cells during DNA replication. Cells which are proliferating should accumulate $[^3H]$ thymidine, and the radioisotope can then be detected by scintillation counting.

The results of this experiment are shown in Figure 8.3. As predicted from the results generated in previous experiments, CD3 crosslinking on control C57/CBA and MRL thymocytes results in significantly increased proliferation after 72 hours in culture, compared to isotype-matched control antibody treated cells in which proliferation is negligible ($p<0.01$). In contrast, induction of proliferation in response to anti-CD3 activation of Fas^{lpr} thymocytes was very low, and significantly impaired compared to both C57/CBA and MRL control thymocytes ($P<0.05$). It seemed reasonable to conclude from these studies that activation induced cell death and proliferation could be induced in normal thymocytes by CD3 stimulation *in vitro*. Not only was activation induced cell death blocked in Fas^{lpr} thymocytes however, these thymocytes also exhibited a proliferative defect in response to CD3 crosslinking. The data also suggest that Bcl-2 transgenic thymocytes have reduced proliferation in response to CD3 crosslinking, compared with control C57/CBA and MRL thymocytes, although in this case the difference was not significant. This is in keeping with previous reports of impaired proliferation in Bcl-2 overexpressing cells (Grierson *et al.*, 1995). The proliferative defect in these cells however was not nearly as striking as that observed in Fas^{lpr} thymocytes. These data may have implications for the low tumour frequency in Fas^{lpr} animals. If Fas:FasL interactions contribute to the anti-CD3 mediated proliferative response, then perhaps any loss of cell death in Fas^{lpr} cells could be compensated by loss of cell activation.

8.2.4 CD3 and CD28 Co-Stimulation Relieved Block in Activation Induced Cell Death and Proliferation in Fas^{lpr} Thymocytes

Although results obtained from studies of anti-CD3 activation have been informative, CD3 crosslinking alone does not promote full activation of T cells. Co-stimulatory signals or other mitogenic stimuli are required for full activation (Chambers & Allison, 1997). One such co-stimulatory signal is ligation of the surface molecule CD28 (Turka *et al.*, 1990), described in more detail in Chapter 1. Co-stimulation with plate-bound antibodies to CD3 and to CD28 has been reported to be essential for full T cell activation (Jenkins, 1994). With this in mind, experiments were set up to investigate the effects of CD3 and CD28 co-stimulation on activation of T cells from control and Fas^{lpr} mice. Antibodies to CD3 and/or CD28 or their isotype-

matched control antibodies were bound to flat-bottomed 96 well plates as before, and thymocytes explanted from either control MRL or Fas^{lpr} mice were cultured on these over a 144 hour period. The viability and total number of live cells was assessed at 24 hour intervals by trypan blue exclusion.

In keeping with previous results, anti-CD3 antibody alone resulted in increased cell death and subsequent proliferation in control thymocytes, while having no effect on the viability of Fas^{lpr} thymocytes. Figure 8.4 shows the results of these experiments. Crosslinking with anti-CD28 antibody alone had no significant effect on the viability of thymocytes from either control or Fas^{lpr} mice. In contrast to CD3 crosslinking alone however, stimulation of both CD3 and CD28 together resulted in rapid and significantly increased cell death in both control and Fas^{lpr} thymocytes. Figure 8.4 shows that this cell death was significantly increased compared to both isotype-matched control and anti-CD3 treated thymocytes after 24 hours, in both MRL ($P<0.01$) and Fas^{lpr} cultures ($P<0.05$). Further, following this initial induction of death, proliferation assessed by live cell number, was observed in both control MRL and Fas^{lpr} thymocyte cultures stimulated with anti-CD3 and anti-CD28 together. The total number of live Fas^{lpr} cells 144 hours following CD3/CD28 crosslinking together, was significantly increased compared to both anti-CD3 treated and isotype-matched control antibody treated cells ($P<0.01$), (Figure 8.4D). There was no difference in viability or live cell number of control MRL thymocytes at 144 hours between anti-CD3/CD28 and anti-CD3 treated cells. The percentage of live cells in anti-CD3/CD28 co-stimulated cultures started to increase more rapidly however (after 48 hours) than in anti-CD3 stimulated cultures, as shown in Figure 8.4B. The graphs also show that anti-CD3 mediated activation induced cell death, and proliferation, appear to occur faster in MRL thymocytes than in the previous experiment (see Figure 8.2). In addition, by 144 hours following incubation with anti-CD3, the viability and live cell total of the anti-CD3 and anti-CD3/CD28 treated MRL cells, appear to level out. It seems that the initial response to activation is elevated compared to the previous experiment, and that background death is decreased, but that by 144 hours, the proliferative response is weakened. It was also observed that by 144 hours, the total number of live anti-CD3 stimulated Fas^{lpr} thymocytes was slightly increased compared with isotype matched control antibody

treated cells. The reason for this observation is not clear, but is consistent with the decreased background death in this experiment compared with the previous experiment. Perhaps, a small population of Fas^{lpr} thymocytes that showed a limited response to anti-CD3 activation survived the initial stages of culture in this experiment. Nevertheless, the proliferative defect in Fas^{lpr} thymocytes in response to CD3 crosslinking was still clearly observed. The kinetics of activation induced cell death and proliferation may vary slightly between each experiment, however the trend is unchanged. The results suggest that CD3/CD28 crosslinking activates other pathways in addition to the Fas pathway to induce cell death in thymocytes, and also that proliferation can be induced through different mechanisms depending on the initial activational stimuli. Co-stimulation of CD28 may allow CD3 signalling to recruit more efficient, Fas independent pathways.

8.2.5 Phenotype of Thymocyte Populations Following CD3 Crosslinking

During T cell development, cells are defined by their expression of either the CD4 or CD8 co-receptor molecules (reviewed by Ellmeier *et al.*, 1999). Immature progenitor T cells bearing neither surface marker, give rise to progenitor double positive T cells which express both CD4 and CD8. 95% of these double positive thymocytes die in the thymus through neglect (reviewed by Cresswell, 1998). Differentiation to mature single positive thymocytes occurs through positive selection of cells that express TCRs with appropriate affinity for MHC/peptide complexes. CD4 single positive thymocytes express TCRs specific for MHC class II, and CD8 single positive thymocytes express TCRs specific for MHC class I.

