STUDIES ON THE CELLULAR IMMUNE RESPONSE TO FELINE LEUKAEMIA VIRUS

A thesis for the degree of Doctor of Philosophy

Submitted by

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ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytotoxicity
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AEC	3-amino-9-ethyl-carbazole
AIDS	Acquired immune deficiency syndrome
ALV	Avian leukosis virus
AKRMLV	AKR murine leukaemia virus
AMV	Avian myelocytomatosis virus
AP	Alkaline phosphatase
APC	Antigen presenting cell
ASLV	Avian sarcoma and leukosis viruses
BaEV	Baboon endogenous virus
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BFA	Brefeldin A
BFU	Burst forming unit
BFV	Bovine foamy virus
BIV	Bovine immunodeficiency virus
BLV	Bovine leukaemia virus
BMTM	Bone marrow transport medium
BSA	Bovine serum albumen
CA	Capsid protein
CAEV	Caprine arthritis-encephalitis virus
CCR	Chemokine receptor
CD	Cluster of differentiation
CDA	Complement-dependent antibodies
CFS	Cell free supernatant
CFV	Chimpanzee foamy virus
CL	Chemiluminescence
CMV	Cytomegalovirus
Con A	Concanavalin A
cpm	Counts per minute
CTL	Cytotoxic T lymphocyte

DAB	Diaminobenzidine
DC	Dendritic cell
DMSO	Dimethylsulphoxide
DSH	Domestic shorthair
dsRAD	dsRNA specific adenosine deaminase
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunospot assay
EM	Electron microscopy
enFeLV	FeLV-related gene sequences
ER	Endoplasmic reticulum
E-RFC	Erythrocyte-rosette forming cells
FAE	FeLV-associated enteritis
FBBB	Fast Blue BB
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FECV	Feline enteric coronavirus
FeEV	Feline endogenous virus
FEF	Feline embryonic fibroblast
FeLV	Feline leukaemia virus
FeLV-FAIDS	FeLV-A isolate that causes feline AIDS
ffu	Focus forming unit
FFV	Feline foamy virus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FrMLV	Friend murine leukaemia virus
FV	Friend retrovirus complex
GaFeSV	Gardner-Arnstein feline sarcoma virus
GALV	Gibbon ape leukaemia virus
GEDELISA	Assay combining PAGE and ELISA
GIT	Gastrointestinal tract

GSA	Group specific antigen
GST	Glutathione S-transferase
HAT	Sodium hypoxanthine, aminopterin and thymidine
HERV	Human endogenous sequence
HFV	Human foamy virus
HIV	Human immunodeficiency virus
HTLV	Human T cell leukaemia virus
HZFeSV	Hardy-Zuckerman feline sarcoma virus
IAP	Intracisternal-A particle
ICAM	Intracellular adhesion molecule
ICS	Intracellular cytokine staining
IFA	Indirect immunofluorescent antibody
IFN-γ	Interferon-γ
IFN-γ-R	IFN-γ receptor
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IN	Integrase
IP	Intraperitoneal
IPC	IFN-producing cells
IPTG	Isopropylthio-β-D-galactoside
IV	Intravenous
JSRV	Jaagsiekte sheep retrovirus
LBT	Lymphocyte blast transformation
LCMV	Lymphocytic choriomeningitis virus
LPA	Lymphocyte proliferation assay
LPS	Lipopolysaccharide
LSA	Lymphosarcoma
LTNP	Long-term non-progressor
LTR	Long terminal repeat
LV	Langur virus
MA	Matrix protein
mAb	Monoclonal antibody

MAF	Macrophage activating factor
MCF	Mink cell focus-inducing
ME	Mercaptoethanol
M:E	Myeloid:erythroid ratio
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
MLR	Mixed leukocyte reaction
MLV	Murine leukaemia virus
MMTV	Mouse mammary tumour virus
MoMLV	Moloney murine leukaemia virus
MoMSV	Moloney murine sarcoma virus
MPD	Myeloproliferative disease
MPMV	Mason-Pfizer monkey virus
MVA	Modified vaccinia virus Ankara
MVV	Maedi-Visna virus
mw	Molecular weight
NBT	Nitro blue tetrazolium
NC	Nucleocapsid
n.d	Not done
NGS	Normal goat serum
NHS	N-hydroxysuccinimide
NK	Natural killer
NMS	Normal mouse serum
NO	Nitric oxide
NOS	Nitric oxide synthase
NRS	Normal rabbit serum
NSS	Normal swine serum
O.D.	Optical density
OPAV	Ovine pulmonary adenocarcinoma virus
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PBS-T	PBS containing 0.05% tween-20
PBS-T-BSA	PBS containing 0.05% tween-20 and 1% BSA
PE	Phycoerythrin
PEG	Polyethylene glycol
PERV	Porcine endogenous retrovirus
PF	Preventable fraction
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PKR	dsRNA activated protein kinase
PLN	Peripheral lymph node
PLV	Puma lentivirus
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear
PR	Protease
PTLV	Primary T-lymphotropic virus
PWM	Pokeweed mitogen
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RANTES	'Regulated on activation, normal T cell expressed and secreted'
RBC	Red blood cell
REV	Reticuloendotheliosis virus
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RT	Room temperature
rVV	Recombinant vaccinia virus
SC	Subcutaneous
SDS	Sodium dodecyl sulphate
SEA	*Staphylococcus aureus* enterotoxin A
SHIV	Simian-human immunodeficiency virus
S.I.	Stimulation index
SIV	Simian immunodeficiency virus

SMRV	Squirrel monkey retrovirus
SnRV	Snakehead retrovirus
SP	Slow progressor
s:p	Sample:positive ratio
SPA	*Staphylococcus aureus* protein A
SPF	Specific pathogen free
STLV	Simian T lymphotropic virus
SU	Surface envelope glycoprotein
2-5A synthetase	2'-5'-oligoadenylate synthestase
TBS	Tris buffered saline
TBS-T	TBS containing 0.05% tween-20
TCF	Tissue culture fluid
TCR	T cell receptor
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
(T,G)AL	($_{L}$-tyrosine-$_{L}$-glutamic acid)-poly-$_{DL}$-alanine-poly-$_{L}$-lysine
TM	Transmembrane envelope protein
TNF	Tumour necrosis factor
Tr	Regulatory T cell
UV	Ultraviolet
UVD	Unintegrated viral DNA
VI	Virus isolation
VNA	Virus neutralising antibodies
Vr	Variable region
WBC	White blood cell
WDSV	Walleye dermal sarcoma virus
WMSV	Woolly monkey sarcoma virus
Y73SV	Y73 sarcoma virus

DECLARATION

The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow, between February 2000, and February 2003. The author was responsible for all results, except where otherwise stated. No part of this thesis has been submitted for any other degree.

Elizabeth M. Graham, April, 2003.

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Colin Nixon, Histopathology Laboratory, University of Glasgow, performed the immunohistochemical staining on formalin-fixed bone marrow sections, which was described in Chapter 5. Ronnie Barron and colleagues in the Clinical Diagnostics Laboratory conducted the haematological analysis on post mortem blood samples, and prepared the bone marrow cytospins used in immunofluorescent staining and cytology analysis (Chapter 5). Ronnie Barron examined and assessed the bone marrow cytology from each cat; his report was presented and discussed in Chapter 5. Alan May and Ronnie Barron expertly took all the original photographs illustrated in this thesis.

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SUMMARY

The aim of this study was to investigate the virus-specific cell-mediated immune responses, particularly virus-specific $CD4^+$ T cells, elicited following oronasal exposure to feline leukaemia virus (FeLV).

In Chapter 1, the general features of the retroviridae are outlined; current knowledge on various aspects of FeLV, including epidemiology, pathogenesis and clinical disease is reviewed. General features of the immune responses to retroviridae are described; in addition, the virus-specific immune response to FeLV, and other retroviridae such as human immunodeficiency virus (HIV), Friend retrovirus complex (FV) and bovine leukaemia virus (BLV), is illustrated in detail. The technologies currently available to detect virus-specific $CD4^+$ T cell responses are described and reviewed. This chapter also outlines the aims of this thesis.

Chapter 2 describes the development and characterisation of monoclonal and polyclonal antibodies to feline interferon-γ (fIFN-γ). These novel reagents were generated as tools to detect virus-specific immune responses using intracellular cytokine staining (ICS), and flow cytometry. The sensitivity of these novel reagents in the detection of both recombinant fIFN-γ using an enzyme linked immunosorbent assay (ELISA) and Western blots, and the production of intracellular fIFN-γ in physiologically stimulated cells using flow cytometry, was illustrated.

A longitudinal FeLV immunopathogenesis study was conducted to monitor the evolution of virus-specific cell-mediated immune responses, particularly virus-specific $CD4^+$ T cells, following oronasal exposure to FeLV. A group of six specific pathogen free (SPF) cats was exposed oronasally to 5×10^5 focus forming units (ffu) FeLV-A/Glasgow-1 aged sixteen weeks, in the expectation that approximately fifty *per cent* of cats would recover, and fifty *per cent* would become persistently infected. However, all six cats became persistently infected. In an attempt to modify the outcome of exposure and improve the chances of recovery, a second group of six SPF cats was exposed to a lower dose of FeLV, 5×10^4 ffu FeLV-A/Glasgow-1, under identical conditions. Five of these cats became persistently infected following

oronasal exposure to FeLV, and one cat ostensibly recovered. Although virus was not detected in the peripheral blood from this cat at any time throughout the study, proviral DNA was detected in the bone marrow and lymphoid tissues post mortem, as a latent infection. An account of the virological parameters measured in these cats throughout the study is given in Chapter 3. Sensitive and sophisticated assays, such as the p27 ELISA, virus isolation (VI), and quantitative reverse transcription-polymerase chain reaction (qRT-PCR), were used to detect antigenaemia, viraemia and proviral DNA, respectively, in the peripheral blood throughout the study, and in the lymphoid tissues post mortem.