A number of recent studies have implied that the response of both immature and mature T cells to CD3 crosslinking or activation through the TCR might differ between cells of different surface phenotype (Basson *et al.*, 1998; Veiga-Fernandes *et al.*, 2000). In addition, further studies of Fas:FasL interactions have highlighted possible differences between cell types in their response to anti-CD3 treatment. Proliferation of T lymphocytes from Fas^{gld} mice which lack functional FasL, in response to anti-CD3 treatment was shown to be depressed compared to those from control mice, and this was shown to be a result of depressed proliferation of CD8

single positive T cells (Suzuki & Fink, 1998). To investigate this idea further in connection with the results presented so far, cultures were set up of explanted control thymocytes in anti-CD3 or isotype control antibody coated 96 well plates. The cells from replicate cultures were pooled at the 0 hour, 48 hour, 72 hour and 144 hour time points in order to perform cell surface analysis. Cells were labelled with antibodies to CD3, CD4 and CD8, or with isotype-matched control antibodies and analysed by flow cytometry.

The results of the analysis of CD4 and CD8 profile are shown in Figure 8.5A. The data show that the CD4/CD8 surface phenotype of control thymocytes changes over the 144 hour period in culture with anti-CD3 stimulation. The percentage of CD4/CD8 double positive thymocytes in the total population decreases over the course of the experiment from 84% to 49%, while the percentage of CD8 single positive thymocytes increases from 3% to 40%. Conversely, the proportions of CD4 single positive thymocytes and double negative thymocytes remain relatively constant throughout the time in culture. There are a number of possible explanations for this phenomenon. As in the thymus during T cell development (reviewed by Ellmeier *et al.*, 1999), double positive cells may differentiate *in vitro*. The growing CD8 single positive population may arise from downregulation of CD4 on double positive thymocytes, thereby accounting for the decrease in the fraction of double positive cells in the total population. What is not clear however, is why the relative level of CD4 single positive cells does not increase. This may be explained by differential responses between CD4 and CD8 single positive cells to activation. It is possible that CD4 thymocytes are not as susceptible to activation induced proliferation as CD8 single positive thymocytes, and therefore the CD8 population proliferates, at the expense of the CD4 single positive population. It is also feasible that CD4 positive cells are more likely than CD8 single positive thymocytes to die in response to activation, and therefore unable to expand as a population, at least *in vitro*. Undoubtedly the explanation for this phenomenon may be a combination of a number of theories. It is entirely reasonable to suggest that the same population of thymocytes may be acutely sensitive to activation induced cell death and proliferation. The signals determining the decision to die or proliferate in cells of similar phenotype are likely to be more complex.

Figure 8.5B shows the relative percentages of CD3 positive cells, as a fraction of the total population, and as a fraction of the live population, as estimated by forward scatter. Over 144 hours in culture, the proportion of thymocytes bearing CD3 was slightly reduced, from 96 to 86%, however in the live population, the fraction of CD3 positive cells decreased from 96% to 65%. Again, there may be a number of explanations for this trend. One possible explanation may be that in this system, surviving or proliferating cells have downregulated surface CD3 as a survival measure. It is also conceivable that cells with low surface CD3 expression are preferentially selected for survival and proliferation, while high CD3 expression marks thymocytes for activation induced cell death. More subtle analysis of the level of CD3 expression on these thymocytes would be required to determine the reason for these results.

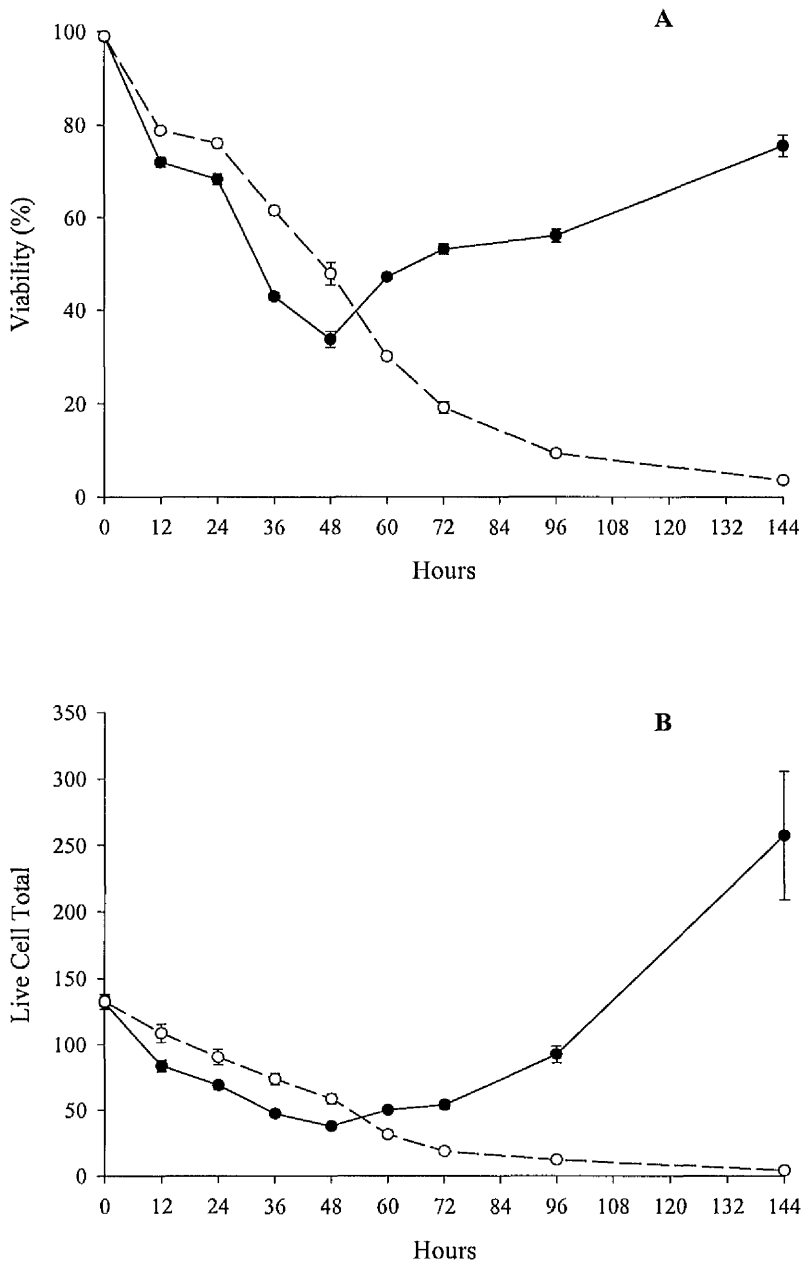


Figure 8.1 Viability and Proliferation of Control Thymocytes at 12 Hour Intervals Over 144 Hours Following CD3 Crosslinking

Thymocytes explanted from control mice were incubated in anti-CD3 (solid line, filled circles) or isotype-matched control antibody coated 96-well plates (broken line, open circles). Antibody was used at 10 μg/ml and cells were cultured at 1.5 x 10^6 cells/ml in flat-bottomed wells. Viability was assessed every 12 hours over a 72 hour period, and thereafter at the time points indicated. Results represent live:dead counts performed in triplicate, expressed as **A**, percentage live over total cells, and **B**, total live cell number. Results represent the average of pooled data from 3 experiments. Error bars show standard errors.

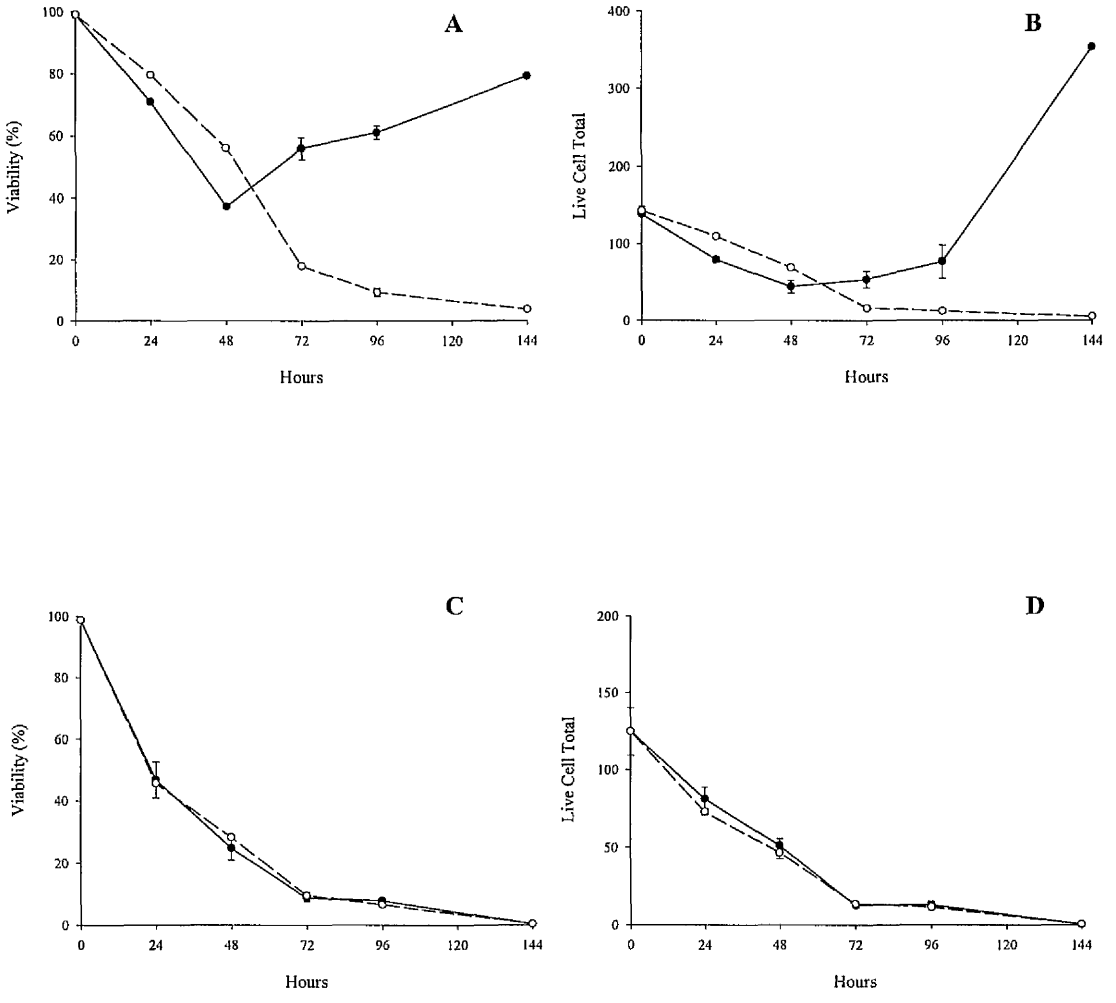


Figure 8.2 Viability of Control MRL and Fas^{lpr} Thymocytes Following CD3 Crosslinking

Thymocytes explanted from control mice (**A** and **B**) or Fas^{lpr} mice (**C** and **D**) were incubated in anti-CD3 (solid line, filled circles) or isotype-matched control antibody (broken line, open circles) coated 96-well plates. Antibody was used at 10 μg/ml and cells were cultured at 1.5 x 10^6 cells/ml in flat-bottomed wells. Viability and total live cell number was assessed over a 144 hour period. Results in **A** and **C** represent live:dead counts, expressed as percentage live over total, performed in triplicate. **B** and **D** show total number of live cells, as an average of triplicate results.