Disease outcomes and proviral DNA loads were correlated with virus-specific $CD4^+$ T cell responses. In Chapter 4, virus-specific cell-mediated immune responses in these cats were investigated using two techniques, the lymphocyte proliferation assay (LPA) and ICS, analysed using flow cytometry. Results obtained in either assay did not correlate with the proviral DNA load; however, the data generated using ICS appeared to be more relevant to the outcome of infection. Latently infected cat E12 failed to develop virus-specific proliferative responses in the peripheral blood; however, the virus-specific upregulation of fIFN-γ in $CD4^+$ T cells was detected using ICS. Conversely, persistently infected cats generated significant virus-specific proliferative responses in the peripheral blood, and transient low frequencies of virus-specific $CD4^+$ T cell responses were detected using ICS in the critical early weeks following exposure.

The bone marrow and lymphoid tissues are important sites of FeLV integration and replication. In Chapter 5, FeLV expression in the post mortem bone marrow and peripheral lymph node (PLN) tissues of these cats is described. In addition, the impact of an active bone marrow infection on haematopoiesis is assessed, using concurrent cytological and haematological analyses of bone marrow and peripheral blood, respectively. Persistently infected cats expressed FeLV in the bone marrow and in PLN tissues; in addition, abnormal haematology and cytology reports were recorded for each of these cats. The latently infected cat did not express FeLV in either the bone marrow or PLN tissues; furthermore, cytology and haematology reports were normal in this cat. In Chapter 6, the results of this thesis are brought together and discussed in the context of current research.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Retroviridae

1.1.1 Introduction

Retroviruses comprise a large family of enveloped RNA viruses, defined by common morphological and replicative properties. Each family member is equipped with a reverse transcriptase (RT) enzyme, which derives double-stranded DNA from the genomic dimer RNA. This distinctive feature of retroviral replication facilitates viral integration into host chromosomal DNA as provirus. Once integrated, proviral DNA is replicated along with the host DNA. The dual processes of reverse transcription and integration contribute to the proficiency of retroviruses in establishing lifelong infections in their hosts (Vogt, 1997a).

Retroviruses are significant causes of cancer and other serious diseases in humans and animals. Since the discovery of the first disease-causing retrovirus, equine infectious anaemia virus (EIAV), in 1905 (Vallée and Carré, 1905), investigators have strived to understand the pathogenesis of retroviral disease, identify the correlates of protective immune responses, and develop novel curative and prophylactic therapies. More recently, workers have sought to manipulate the retrovirus to advance other areas of medicine and science.

The pathogenesis of retroviral disease is not completely understood, and its elucidation remains an important aspect of viral research. Although retroviral disease can be investigated most effectively in its natural host, experimental animal models have an important role, particularly in investigating human retroviral disease. Thus, feline immunodeficiency virus (FIV) infection in the cat is a useful model for human immunodeficiency virus (HIV) infection (Willett *et al.*, 1997; Hosie *et al.*, 1998; Flynn *et al.*, 2000b), while the murine leukaemia virus (MLV) complex provides an important model to study genetic resistance to retroviral infection, the evolution of oncogenesis and tumour immunity (Adams *et al.*, 1985; Beaty *et al.*, 1999; Dittmer *et al.*, 2001).

Their unique life cycle and ability to flout antiviral defences make retroviruses

successful intracellular pathogens. Protective vaccines are proving elusive to investigators while treatments for retroviral infection are rarely curative. However, while considerable success has been achieved, particularly in regard to vaccination against feline leukaemia virus (FeLV) (Marciani *et al.*, 1991; Hanlon *et al.*, 2001), and FIV (Hosie *et al.*, 2000; Dunham *et al.*, 2002), protective regimes have yet to be established for many retroviral infections.

Interestingly, the fields of molecular biology and biotechnology have exploited the retroviral life cycle in developing novel reagents and techniques. Retroviruses are proving to be useful vectors in gene transfer (Rosenberg *et al.*, 1990), while reverse transcriptase enzymes from avian and murine retroviruses are used extensively to generate cDNA copies of RNA for molecular cloning and sequencing (Vogt, 1997a).

1.1.2 History

Retroviruses were first discovered because of their ability to cause disease, particularly cancer (Temin, 1992). However, the first retrovirus to be described, EIAV, was in fact non-oncogenic (Vallée and Carré, 1905). In 1908, a Danish team (Ellermann and Bang, 1908) demonstrated that leukosis in chickens was caused by a virus now known as the avian leukosis virus (ALV). Shortly afterwards, Rous (1911) described the experimental transmission of sarcoma in chickens using cell-free material (Rous sarcoma virus, RSV). A further twenty years would elapse before viral-induced tumours were described in mammalian species, when a filterable agent transmitted in milk was reported to cause murine mammary carcinoma (Bittner, 1936). Over the following decades, tumour-causing retroviruses were identified in many outbred mammalian species: FeLV (Jarrett *et al.*, 1964b); gibbon ape leukaemia virus (GALV) (Theilen *et al.*, 1971); bovine leukaemia virus (BLV) (van Der Maaten *et al.*, 1972), and Jaagsiekte sheep retrovirus (JSRV) (Verwoerd *et al.*, 1983). The first oncogenic human retrovirus, human T cell leukaemia virus-1 (HTLV-1) was isolated in 1980 (Poiesz *et al.*, 1980). More recently, retroviruses with oncogenic properties that principally cause immunodeficiency diseases have been identified: HIV (Barré-Sinoussi *et al.*, 1983) and FIV (Pedersen *et al.*, 1987).

Originally, murine and avian RNA leukaemogenic viruses were believed to be transmitted solely as Mendelian traits (Aaronson and Todaro, 1968; Huebner and Todaro, 1969; Hardy *et al.*, 1973). The vertical transmission of retroviruses was first described in 1951, when MLV was isolated from a strain of mice selected for high frequency of leukaemia (Gross, 1951). Citing the results of prior studies using murine embryonic cells, Huebner (1969) hypothesised that viral information in the form of an oncogene-bearing virogene was present within the genome of most vertebrates. Huebner believed that the virogene was transmitted from animal to progeny animal, and from cell to progeny cell, and proposed that oncogenic reagents and carcinogens precipitated activation of the oncogene. However, the discovery of FeLV in 1964, and the subsequent revelation that this retrovirus could be horizontally transmitted, proved significant landmarks in the field of retroviral research (Jarrett *et al.*, 1964b; Hardy *et al.*, 1973). All disease-inducing retroviruses are now known to be horizontally transmitted (Hardy, 1993).

1.1.3 Taxonomy

Historically, retroviruses within the retroviral family were divided into types A, B, C and D, based on their morphological appearance in cells using electron microscopy (EM). Recently, the availability of genomic sequence data has enabled the retroviruses to be regrouped according to sequence conservation; however, these divisions often reflect the morphological similarities identified earlier. Retroviruses are now classified under the following genera: alpha (α), beta (β), gamma (γ), delta (δ) and epsilon (ε) retroviruses, lentiviruses and spumaviruses (Mayo and van Regenmortel, 2000) (Table 1.1).

1.1.4 Retroviral Genomic Organisation

All retroviral genomes consist of two identical single-stranded positive sense molecules of RNA, which are physically linked by hydrogen bonds to form a dimer. A third molecule of nucleic acid, a specific type of tRNA used as a primer for reverse transcription, is present freely within the nucleocapsid (Vogt, 1997b). The diploid genome consists of three principal genes, *gag*, *pol* and *env*. The gene order in all retroviruses is 5'-*gag-pol-env*-3'. Some retroviruses possess additional 'accessory'

Table 1.1 Taxonomy of selected retroviruses

Derived from the International Committee on Taxonomy of Viruses (ICTV) {http://www.ncbi.nlm.nih.gov/ICTV/}.

Genus	Species	Isolates or Strains
Alpharetrovirus	Avian leukosis virus (ALV) Avian myelocytomatosis virus (AMV) Rous sarcoma virus (RSV) Y73 sarcoma virus (Y73SV)	
Betaretrovirus	Langur Virus (LV) Mason-Pfizer monkey virus (MPMV) Mouse mammary tumor virus (MMTV) Ovine pulmonary adenocarcinoma virus (OPAV) Squirrel monkey retrovirus (SMRV)	
Gammaretrovirus	Feline leukaemia virus (FeLV)	FeLV strain A/Glasgow-1 FeLV strain B/lambda-B1 FeLV strain C/FA27 FeLV strain C/FS246 FeLV strain C/Sarma
	Gardner-Arnstein feline sarcoma virus (GAFeSV) Gibbon ape leukaemia virus (GALV) Hardy-Zuckerman feline sarcoma virus (HZFeSV) Moloney murine sarcoma virus (MoMSV)	
	Murine leukaemia virus (MLV)	AKR murine leukaemia virus (AKRMLV) Friend murine leukaemia virus (FrMLV) Moloney murine leukaemia virus (MoMLV)
	Pig endogenous retrovirus (PERV) Reticuloendotheliosis virus (REV)	
	Woolly monkey sarcoma virus (WMSV)	Simian sarcoma virus

Genus	Species	Isolates or Strains
Deltaretrovirus	Bovine leukaemia virus (BLV)	
	Human T-lymphotropic virus-1 (HTLV-1)	Simian T-lymphotropic virus-1 (STLV-1)
	Human T-lymphotropic virus-2 (HTLV-2)	Simian T-lymphotropic virus-2 (STLV-2)
	Primate T-lymphotropic virus-1 (PTLV-1)	
	Primate T-lymphotropic virus-2 (PTLV-2	
	Primate T-lymphotropic virus-3 (PTLV-3)	Simian T-lymphotropic virus-3 (STLV-3)
Epsilonretrovirus	Snakehead retrovirus (SnRV)	
	Walleye dermal sarcoma virus (WDSV)	
Lentivirus	Bovine immunodeficiency virus (BIV)	Jembrana disease virus
	Equine infectious anaemia virus (EIAV)	
	Feline immunodeficiency virus (FIV)	Feline immunodeficiency virus Petaluma (FIV-P) Feline immunodeficiency virus Oma (FIV-O)
	Panther lentivirus	
	Puma lentivirus 14 (PLV-14)	
	Puma lentivirus 21 (PLV-21)	
	Caprine arthritis-encephalitis virus (CAEV)	
	Maedi-Visna virus (MVV)	
	Human immunodeficiency virus (HIV)	
	Human immunodeficiency virus type 1 (HIV-1)	
	Human immunodeficiency virus type 2 (HIV-2)	
	Simian immunodeficiency virus (SIV)	

Genus	Species	Isolates or Strains
Spumavirus	Bovine foamy virus (BFV) Chimpanzee foamy virus (CFV) Feline foamy virus (FFV)	

genes encoding regulatory non-structural proteins, and are described as 'complex'. Accessory genes regulate and co-ordinate retroviral gene expression. The HIV genome encompasses a number of accessory genes including *tat*, *rev*, *vif*, *vpr* and *nef*. The *tat* gene activates transcription, while *rev* regulates RNA splicing. The products of the *nef*, *vpr* and *vif* genes facilitate viral infectivity (Vogt, 1997b). The genome of retroviruses possessing only the genes required for replication is termed 'simple', such as in FeLV (Vogt, 1997a) (Fig. 1.1).