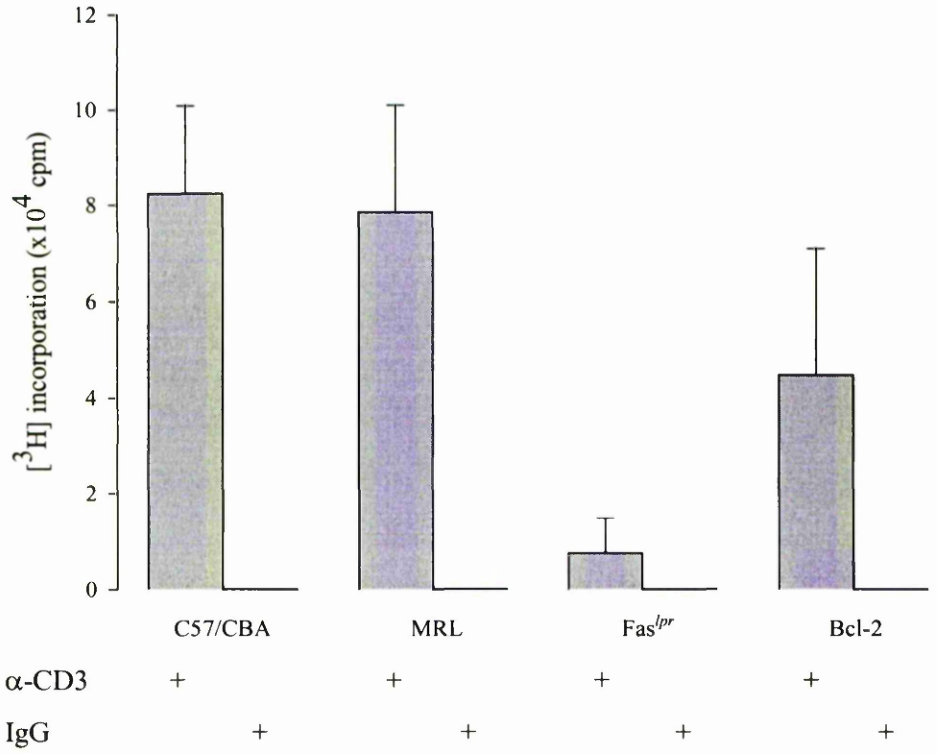


Figure 8.3 Proliferation of Control and Fas^{lpr} Thymoctyes Following CD3 Crosslinking

Thymocytes explanted from C57/CBA, MRL, Fas^{lpr} and Bcl-2 transgenic mice were incubated at 1.5 x 10^6 cells/ml in anti-CD3 (10μg/ml, grey bars) or isotype-matched control antibody (black bars) coated 96 well flat-bottomed plates, as indicated. Following 72 hours incubation, cells were pulsed with [^{3}H] thymidine. Proliferation of cells was measured by incorporation of [^{3}H] thymidine over a 16 hour period, and recorded release of radioactive counts from each cell culture. Results show mean of counts performed in quadruplicate.

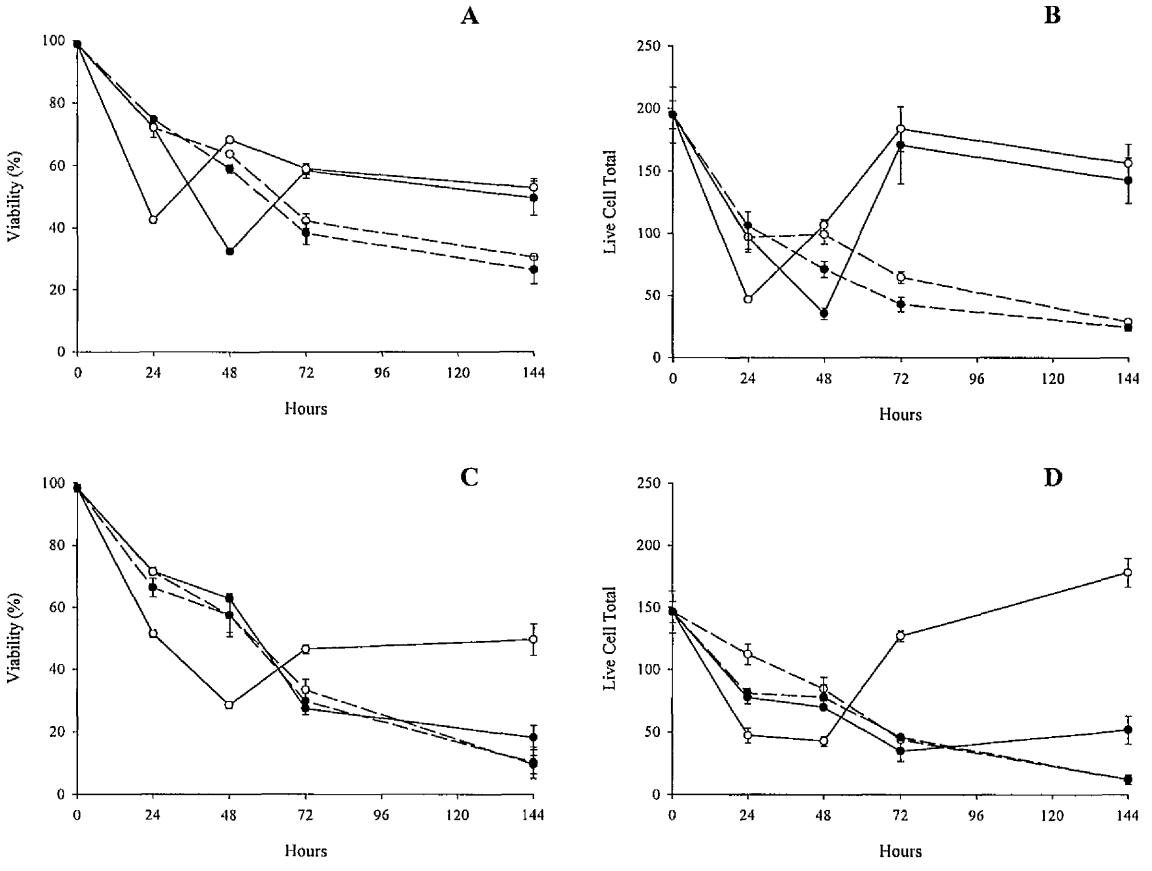


Figure 8.4 Viability of Control and Fas^{lpr} Thymocytes Over 144 Hours Following CD3/CD28 Crosslinking

Thymocytes explanted from control mice (**A** and **B**) or Fas^{lpr} mice (**C** and **D**) were incubated in anti-CD3 (solid line, filled circles), anti-CD28 (broken line, open circles), both anti-CD3 and anti-CD28 (solid line, open circles) or isotype control antibody (broken line, closed circles) coated 96-well plates. Antibodies were used at 10 μg/ml and cells were cultured at 1.5 x 10^6 cells/ml in flat-bottomed wells. Viability (**A** and **C**) was assessed at 24 hours intervals over a 144 hour period. Results represent live:dead counts, performed in triplicate, expressed as an average percentage of live over total. **B** and **D** show curves for total cell numbers, as an average of triplicate results.