Gag proteins, comprising nucleocapsid (NC), capsid (CA) and matrix (MA) proteins, form the internal virion structure. The *pro* gene lies between the *gag* and *pol* genes, and encodes the viral protease (PR). *Pol*-encoded proteins, RT and integrase (IN), facilitate viral replication. Finally, the surface (SU) and transmembrane (TM) components of the outer glycoprotein envelope are products of the *env* gene (Fig. 1.2; Table 1.2).

The NC core comprises the RNA genome as well as NC proteins. These proteins are closely associated with the genome and are involved with RNA packaging, at least *in vitro* (Coffin, 1992). Surrounding the core lies an internal virion shell made up of the CA protein, the exact structural and biological functions of which have yet to be identified. The MA protein lines the inside of the viral envelope and may interact with the Env proteins in order to facilitate viral budding from the host cell membrane (Vogt, 1997b). The *pro* gene product, PR, generates mature Gag and Pol proteins through proteolytic cleavage of the precursor proteins (Vogt, 1996). This process occurs either late in assembly or immediately after budding.

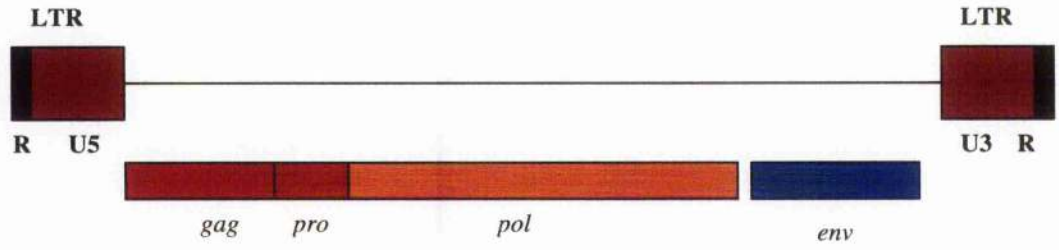
The viral RT has RNA and DNA polymerase activity, in addition to RNA endonucleolytic activity, and generates double-stranded DNA from the single strand of viral RNA. The IN protein demonstrates both nucleolytic and ligation activity and is responsible for the integration of proviral DNA into cellular DNA (Katz and Skalka, 1994).

The viral envelope is a lipid bilayer, which is derived from the host cell membrane during the budding process. The envelope surface contains SU and TM glycoproteins, which are derived from a common precursor polypeptide (Vogt,

Fig. 1. 1 Genetic organisation of prototypic retroviruses

A 'simple' retroviral genome (A) contains four major coding regions, *gag*, *pro*, *pol*, *env*; a 'complex' retroviral genome (B) contains four major coding domains in addition to information for six regulatory proteins. Based on Vogt, V. M. 1997. Retroviral Virions and Genomes, pp 27-69 *in* J. M. Coffin, S. H. Hughes, and H. E. Varmus, editors. Retroviruses. Cold Spring Harbor Laboratory Press, New York.

A FeLV


LTR
LTR
R
U5
U3
R
gag
pro
pol
env

B HIV

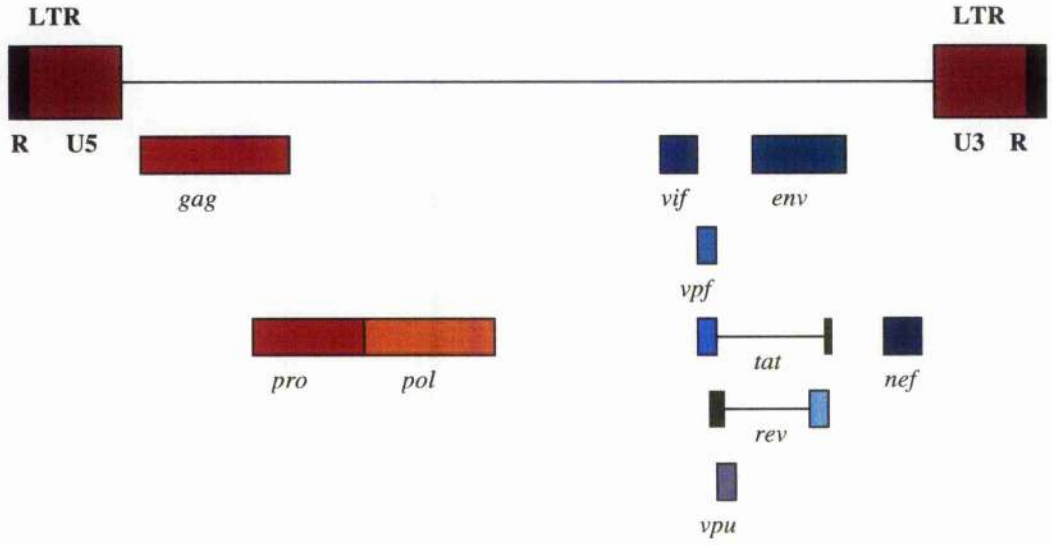

LTR
LTR
R
U5
U3
R
gag
vif
env
vpf
pro
pol
tat
nef
rev
vpu

Fig. 1.2 Schematic cross-section through a retroviral particle

Based on Vogt, P. K. 1997. Historical Introduction to the General Properties of Retroviruses, pp 1-25 *in* J. M. Coffin, S. H. Hughes, and H. E. Varmus, editors. Retroviruses. Cold Spring Harbor Laboratory Press, New York.

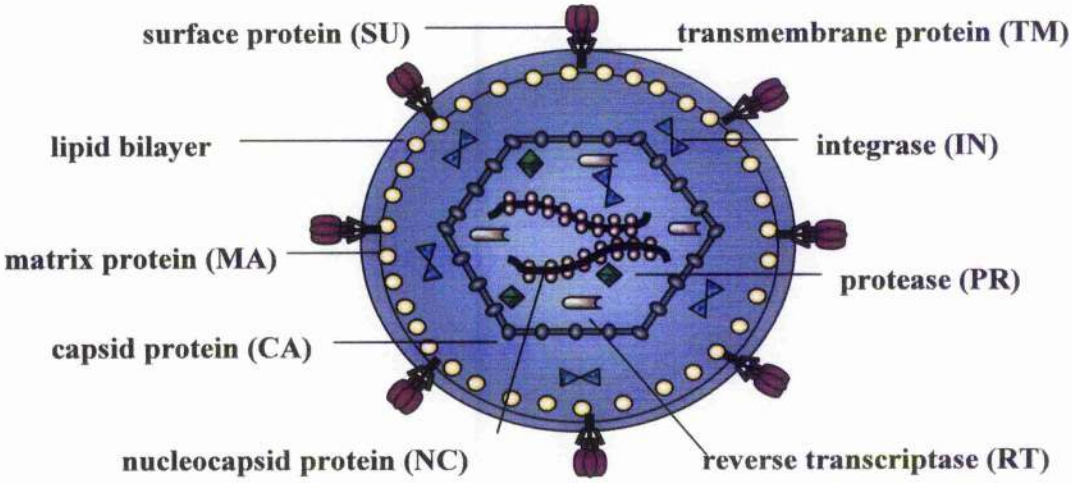

surface protein (SU)
transmembrane protein (TM)
lipid bilayer
integrase (IN)
matrix protein (MA)
protease (PR)
capsid protein (CA)
nucleocapsid protein (NC)
reverse transcriptase (RT)

Table 1.2 Retroviral proteins, nomenclature and function

Name	Protein	Function
MA	Matrix	*gag* encoded protein; lines envelope
CA	Capsid	*gag* encoded; protects the core; most abundant protein in virus particle
NC	Nucleocapsid	*gag* encoded; protects the genome; forms the core
PR	Protease	Essential for Gag and Pol protein cleavage during maturation
RT	Reverse transcriptase	Reverse transcribes the RNA genome; has RNAse activity
IN	Integrase	*pol* gene encoded; needed for integration of the provirus
SU	Surface glycoprotein	The outer envelope glycoprotein; major virus antigen
TM	Transmembrane protein	The inner component of the mature envelope glycoprotein

1997b). The SU protein mediates virus-host cell attachment via the host cell receptor, and thus entry into the host cell. In addition, the SU, due to its external location, contains the major antigenic determinants for virus neutralising antibodies (VNA). The TM protein anchors the envelope protein complex to the virus envelope and mediates envelope fusion with the host cell membrane.

1.2 Feline Leukaemia Virus

1.2.1 Discovery of FeLV

1.2.1.1 Transmission of FeLV

Feline leukaemia virus was first associated with feline leukaemia in 1964 (Jarrett *et al.*, 1964b). Four kittens, injected at birth with cell-free material from a field case of thymic lymphosarcoma (LSA), died within eighteen months; three of these kittens were subsequently diagnosed with LSA. This was the first report of the horizontal transmission of spontaneous LSA. In 1967, a similar experiment was conducted; cats were inoculated with cell-free tumour extracts and found to transmit disease. The presence of virus within all experimentally induced cases of LSA was confirmed using EM techniques (Rickard *et al.*, 1967). Kawakami *et al.* (1967) reported similar findings using virus isolated from plasma. In 1969, Rickard *et al.* used cellular and cell-free thymic tumour extracts derived from a cat with spontaneous lymphocytic leukaemia to inoculate newborn kittens. Eighty-two *per cent* of kittens died from leukaemia, seven *per cent* died from other diseases and ten *per cent* survived. Two uninoculated in-contact controls died from leukaemic lesions and C-shaped nucleocapsid cores (type-C particles) were demonstrated in tissues post mortem.

1.2.1.2 Identification of FeLV in Neoplastic Tissues

Using transmission electron microscopy (TEM), type-C viral particles were identified within the neoplastic tissue from a kitten in the original transmission study (Jarrett *et al.*, 1964b), and purported to be the aetiological agent (Jarrett *et al.*, 1964a). Furthermore, virus-like particles were demonstrated in three-quarters of spontaneous feline LSA cases involving spleen, thymus or mesenteric lymph nodes

(MLN), using EM imaging (Laird *et al.*, 1967). Type-C particles were apparent within extracellular spaces and intracellular vacuoles; in addition, particles were observed budding from plasma membranes, which suggested that the tumour cells were actively producing virus.

1.2.1.3 Isolation of FeLV from Plasma

In 1967, FeLV was isolated from the plasma of cats, suffering from both naturally and experimentally induced leukaemia, using sucrose-density gradients (Kawakami *et al.*, 1967). The isolated virus displayed characteristic of type-C particles, similar to MLV, with a buoyant density of 1.15-1.17 g/cm^2 (O'Connor *et al.*, 1964). Electron micrograph preparations of the isolated virus showed typical morphological features of immature and mature type-C particles. Mature virions measured approximately 115 μm in diameter, with an outer envelope and inner dense core. The isolated viral particles were effective and rapid inducers of LSA in kittens less than four days old.

1.2.1.4 Detection of Group Specific Antigen

The group specific antigen (GSA) represented the internal component of avian and murine leukaemia viruses, which occurred freely in leukaemic and other infected cells. The GSA described in the early years of FeLV research corresponds to the FeLV CA protein p27. Feline leukaemia virus was recognised as an oncogenic virus morphologically similar to MLV and ALV, prompting suggestions that these viruses might be related. Using the Ouchterlony immunoprecipitation technique, Geering *et al.* (1968) demonstrated that FeLV shared the GSA of MLV, but not ALV. Feline leukaemia virus GSA was subsequently detected in twenty-five out of thirty-three cats with LSA, using precipitating rabbit antiserum raised against purified virus in immunodiffusion tests. Electron microscopy was used to verify the presence of FeLV in cats considered positive by immunodiffusion (Hardy *et al.*, 1969).

1.2.1.5 Culture of FeLV

The virus was initially cultured in spleen, thymus, liver, heart and kidney cells from

newborn kittens *in vitro*, as well as in embryonic lung cells. The cells were cultured with cell-free homogenates of leukaemic cells and platelets from spontaneous leukaemic cases (Jarrett *et al.*, 1968). In each culture system, virus could be detected within most cells after eleven days, and was present until the termination of the experiment at day twenty-nine. Virus was observed budding from cell membranes, suggesting active replication. Thereafter, a culture system based on feline embryonic cells of the FEA strain was used; cells were propagated using Eagles' minimal essential medium (MEM) supplemented with ten *per cent* foetal calf serum (FCS) (Jarrett *et al.*, 1972; Jarrett *et al.*, 1973).

1.2.2 Taxonomy

Feline leukaemia virus is classified as a γ-retrovirus. This group includes many viruses within the MLV family as well as endogenous retroviral sequences in porcine cells (PERV), GALV and the reticuloendotheliosis virus. Feline leukaemia virus displays type-C particles on budding from infected cells using EM techniques (Fig. 1.3). On maturation, the core appears spherical and is centrally located within the virion (Coffin, 1992).

1.2.3 Evolution

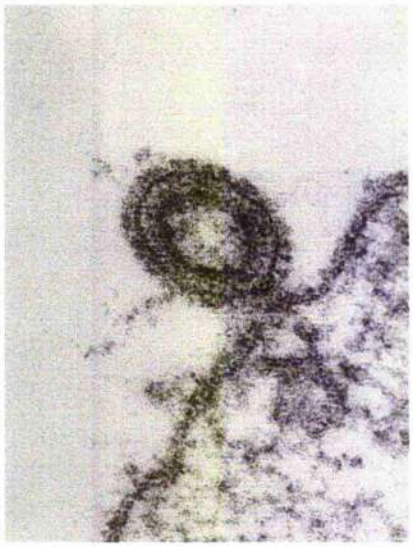
At least three groups of endogenous FeLV-related cat viruses have been detected in the cellular DNA from all domestic cats and some close relatives (Benveniste *et al.*, 1975; Hardy, 1993). These endogenous viruses are genetically transmitted, do not replicate in cats, and cannot cause disease by themselves (Hardy, 1993). However, some recombine with exogenous FeLV, giving rise to pathogenic viruses with an altered host range (Stewart *et al.*, 1986). Feline endogenous retroviral sequences are related to endogenous viruses in other species, such as rodents and primates, from which these endogenous sequences, and ultimately the pathogenic and contagious exogenous FeLV, are believed to be derived (Benveniste *et al.*, 1975).

Feline leukaemia virus-related gene sequences (enFeLV) have been detected in cellular DNA of specific pathogen free (SPF) domestic cats (*Felis catus*) and in DNA of three other closely related felidae: the jungle cat, sand cat and European wildcat

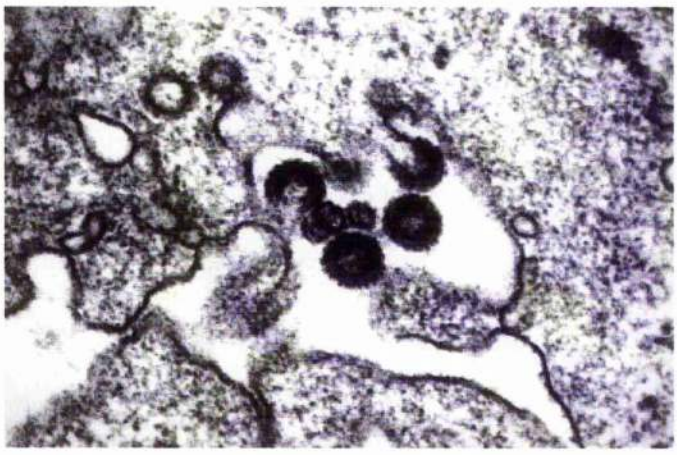
Fig. 1.3 TEM demonstrating FeLV budding from membrane of an infected cell

Transmission electron micrograph images of FeLV during the final stages of budding (A and B). Mature virions are apparent within the extracellular space (B). Images prepared by Laird, H.M., University of Glasgow.

A



B



(Benveniste *et al.*, 1975). These species all originated from the Mediterranean basin and are closely related genetically. More distantly related felidae, originating from the Americas, Sub-Saharan Africa or Southeast Asia, lack these FeLV-related genes. Analysis of rodent cellular DNA, particularly from the rat, but also from mouse and hamster, revealed that they also contained FeLV-related sequences (Benveniste *et al.*, 1975). Therefore, it appears that the FeLV-related sequences were acquired by cats subsequent to the initial felidae evolutionary divergence, but before the separation of the four closely related felidae described above. The source of enFeLV would appear to be the rat (Sherr *et al.*, 2002) (Fig. 1.4). The enFeLV exist as full-length proviruses; however, they contain many stop codons, which prevent their expression as whole infectious viruses. However, the *env* gene of these viruses may participate in recombinational events with exogenous FeLV to produce novel variant viruses (Jarrett, 2001).

The endogenous feline RD-114 virus, referred to as feline endogenous virus (FeEV), displays a similar viral sequence to an infectious endogenous virus in baboons, baboon endogenous virus (BaEV), implying a cross-species infection by an ancestor virus of BaEV (McAllister *et al.*, 1972; Hardy, 1993). The FeEV is present in feline cellular DNA as a complete provirus, which produces infectious virus if expressed. However, the virus is xenotropic; thus, it cannot reinfect cat cells and is considered non-pathogenic (Jarrett, 2001). The existence of a third feline endogenous virus distantly related to a primate virus MAC-1, has also been implied, but not characterised (Todaro *et al.*, 1978; Bonner and Todaro, 1979). However, the MAC-1 sequences in feline cells do not give rise to infectious replicating virus particles (Hardy, 1993).

1.2.4 Genomic Organisation

The genetic structure of FeLV parallels that of other simple retroviruses, such as the MLV complex (Hardy, 1993) (Fig. 1.5). Only the genome of an isolate of FeLV subgroup A, which causes feline acquired immune deficiency syndrome (FeLV-FAIDS), has been sequenced in its entirety (Donahue *et al.*, 1988) (Accession nos M18247 and M19392). Feline leukaemia virus gene products have yet to be fully characterised but deficiencies in the knowledge base have been plugged through

Fig. 1.4 Phylogenetic tree depicting retrovirus species

Phylogenetic tree depicting a number of retrovirus species drawn from comparisons of reverse transcriptase sequences. Taken from Vogt, V. M. 1997. Retroviral Virions and Genomes, pp 27-69 *in* J. M. Coffin, S. H. Hughes, and H. E. Varmus, editors. Retroviruses. Cold Spring Harbor Laboratory Press, New York.