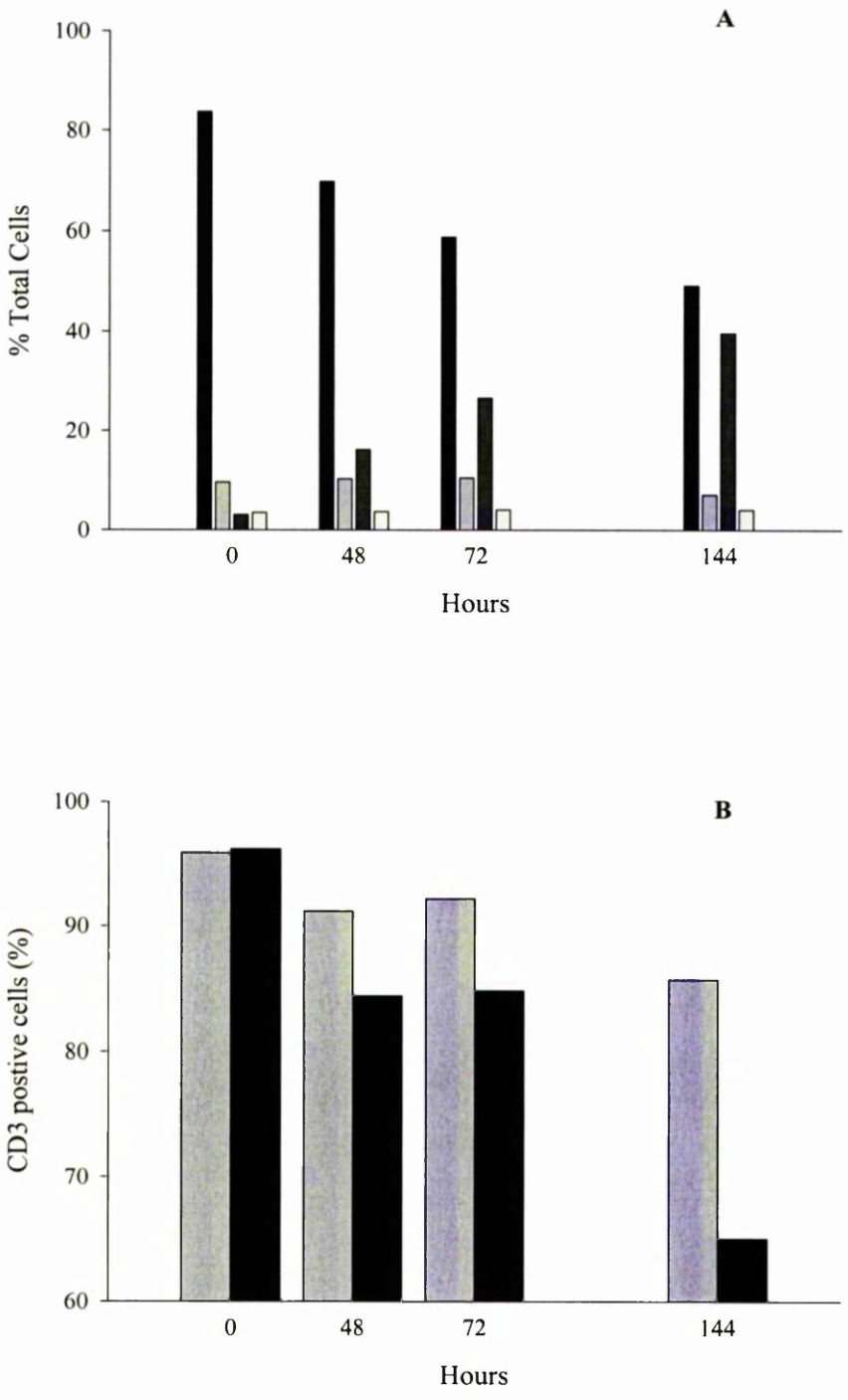


Figure 8.5 Phenotype of CD3 Stimulated Control MRL Thymocytes

Thymocyte populations over a course of time following CD3 crosslinking. **A,** shows CD4/CD8 double positive (black bars), CD4 positive (mid-grey bars), CD8 positive (dark grey bars) and double negative (light grey) thymocytes as a percentage of the total cell population. **B,** shows CD3 positive cells as percentage of the total population (grey bars), or of the live cell population (black bars).

8.3 DISCUSSION

8.3.1 Deficiency in Activation Induced Cell Death in Fas^{lpr} T cells

The results generated from investigation of activation induced cell death in control and Fas^{lpr} thymocytes demonstrated that thymocytes lacking the Fas pathway were defective in their apoptotic response to activation, or at least to stimulation by CD3 crosslinking. These studies were carried out on thymocytes from young disease-free animals. The results are consistent however, with other reports of anti-CD3 induced cell death being defective in splenocytes from Fas^{lpr} mice (Russell *et al.*, 1993), and of lack of response to TCR stimulation in the abnormal T cell population in the periphery of Fas^{lpr} mice (Sy *et al.*, 1988). The results also fit in with previous reports of activation induced cell death being mediated by Fas:FasL interactions (Ramsdell *et al.*, 1994; Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). Further studies have described the upregulation of Fas and FasL, and subsequent cell death following TCR activation (Brunner *et al.*, 1996; Latinis *et al.*, 1997; Oberg *et al.*, 1997), and also activation of the Fas pathway caspases 8 and 3 (Jiang *et al.*, 1999). Although other apoptotic molecules, most recently that transcribed by the p53 related gene, p73, have been implicated in TCR mediated apoptosis (Irwin *et al.*, 2000; Lissy *et al.*, 2000), no evidence of a Fas independent pathway was observed in the above studies.