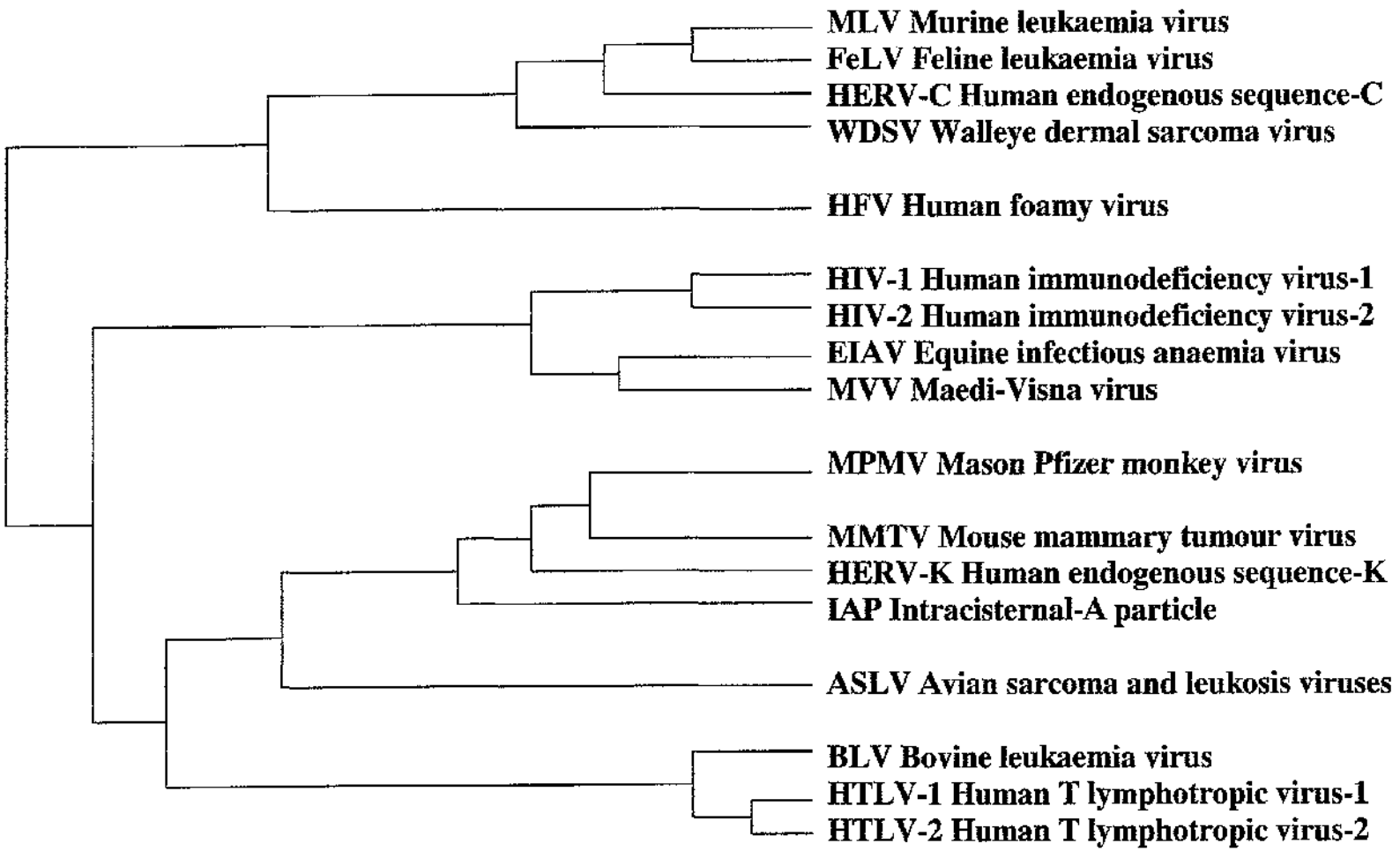
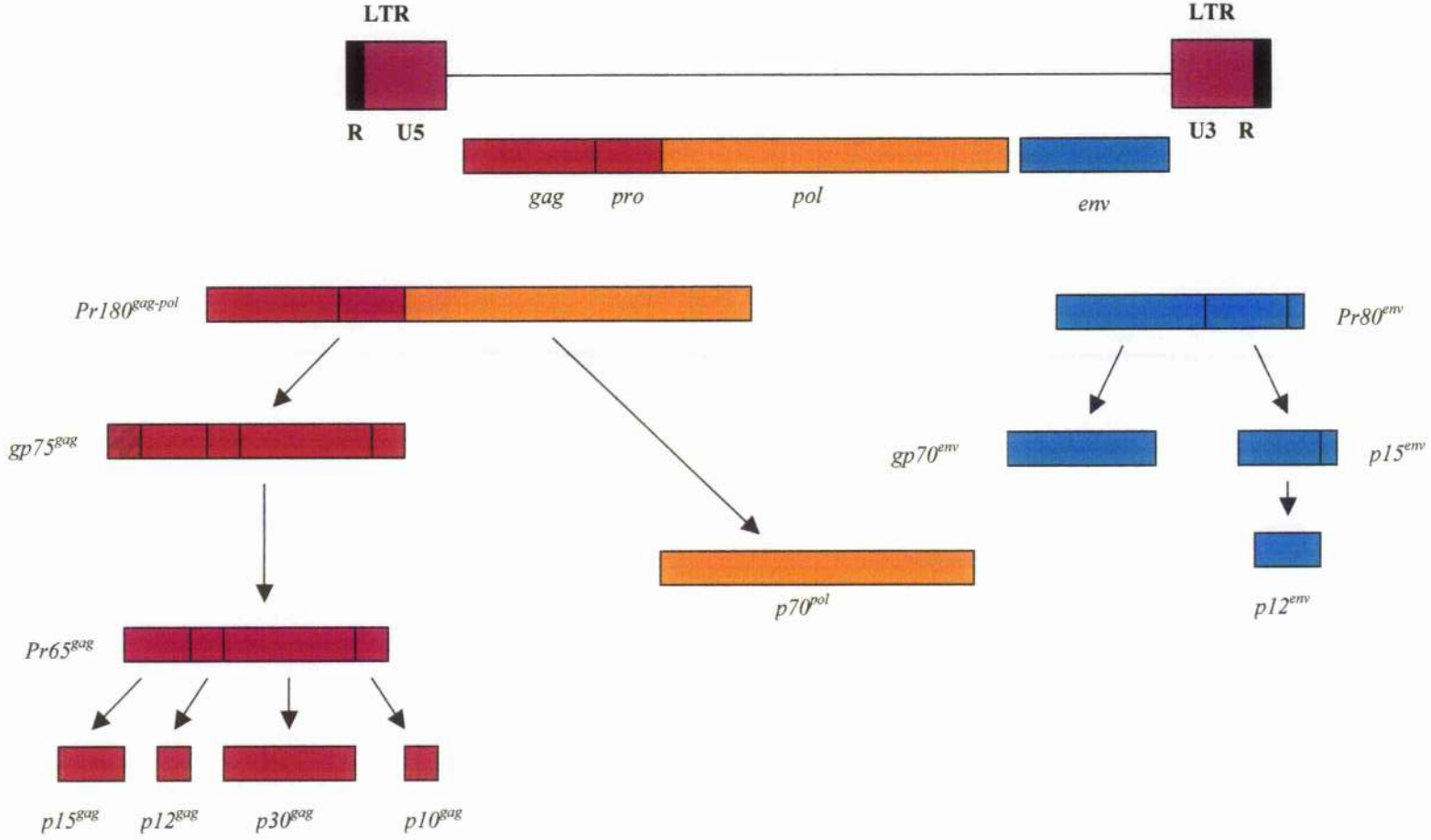

MLV Murine leukaemia virus
FeLV Feline leukaemia virus
HERV-C Human endogenous sequence-C
WDSV Walleye dermal sarcoma virus
HFV Human foamy virus
HIV-1 Human immunodeficiency virus-1
HIV-2 Human immunodeficiency virus-2
EIAV Equine infectious anaemia virus
MVV Maedi-Visna virus
MPMV Mason Pfizer monkey virus
MMTV Mouse mammary tumour virus
HERV-K Human endogenous sequence-K
IAP Intracisternal-A particle
ASLV Avian sarcoma and leukosis viruses
BLV Bovine leukaemia virus
HTLV-1 Human T lymphotropic virus-1
HTLV-2 Human T lymphotropic virus-2

Fig. 1.5 FeLV genome and gene products

Derived from Neil, J. C. and Onions, D. 1985. Feline leukaemia viruses: molecular biology and pathogenesis. *Anticancer Research*, **5**: 49-64.


LTR
LTR
R
U5
U3
R
gag
pro
pol
env
Pr180gag-pol
gp75gag
Pr65gag
p15gag
p12gag
p30gag
p10gag
p70pol
Pr80env
gp70env
p15env
p12env

extrapolation from the closely related, and more completely characterised, MLV complex (Neil and Onions, 1985).

Feline leukaemia virus *gag* gene products comprise both glycosylated and non-glycosylated proteins. Approximately half of the principal Gag precursor protein, Pr65, remains in the cell cytosol to be assembled into progeny virus, whereas the remainder is translocated to the lumen of the endoplasmic reticulum (ER) where it is glycosylated and transported to the plasma membrane (Jarrett, 2001). The glycosylated Pr65 is cleaved at the cell surface releasing a soluble glycosylated gp40. The gp40 protein contains determinants recognised by p27, p12 and p10 antisera and thus is likely to contain peptide sequences from p27, p12 and p10 Gag proteins (Neil *et al.*, 1980b). Glycosylated Gag proteins contribute to murine retrovirion assembly (H. Fan, personal communication). However, the role of these proteins in FeLV infection is yet unclear. It has been suggested that anti-Gag antibodies may contribute to the formation of immune complexes, or that the large amounts of soluble glycosylated protein released from FeLV-infected cells may trigger anergic responses (Jarrett, 1999). Within the cytosol, the non-glycosylated Gag protein, Pr65, is further cleaved into p10, p12, p15 and p30 proteins (Okasinski and Velicer, 1976; Khan and Stephenson, 1977). The largest of these mature proteins, p30, forms the internal virion shell, or CA. The p15 entity functions as the MA protein (Hardy, 1993). While the role of the smaller p10 and p12 proteins has yet to be fully elucidated, these may be involved in packaging and binding of the nucleic acid as NC proteins (Neil and Onions, 1985).