8.3.2 Fas^{lpr} T Cells are Deficient in T Cell Receptor Activation Induced Proliferation

The results of investigating TCR activation in control and Fas^{lpr} T cells not only demonstrated the lack of activation induced cell death when the Fas pathway is absent but also highlighted a potential role for Fas:FasL interactions in activation induced proliferation, suggesting that Fas may not simply signal through a death pathway. Following an initial induction of death in control thymocytes, TCR activation was also shown to induce proliferation, however neither increased death nor proliferation were observed in Fas^{lpr} thymocytes. The result highlighted a proliferative defect in response to activation by CD3 crosslinking in thymocytes

lacking functional Fas. The explanation for this is not clear. No previous reports have been made of a defect in activation induced proliferation in thymocytes from young Fas^{lpr} animals, however evidence presented in other reports has implied involvement of Fas:FasL interactions in the proliferation of T cells.

Although the T cells which accumulate in older Fas^{lpr} mice are of abnormal phenotype (Morse *et al.*, 1982), and are functionally very limited in response to antigenic stimulation (Davignon *et al.*, 1985, reviewed by Altman, 1994), this does not explain the aberrant response to CD3 crosslinking that was observed in the experiments carried out in this chapter, since the thymocytes were explanted from young Fas^{lpr} animals before the emergence of a large population of phenotypically abnormal cells. The data here show a proliferative defect in response to CD3 crosslinking in thymocytes from young 'healthy' Fas^{lpr} animals. These results are supported by an earlier investigation into mitogenic stimulation of splenocytes from Fas^{lpr} mice, which showed a decreased proliferative response even in young mice, which became more pronounced in ageing mice (Froidevaux *et al.*, 1991).

A role for Fas in activation induced proliferation is also supported by a study of antibodies against Fas, which demonstrated that an immobilised anti-Fas antibody, rather than inducing apoptosis was able to co-stimulate anti-CD3 antibody mediated proliferation of human T cells *in vitro* (Alderson *et al.*, 1993). Additional evidence that Fas may act as a co-stimulatory receptor was generated in an *in vivo* study which showed that during thymocyte development, the Fas^{lpr} mutation caused a decrease in the number of CD4/CD8 double positive cells in the thymus, and in total thymic cellularity, and suggested that Fas signalling was involved in the generation and positive selection of CD4/CD8 T cells (Kurasawa *et al.*, 2000). Furthermore, recent studies have suggested a role for caspases in T cell proliferation. Kennedy *et al.* (1999) reported that caspase 8 activation, possibly via TCR mediated upregulation of FasL, was required for T cell proliferation. Caspase 8 was cleaved rapidly after CD3 crosslinking of Jurkat T cells, and inhibition of caspase 8 activation caused a block in CD3 induced proliferation (Kennedy *et al.*, 1999). The study also showed that soluble FasL was able to augment CD3 induced proliferation of human T cells (Kennedy *et al.*, 1999).

Further evidence associating Fas:FasL interactions with T cell proliferation was found in studies of mice deficient in FADD. In common with our system, activation induced proliferation *in vitro* was shown to be impaired in FADD null T cells (Zhang *et al.*, 1998), and in T cells expressing a dominant negative FADD (Newton *et al.*, 1998; Strasser & Newton, 1999). Thymocytes from FADD-DN transgenic mice were also found to have impaired proliferation *in vivo*, and activation induced proliferation of these cells *in vitro* was inhibited in a p53 dependent manner (Zornig *et al.*, 1998). If the block in activation induced proliferation in Fas^{lpr} mice is dependent on p53, this might explain why in Fas^{lpr} mice null for *Trp53*, lymphoproliferation appears to be enhanced and accelerated, compared to that observed in Fas^{lpr} mice wild type for *Trp53* (see Chapter 6, Cameron *et al.*, unpublished).

The involvement of Fas signalling in proliferation has been observed in systems other than activated T cells. Interestingly, although Fas specific antibodies are normally lethal in mice due to induction of hepatic failure, following partial hepatectomy in mice, anti-Fas stimulation accelerated regeneration of the liver. Further, liver regeneration was delayed in Fas^{lpr} mice suggesting that Fas engagement may promote proliferation of hepatocytes (Desbarats & Newell, 2000). Fas signalling has also been implicated in proliferation of fibroblasts. Aggarwal *et al.* (1995) demonstrated that engagement of the Fas receptor on human diploid fibroblasts resulted in proliferation of those cells in a dose dependent manner (Aggarwal *et al.*, 1995). A later study demonstrated that the anti-Fas antibody could induce either proliferation or cell death of human dermal fibroblasts, depending on the level of surface Fas expression (Freiberg *et al.*, 1997). These results were again reinforced by studies in FADD deficient cells. Fibroblasts expressing dominant negative FADD also showed a decreased proliferative capacity, suggesting that this property of Fas signalling is not restricted to T cells (Hueber *et al.*, 2000).

Finally, Fas has been implicated as a growth promoter in some tumour cells. In one study investigating the possibility of exploiting anti-Fas antibodies as anti-tumour therapies, growth of a mitogen activated B cell tumour cell line was reported to be significantly enhanced by treatment with anti-Fas antibody (Owen-Schaub *et al.*, 1993). Further, a study characterising two T cell hybridomas generated from Fas^{lpr}

mice showed that in addition to lacking the potential to undergo activation induced cell death in response to anti-CD3 activation, cells from one of these lines were also temporarily growth arrested at the border between G1 and S phase, although the block was overcome in some cells which eventually proliferated (Cui *et al.*, 1996). As well as reinforcing the results in this chapter, these data may also explain why establishment of lymphoma cell lines homozygous for the Fas^{lpr} mutation was so unsuccessful.

8.3.3 Mechanisms by Which Fas:FasL Interactions May Influence Proliferation

Although the results shown in this chapter demonstrate a role for Fas:FasL interactions in T cell proliferation, they do not illustrate how these molecules might regulate proliferation. The fact that CD8+ single positive thymocytes appear to be most susceptible to activation provides a clue as to how Fas:FasL interactions may function. A recent study reported data concerning the reverse signalling capacity of FasL in CD8+ cytotoxic T lymphocytes (Suzuki & Fink, 1998). In that report, murine CTL lines lacking FasL were shown to have diminished proliferation in response to antigenic activation, however CD4+ cells had a normal proliferative response (Suzuki & Fink, 1998). Further, blocking Fas:FasL interactions also caused a diminished proliferative response to antigenic stimulation in CTLs, while stimulating FasL on CTLs using plate-bound Fas-Ig was able to co-stimulate sub-optimal anti-CD3 activated proliferation (Suzuki & Fink, 1998). These results suggest that Fas may be the stimulatory ligand which induces a proliferative signal through FasL.

It is reasonable to speculate that in the experiments carried out in this chapter, activation achieved through CD3 crosslinking was sub-optimal, and that may explain why proliferation was not induced in thymocytes in which FasL ligation could not occur. Some evidence to support these data has been generated in mice expressing a T cell specific dominant negative TGF-β receptor (Lucas *et al.*, 2000). These mice develop a CD8+ T cell lymphoproliferative disorder, which is due to increased proliferation of CD8 single positive T cells, and not resistance to activation induced

cell death as in Fas^{lpr} mice (Lucas *et al.*, 2000). This study is particularly significant in light of the fact that TGF-β is reported to inhibit FasL expression (Genestier *et al.*, 1999), suggesting that in mice lacking TGF-β function, there may be enhanced levels of FasL. These results add to the evidence which indicates that CD8+ T cells are particularly susceptible to proliferation through FasL stimulation. In contrast, a separate study showed that engagement of FasL on CD4+ single positive cells led to cell cycle arrest and death (Desbarats *et al.*, 1998). From the experiments carried out in this chapter, activation had no visible effect on the CD4+ single positive population, but these cells may have been arrested. Nevertheless it is apparent that CD8+ single positive cells were highly responsive to CD3 crosslinking when Fas:FasL interactions were possible, while there was no detectable response to CD3 crosslinking in any Fas^{lpr} T cells. There is no clear-cut reason for the difference in response to activation between CD4 and CD8 single positive T cells, but it may be linked to the relative balance between TCR stimulation and other signals, and the role these signals play in regulating differentiation to the CD4 or CD8 lineages.

8.3.4 CD3 and CD28 Co-Stimulation Relieves Block in Activation Induced Cell Death and Proliferation in Fas^{lpr} T Cells

CD28 has been described as a co-stimulatory receptor for TCR/CD3 mediated events (Turka *et al.*, 1990). Reports have suggested that anti-CD3 treatment alone provides sub-optimal activation. Both CD28 and FasL ligation have now been shown to mediate a co-stimulatory signal which can augment the response of cells to CD3 ligation in certain circumstances (Clements *et al.*, 1993; Suzuki & Fink, 1998). The results in the first part of this chapter show that while anti-CD3 treatment alone induces cell death followed by proliferation in control MRL cells, Fas^{lpr} thymocytes are unresponsive to anti-CD3 treatment. Following CD3 and CD28 co-stimulation however, cell death and proliferation were observed in both control MRL, and Fas^{lpr} thymocytes. CD28 co-stimulation allowed full activation of cell death and then proliferation in Fas^{lpr} cells, albeit delayed compared to control cells. The data presented in this chapter is supported by another study which investigated activation of the abnormal CD4/CD8 double negative peripheral T cell population in Fas^{lpr} mice (Clements *et al.*, 1993). This study reported the ability of CD28 to co-stimulate CD3

activated proliferation in these abnormal Fas^{lpr} CD4/CD8 double negative T cells which were otherwise unresponsive to activation (Clements *et al.*, 1993).

It is not clear how CD28 ligation can bypass the requirement for Fas:FasL interactions in activation induced proliferation. It seems reasonable to speculate however that Fas is required for activation induced cell death and proliferation, as stimulated by CD3 crosslinking, and that CD28 co-stimulation allows activation signalling to occur through an alternative, Fas independent pathway. Nevertheless, there are other more complex explanations which must be considered.

A number of reports have suggested a link between FasL and CD28. One study in peripheral blood T cells (PBTCs) and bone marrow T cells (BMTCs) demonstrated that CD3/CD28 co-stimulation resulted in induction of FasL (Sato *et al.*, 1999). Further, co-stimulation through CD28 was reported to be necessary for maximal induction of the FasL gene in response to CD3 crosslinking (Norian *et al.*, 2000). It appears that signalling through CD28 can induce FasL expression. A conflicting study reported that CD28 signalling alone induced FasL expression and cell death in T cell hybridoma cells, but in combination with CD3 crosslinking, CD28 stimulation prevented FasL expression, upregulated Bcl-X_L, and caused activation induced apoptosis to be downregulated (Collette *et al.*, 1998). This is in contrast to the results obtained in our system, since CD28 stimulation alone had no effect on either control or Fas^{lpr} cells, while CD3/CD28 co-stimulation resulted in increased activation induced cell death. Regulation of FasL by CD3/CD28 co-stimulation may depend on the cell type and the surrounding environment however. It is also difficult to explain how CD28 dependent upregulation of FasL might effect a stimulatory signal in Fas^{lpr} T cells. Previous results using the anti-FasL antibody which are described in Chapter 6, have indicated that FasL may have Fas independent functions, so it is possible that reverse signalling through FasL may occur in the absence of Fas, and that ligation of CD28 may facilitate this event.