Feline leukaemia virus *pol* gene products have not been characterised. However, the closely related MLV *pol* gene encodes PR, RT and IN proteins (Levin *et al.*, 1984). The *env* gene products are derived from a single large precursor protein Pr80, which yields a glycosylated SU protein gp70 (Bolognesi, 1974), and a smaller non-glycosylated TM protein, p15 (p15E). The FeLV TM protein p15E undergoes further processing to yield p12. These smaller hydrophobic proteins are buried within the outer viral membrane and are disulphide-linked to SU protein gp70 (Neil *et al.*, 1980a), several hundred of which make up the outer virus envelope (Neil and Onions, 1985).

1.2.5 Feline Leukaemia Virus Subgroups

Three subgroups of FeLV have been identified, FeLV-A, -B and -C, based on differences in their SU protein gp70 (Jarrett and Russell, 1978). A distinction was made based on the results of interference tests (Sarma and Log, 1971; Sarma and Log, 1973), neutralisation tests using subgroup-specific antisera (Sarma and Log, 1973; Russell and Jarrett, 1978b), and viral host cell range (Jarrett *et al.*, 1973; Sarma *et al.*, 1975).

Sarma and Log (1971) reported that when FeLV-infected feline embryonic fibroblast (FEF) cells were superinfected with a closely related pseudotype virus derived from an identical FeLV strain, the FEF cells were resistant to viral transformation in a phenomenon known as viral interference, where both viruses are assumed to use the same cellular receptor. However, when the FEF cells were superinfected with a pseudotype virus derived from a different FeLV strain to the original infecting strain, no interference occurred. From the results of this study, Sarma proposed the existence of three FeLV subgroups, A, B and C. Virus neutralisation studies were conducted using subgroup-specific antisera in order to confirm the interference data (Sarma and Log, 1973). The findings were identical, verifying the existence of three major FeLV envelope variations corresponding to three FeLV subgroups. In his original interference study, Sarma and Log (1971) demonstrated that FeLV-A induced viral interference only when used in conjunction with homologous pseudotype viruses, whereas FeLV subgroup B and C viruses were able to induce interference to the pseudotype viruses derived from FeLV-A, in addition to their homologous pseudotype viruses. These findings suggested that subgroups B and C might contain FeLV-A sequences. Supporting data were provided from neutralisation studies testing viral isolates prepared from both lymphomatous cats and healthy cats in multiple cat households. Subgroup A was identified in every FeLV isolate, whereas subgroups B and C occurred only in combination with subgroup A viruses (Jarrett *et al.*, 1978). In a subsequent study, FeLV-B was reported to occur in approximately forty-two *per cent* of field isolates in combination with FeLV-A, while FeLV-C was identified in only one *per cent* of isolates, occurring either as FeLV-AC or as FeLV ABC (Jarrett *et al.*, 1984). Furthermore, kittens infected with isolates containing FeLV-B alone were unable to transmit the

virus to contact cats (Jarrett and Russell, 1978), suggesting that horizontal transmission could not occur without FeLV-A.

The unexpectedly high distribution of neutralising antibodies to FeLV-C in cats from FeLV-infected multiple cat households, FeLV-positive experimentally infected cats and lymphomatous cats (Russell and Jarrett, 1978a), compared to the percentage of field isolates which contained subgroup C (Jarrett *et al.*, 1978), raised the possibility that these antibodies were evoked by a determinant expressed by an endogenous FeLV-like gene present in all cat cells. It was suggested that the rare FeLV-C isolates arose *de novo* as a result of recombination between FeLV-A and these endogenous genes (Russell and Jarrett, 1978b). However, further studies disproved this conjecture (Rigby *et al.*, 1992). The phenotypic determinants of subgroup C isolates were isolated to a single variable region (Vr) of FeLV SU protein gp70. The sequence of this region varied from one naturally occurring isolate to another implying that each isolate arose through mutation of FeLV-A (Rigby *et al.*, 1992).

Neutralisation studies conducted to investigate the antigen specificities of VNA to the three FeLV subgroups showed that all FeLV-A viruses used in the experiment were neutralised to the same extent by two antisera raised to FeLV-A/Glasgow 1. These findings suggested that subgroup A virus was very stable antigenically (Russell and Jarrett, 1978b), and supported growing speculation that FeLV-A might be the prototype virus from which subgroups B and C were derived (Russell and Jarrett, 1978a). It had already been shown that subgroups FeLV-A, B and C were very closely related (Levin *et al.*, 1976), demonstrating eighty-five *per cent* genetic homology. A later study examined the more divergent sequence of the FeLV-A *env* gene and compared the region in two FeLV-B isolates (Stewart *et al.*, 1986). The authors reported that the FeLV-B probes prepared to the region in question hybridised only to FeLV-B isolates and endogenous FeLV-related sequences, supporting the hypothesis that FeLV-B was derived from recombination between FeLV-A and proviral elements within the feline genome.

Data derived from the studies described above and subsequent work (Roy-Burman, 1996; Betchel *et al.*, 1999) has greatly improved our understanding of the origin of the three principal FeLV subgroups. From all the available data, it is clear that

FeLV-A serves as the prototype virus from which other phenotypic variants are derived. Feline leukaemia virus-A is the most frequently occurring subtype in nature, and is highly conserved antigenically. In addition, FeLV-A is ecotropic, and has the most restricted host range, growing almost exclusively in feline cells (Hardy, 1993). Subgroup FeLV-B is polytropic and arises *de novo* from recombination events between FeLV-A and endogenous *env* sequences; various recombinant genotypes may arise depending on the site of recombination. Feline leukaemia virus-C is also polytropic and is derived from mutational events within FeLV-A.

Certain subgroups have been linked with particular diseases under experimental conditions. Aplastic anaemia was shown to be associated with FeLV-C (Mackey *et al.*, 1975; Onions *et al.*, 1982). Additionally, a higher prevalence of FeLV-AB than FeLV-A alone was reportedly associated with LSA (Jarrett *et al.*, 1978).

1.2.6 Epidemiology

1.2.6.1 Prevalence of FeLV Infection

In 1989, a study was published reporting the prevalence of FeLV in the U.K. (Hosie *et al.*, 1989). The data were acquired from over two thousand blood samples submitted to the University of Glasgow from sick and healthy cats. Samples were screened using a commercial capture enzyme linked immunosorbent assay (ELISA) that detected the presence of the FeLV CA protein p27 in plasma (Leukassay F ELISA, C-Vet Veterinary Products Ltd, Lancashire, U.K.). Positive results were confirmed using virus isolation (VI) techniques on FEA cells (Jarrett *et al.*, 1982a). The prevalence of FeLV infection was reported to be eighteen *per cent* in sick cats, and five *per cent* in healthy cats. The incidence of FeLV was not greater in either sex, and the highest probability of infection was among cats aged between one and five years. Currently, the prevalence of FeLV infection in healthy cats within the U.K. is estimated to be approximately one *per cent* (Addie, D. and Jarrett, O., personal communication, 2002). This dramatic reduction in the prevalence of FeLV is associated with the recent availability of several commercial vaccines (Section 1.3.2.5), and the success of 'test and removal' policies in catteries.

1.2.6.2 Transmission of FeLV

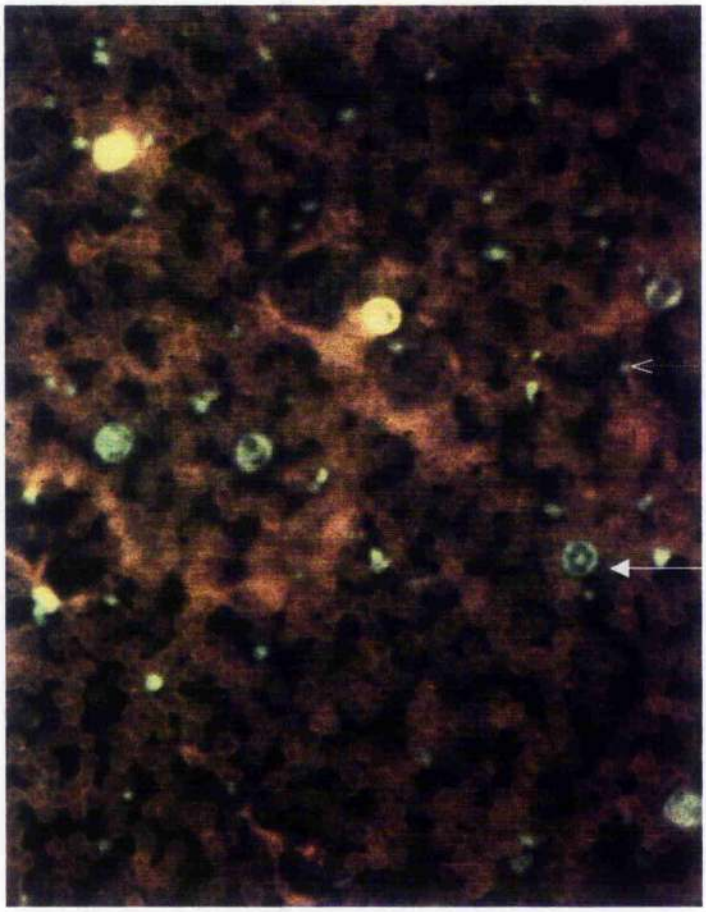
Following the discovery of FeLV in 1964, epidemiological and experimental data rapidly identified FeLV as an exogenous horizontally transmitted virus. In an early FeLV transmission experiment, two uninoculated cats housed with FeLV-inoculated cats died (Rickard *et al.*, 1969). Leukaemic lesions were discovered in both cats post mortem, in which type-C particles were identified. Type-C particles were also identified in plasma pellets and bone marrow samples from a third uninoculated in-contact cat. Blood from this third cat was injected into an experimental kitten that subsequently developed LSA, from which type-C particles were identified. Furthermore, spontaneous infections arose in laboratory cat colonies (Jarrett *et al.*, 1972; Essex *et al.*, 1977; Hoover *et al.*, 1977a; Pedersen *et al.*, 1977). In one incident, six out of twenty-five uninoculated controls living in the same isolation cage as inoculated littermates, died. Half of these had evidence of FeLV infection (Rickard *et al.*, 1969).