There is a possibility that CD28 co-stimulation can be replaced by other signalling pathways. For example the results presented here suggest that Fas:FasL signalling may be able to co-stimulate anti-CD3 activated T cell proliferation. This may happen

either by signalling through Fas, or by reverse signalling of Fas on FasL. The downstream targets of CD28 might provide clues as to how co-stimulation is effected (reviewed by Rudd, 1996). One target for activation upon ligation of CD28 is the PI3 kinase/Akt pathway (Parry *et al.*, 1997). In fact, Akt may substitute for CD28 co-stimulation under certain conditions (Kane *et al.*, 2001). This result is supported by the finding that ligation of α4 and α5 integrins, on the surface of fetal thymocytes was able to co-stimulate anti-CD3 activated proliferation (Halvorson *et al.*, 1998). This is relevant, since integrin signalling is known to stimulate PI3 kinase (Shimizu & Hunt, 1996). High expression of α4 and α5 integrins has been reported on CD4/CD8 double negative T cells (Halvorson & Coligan, 1995). Since the elevated population of abnormal T cells in older Fas^{lpr} mice consists predominantly of CD4/CD8 double negative T cells, then these data might help to explain why the protective effects of cell contact were particularly highlighted in Fas^{lpr} tumour cell lines (see Chapter 7). It could be hypothesised that Fas^{lpr} cells have a growth disadvantage *in vitro* due to a failure in activation induced proliferation. This may be overcome however, by integrin mediated stimulation of the PI3K/Akt survival signal which can overcome the requirement for CD28 co-stimulation in Fas^{lpr} cells.

FUTURE WORK

The results described here demonstrate that loss of Fas signalling does not represent a synergistic event in either c-*MYC* or MuLV induced T cell lymphomagenesis. This is despite the fact that Fas signalling is a major pathway involved in CTL and NK cell mediated immune defence. It might be expected that loss of Fas signalling would promote lymphomagenesis however this is not the case. There may be a number of reasons why the lack of functional Fas in Fas^{lpr} animals does not act to restrict lymphoma development, and future work should concentrate on elucidating these mechanisms.

Ligation of Fas by FasL might not always result in death of malignant or virally transformed cells expressing Fas on their surface. Results from other groups and from transplantation experiments described here have suggested that FasL on the surface of tumour cells might confer immune privilege on those cells. Tumours arising in Fas^{lpr} mice may not be as capable of protecting themselves from immune attack, since infiltrating host T cells do not express Fas. Consequently rejection of tumours in mice lacking Fas might be increased. Corresponding transplantation experiments carried out in Fas^{lpr} and Fas^{gld} mice, whose cells lack FasL, may help determine the importance of Fas and FasL expression on tumours and on host T cells, in the process of tumourigenesis. For example, if FasL expression on tumour cells enhanced survival of the tumour, then we might expect Fas^{gld} tumours to have a considerable growth advantage in control MRL or in Fas^{gld} mice, compared with Fas^{lpr} hosts. In addition, it would be informative to study surface FasL expression on tumours and relate FasL expression to tumour latency.

An obvious explanation for that lack of collaboration between deregulated *MYC* and loss of Fas in T cells, is that MYC does not require the Fas pathway to mediate apoptosis. Results presented here suggest that MYC induced apoptosis can occur in the absence of both Fas and functional p53. Future experiments will continue to examine MYC induced apoptosis in the absence of Fas and p53, and to elucidate the alternative mechanisms by which MYC can induce apoptosis when Fas and p53 are lost. It may be that MYC can simply sensitise cells to apoptosis mediated by other

signals, or can induce a third, Fas and p53 independent apoptotic pathway. Further work should investigate MYC induced apoptosis in the absence of other candidate apoptotic signals, for example, those mediated by members of the TNF receptor family. In addition, it is important to understand how MYC may amplify apoptotic signals. Studies of the mitochondrial apoptotic cascade and cytochrome *c* release following MYC upregulation will be essential to achieve this.

It may be that genetic events which result in upregulation of survival signals, rather than loss of apoptotic pathways are more important in *MYC* induced tumourigenesis. Cell contact was shown to enhance survival of some cell lines in response to MYC upregulation, in a PI3 kinase dependent manner. Future work may investigate the pathways downstream of PI3 kinase in these cell lines, with the expectation that these signals may be the target of mutagenic events during *MYC* induced tumourigenesis. Future work may also examine the roles played by the major adhesion molecules and the pathways stimulated by them, in contact mediated protection from apoptosis. Preliminary research has been carried out, into various integrin pathways and CD44 signalling and their effect, if any, on MYC induced apoptosis.

In addition to upregulation of survival signals, genetic events that result in promotion of cell proliferation represent an important event in tumourigenesis. T cell receptor activation induced proliferation was shown to be limited in thymocytes from Fas^{lpr} mice, suggesting that Fas:FasL interactions may have a role in promoting proliferation in certain contexts. This may explain why tumourigenesis is not accelerated in Fas^{lpr} mice. Future work will investigate the mechanisms by which Fas:FasL interactions might stimulate, or co-stimulate proliferation. Comparison of activation of thymocytes from Fas^{lpr} and Fas^{gld} in mixed culture may be useful, to determine whether Fas signals through FasL or vice-versa to promote proliferation. It will also be necessary to examine the effectors downstream of Fas:FasL interactions, and of CD28 stimulation, and consider how these two stimuli might be functionally analogous.

It has been hypothesised that the block in activation induced proliferation in Fas^{lpr} cells might be dependent on p53 and this may explain why lymphoproliferative

symptoms are increased in Fas^{lpr} mice on a *Trp53* null background. Additional experiments are required to examine the response to activation in cells from Fas^{lpr} mice null for p53, in order to confirm or disprove this hypothesis. It will be necessary to carry out cell cycle analysis on explanted T cells from Fas^{lpr} animals compared to control strain animals, to explore any differences in proliferation of T cells *in vivo* in Fas^{lpr} animals compared to control strain animals. Finally, analysis of the response to activation of tumour cells from Fas^{lpr} mice compared to tumour cells from control strain MRL mice should also be carried out. This should increase our understanding of the proliferative defect in Fas^{lpr} T cells, and help determine the importance of activation induced proliferation, or lack of, in the development and progression of lymphoma.

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