The development of a rapid diagnostic test to determine FeLV status provided conclusive proof that FeLV was a horizontally transmitted disease. In 1973, Hardy *et al.* developed a serological test to detect FeLV GSA in blood smears using rabbit anti-serum prepared against FeLV GSA (Fig. 1.6). Using this novel indirect immunofluorescent antibody (IFA) test, Hardy *et al.* (1973) demonstrated that normal cats living with two or more cats with LSA, were twice as likely to be FeLV-positive than cats living with only one cat with either LSA or an FeLV-associated disease. Two thirds of the LSA-positive cats that were living in households with an average of 3.1 cats with LSA were unrelated to the other diseased cats. The IFA test also demonstrated that apparently normal cats living in households with a known history of LSA had a high risk of becoming infected, and that overt FeLV in normal cats living under other circumstances was very rare (0.14%). A follow-up study investigating the fate of those FeLV-positive cats living under apparently normal circumstances discovered that these cats had an eight hundred and eighty-eight times greater incidence of developing LSA, compared to normal cats. Sixteen *per cent* of these cats developed LSA and over seven *per cent* died of non-responsive anaemia.

In utero infections also occur, where infected queens transfer FeLV to their foetuses

Fig. 1.6 Positive IFA test on PBMC smear

Indirect immunofluorescence test illustrating the presence of the FeLV CA protein p27 in neutrophils (white arrow) and platelets (dashed arrow, open head) in the peripheral blood, using rabbit antiserum against the FeLV CA protein p27.



(Hardy, 1981a). However, most of these infections will result in foetal or neonatal death. A small proportion will survive but remain persistently viraemic (Pedersen, 1988).

1.2.6.3 Sources of FeLV Infection

Prolonged intimate contact between a viraemic cat and a normal cat is required for horizontal transmission. A number of factors contribute to this requirement: the poor environmental stability of the virus (Francis *et al.*, 1979), the relatively large dose of virus required for infection via the oronasal route, and age resistance (Hoover *et al.*, 1976).

Saliva is considered the most likely vehicle for spreading the virus (Hardy *et al.*, 1973; Pedersen, 1988; Hoover and Mullins, 1991; Hardy, 1993; Jarrett, 2001). Cats are social animals and spend hours grooming themselves and their companions. Large amounts of infectious virus are present in the salivary glands of viraemic cats (Francis *et al.*, 1977; Hoover *et al.*, 1977b); FeLV has been identified in the parotid glands of six FeLV-positive cats with LSA (Hardy *et al.*, 1969). It was considered that aerosols of the virus created from nasal and salivary secretions emitted by persistently viraemic cats might also be an important route for dissemination of the virus (Hardy *et al.*, 1973). However, as Francis *et al.* (1977) observed, persistently viraemic cats are usually clinically healthy, and rarely cough and sneeze. Furthermore, FeLV-naïve cats housed in the same room, but not in contact with, FeLV-viraemic cats, did not become infected (Hoover *et al.*, 1977a).

Feline leukaemia virus GSA can be detected in the kidneys from seventy *per cent* of cats suffering from LSA; GSA has been identified in the infiltrating neoplastic lymphocytes, and forms discrete deposits on glomerular basement membranes. From these sites, infectious virus and infected transformed cells can pass into the urine (Hardy *et al.*, 1973); however, infectious virus is poorly preserved in urine (Hoover *et al.*, 1977a). Feline leukaemia virus is also found free in plasma; therefore, it is possible that blood-sucking parasites could acquire and transmit FeLV. Bite wounds are a more efficient means of transmitting the virus than the oronasal route as large amounts of virus can be injected directly into the bloodstream. However, blood

transfer does not appear to be an important factor in transmission of FeLV (Hoover and Mullins, 1991). Infection can also be transmitted using contaminated surgical instruments and infected blood transfusions (Pedersen, 1988).

1.2.7 Pathogenesis of FeLV Infection

The epidemiological data suggest that most cats acquire FeLV via the oronasal route. In the acute stage of infection, virus initially replicates in lymphocytes and macrophages within the tonsils, before being conveyed via efferent lymphatics to the draining lymph nodes of the head and neck (Rojko *et al.*, 1979). Virus replicates extensively within the lymph node; then, after two to twelve days, infected lymphocytes and macrophages disseminate the virus via the blood to bone marrow; thymus; spleen; intestine; MLN and peripheral lymph nodes (PLN) (Rojko and Kociba, 1991). Feline leukaemia virus replicates easily in the actively dividing cells of bone marrow precursor cells, gut endothelial crypt cells and germinal centre B cells. At this point, unless the immune response has curtailed virus growth, FeLV will spread to epithelial and glandular tissues throughout the cat, which usually include salivary, tonsillar, pharyngeal, urinary bladder, gastric, intestinal, pancreatic, endometrial and endothelial tissues (Hoover *et al.*, 1977b; Rojko *et al.*, 1978; Rojko *et al.*, 1979; Rojko and Kociba, 1991).

Immunohistochemical staining of formalin-fixed tissue sections from cats suffering from an FeLV-associated disease, using anti-FeLV monoclonal antibodies (mAb), gave a valuable insight into the nature and distribution of FeLV-infected cells (Kovacevic *et al.*, 1997). Feline leukaemia virus CA protein p27 and SU protein gp70 were detected in the bone marrow from some, but not all, FeLV-infected cats in the same study. Where present, these viral proteins were chiefly associated with megakaryocytes. In lymph nodes, expression of the FeLV CA protein p27 was mainly restricted to the follicles, in association with the follicular dendritic cell (FDC) population, whereas FeLV SU protein gp70 was detected in the lymph node follicles, paracortex and medulla. In spleen, both FeLV CA protein p27 and SU protein gp70 expression was confined to the follicles. Where FeLV proteins were detected in the small intestine, p27-positive cells were found only in crypt epithelial cells, whereas FeLV SU protein gp70 was detected in virtually all mucosal

lymphocytes.

In acute infection, FeLV appears to initially replicate in the lymphoreticular lymphocyte and macrophage populations (Rojko *et al.*, 1979). However, in persistently viraemic cats, the main source of FeLV appears to be the follicular B cell (Rojko *et al.*, 1979; Rojko *et al.*, 1981). Peripheral blood mononuclear cells from kittens and adult SPF cats were exposed to FeLV *in vitro* following a two-day pre-incubation period. Both B and T cells were found to be permissive to FeLV infection. However, lymphocytes from SPF kittens replicated fifty times as much FeLV as SPF adult cats *in vitro* (Rojko *et al.*, 1981). In addition, macrophages from kittens are five times more susceptible to FeLV than macrophages from adult cats *in vitro*, and this effect was enhanced one hundred fold by the addition of corticosteroid (Hoover *et al.*, 1981). However, macrophages from recovered cats were not any more resistant than those from naïve cats. The inhibition of macrophage function through the administration of silica *in vivo* was associated with the suppression of cell-mediated immune responses (Hoover *et al.*, 1981).

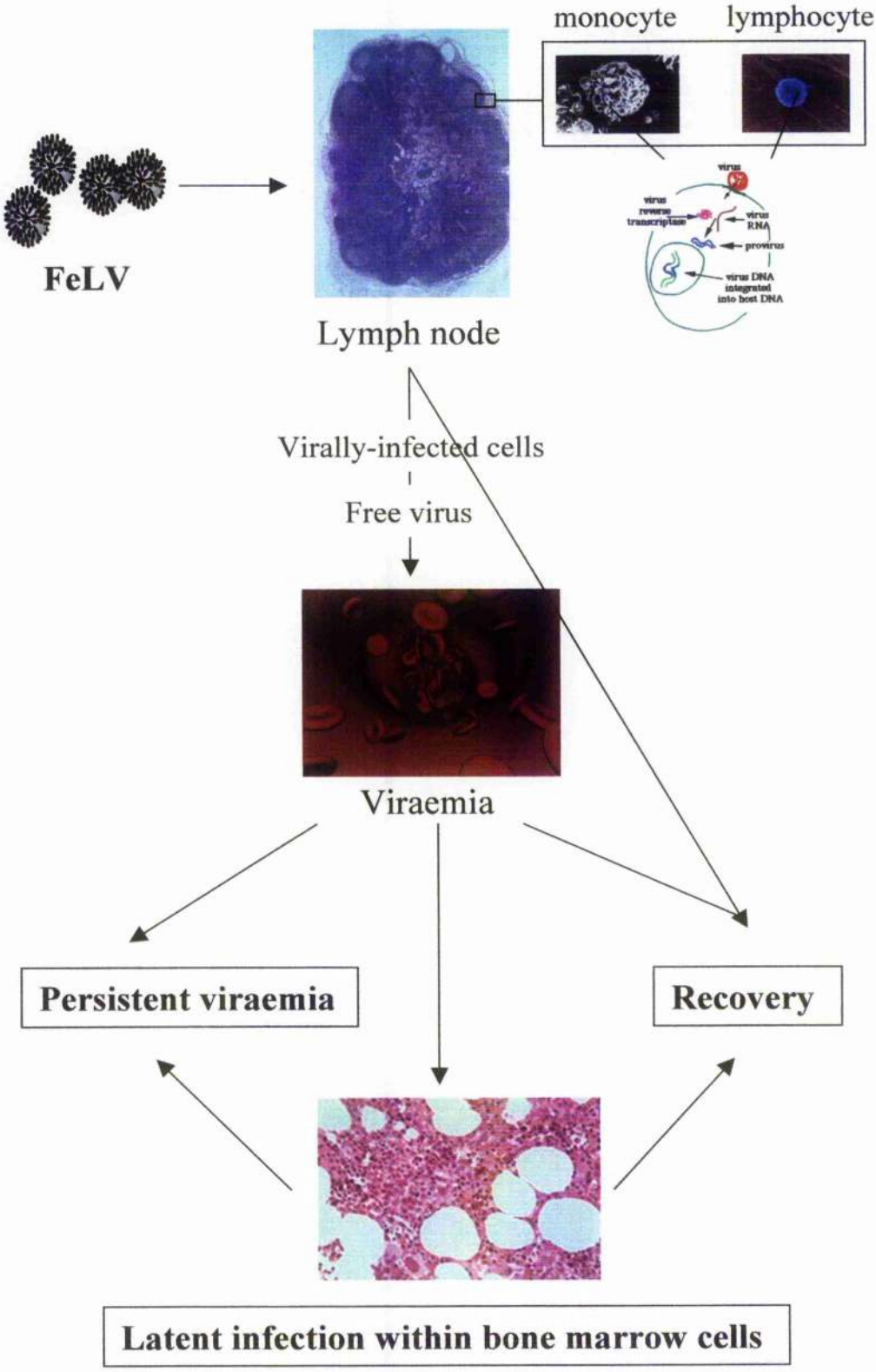
1.2.8 Outcome Following Exposure to FeLV

There are three well-defined outcomes following exposure to FeLV, namely spontaneous recovery, persistent viraemia and the development of a latent infection. If a virus-specific immune response develops during the acute phase of infection, FeLV replication is halted and virus expression abrogated. If sufficiently prompt, the antiviral response may prevent virus being borne into the blood from infected lymph nodes of the head and neck so that a viraemia does not occur. The immune response may emerge later, following a transient viraemia but before widespread dissemination of the virus, typically four to eight weeks following exposure (Hoover *et al.*, 1977b; Rojko *et al.*, 1979). Where the immune response has successfully abrogated virus expression, those cats are deemed to have recovered from infection (Fig. 1.7).

However, cats that experience a transient viraemia before recovering are highly likely to retain virus as a latent proviral infection within bone marrow or lymph node cells for a variable duration (Rojko *et al.*, 1982b; Madewell and Jarrett, 1983). Most

Fig. 1.7 Possible outcomes following exposure to FeLV

There are three possible outcomes following exposure to FeLV, namely recovery, persistence or latency.


monocyte
lymphocyte
virus
virus
reverse
transcriptase
virus
RNA
provirus
virus DNA
integrated
into host DNA
FeLV
Lymph node
Virally-infected cells
Free virus
Viraemia
Persistent viraemia
Recovery
Latent infection within bone marrow cells

latently infected cats were observed to eliminate virus within thirty months of exposure (Pacitti and Jarrett, 1985). Provirus persists in bone marrow monocytic precursor cells and certain T cell populations (Rojko *et al.*, 1982b). Viral proteins are not expressed; therefore, the infection is not detectable unless biopsy or post mortem specimens of bone marrow or lymph node are cultured *in vitro* (Madewell and Jarrett, 1983; Pedersen *et al.*, 1984). It is possible to reactivate latent virus *in vivo* through the administration of pharmacologic doses of corticosteroids (Rojko *et al.*, 1982b; Pedersen *et al.*, 1984), or through pregnancy (Pacitti *et al.*, 1986). It has been reported that cats with latent infections have an increased incidence of FeLV-associated disease and lymphomas, compared to non-infected cats (Lutz *et al.*, 1980a; Rojko *et al.*, 1982b; Lafrado and Olsen, 1986).

A rare outcome following FeLV exposure has been described as an atypical or discordant infection (Lutz *et al.*, 1982). Affected cats give conflicting FeLV status results using VI and p27 ELISA diagnostic tests, being typically antigenaemic but aviraemic. These discordant cats are most likely latently infected, and occasionally express viral proteins that are detected using the p27 ELISA (Kahn *et al.*, 1980; Madewell and Jarrett, 1983). Localised or sequestered infections have also been identified using immunofluorescence. An experimentally infected cat in which neither antigenaemia or viraemia was detected, expressed FeLV CA protein p27 in spleen, but not bone marrow (Hayes *et al.*, 1989).

Cats that are unable to contain viral replication by sixteen weeks are considered persistently viraemic. These cats will die from an FeLV-associated disease within two to three years. Persistently viraemic cats secrete contain large amounts of virus in saliva and nasal secretions, and are important sources of infection for FeLV-naïve cats.

1.2.9 Factors Influencing Outcome Following Exposure to FeLV

Virus isolate, dose, host susceptibility, age at time of exposure and route of exposure all interact to influence the outcome following exposure to FeLV (Hoover *et al.*, 1976).

1.2.9.1 Age-Related Resistance

Age at the time of exposure to FeLV is an important factor influencing the outcome of FeLV infection in cats. All newborn kittens are susceptible to experimental FeLV infection, unless protected by maternal antibodies (Kawakami *et al.*, 1967; Rickard *et al.*, 1969; Hoover *et al.*, 1976; Jarrett *et al.*, 1977). Resistance to FeLV increases with age. Under experimental conditions when viral dose, isolate and route are consistent, only a minority of cats are susceptible to experimental infection by four months of age (Jarrett *et al.*, 1982a). Age-related resistance may reflect the susceptibilities of particular cell subsets to FeLV infection; it has been shown *in vitro* that lymphocytes and macrophages from kittens were more susceptible to FeLV infection than those from adult cats (Hoover *et al.*, 1981; Rojko *et al.*, 1981). Furthermore, kittens are born with greater CD4:CD8 ratios (approximately 3.5:1) than adults (approximately 1.5:1). Changes in the CD4:CD8 ratio appear to be primarily associated with increased $CD8^+$ T cell numbers, which appears to be sensitive to antigen exposure. (Sellon *et al.*, 1996). An adult ratio may not be reached until the cat is one year old. Although neonatal kittens have high numbers of $CD4^+$ T cells, kittens less than ten weeks of age produce lower levels of interleukin (IL)-2 in response to concanavalin A (Con A), compared to adult cats, indicating that these cells are unable to function to capacity (Tompkins, M.B., unpublished data).

1.2.10 Clinical Disease Associated with FeLV

Feline leukaemia virus replicates in all nucleated cells in feline bone marrow (Hardy, 1981b), and favours other rapidly dividing cells elsewhere, such as lymphoid, myeloid, mucosal and epithelial cells (Hardy, 1993). Therefore, the potential for degenerative and proliferative disease is great (Fig. 1.8).

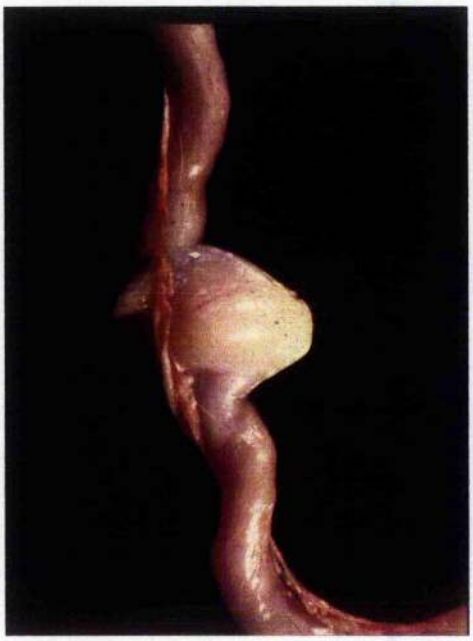
1.2.10.1 Oncogenesis

Feline leukaemia virus is a simple retrovirus, bearing only the genes necessary for replication. As such, the virus is without specific oncogenes. However, FeLV is responsible for most feline haematopoietic tumours, which accounted for one third of all feline tumours in the U.S. (Dorn *et al.*, 1968; Hardy, 1981b). All haematopoietic

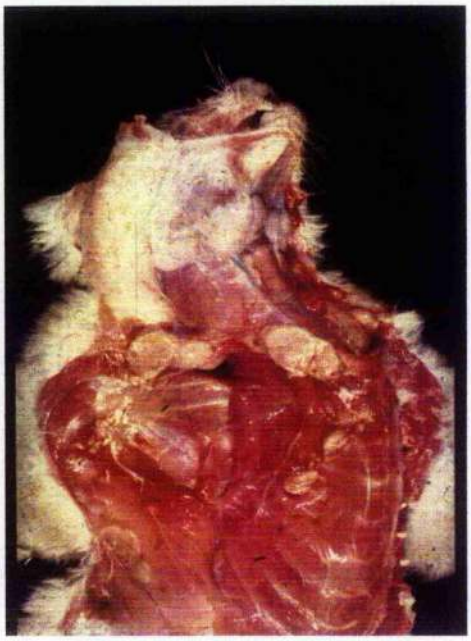
Fig. 1.8 Images illustrating FeLV-associated disease

Fig. 1.8.A and B demonstrate two forms of feline lymphosarcoma, alimentary (A) and multicentric (B). Myelogenous leukaemic cells are demonstrated in blood smear (C). In Fig. 1.8.D bone marrow from a normal cat (upper) is compared with bone marrow from a FeLV-viraemic cat (lower), where a paucity of erythrocytes indicates a severe non-regenerative anaemia.

A



B



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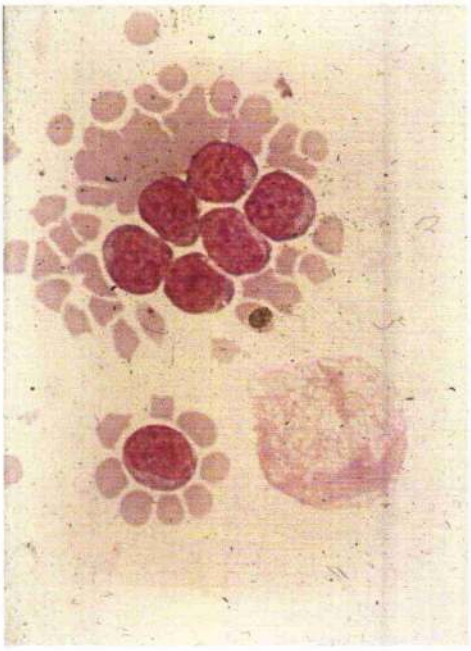
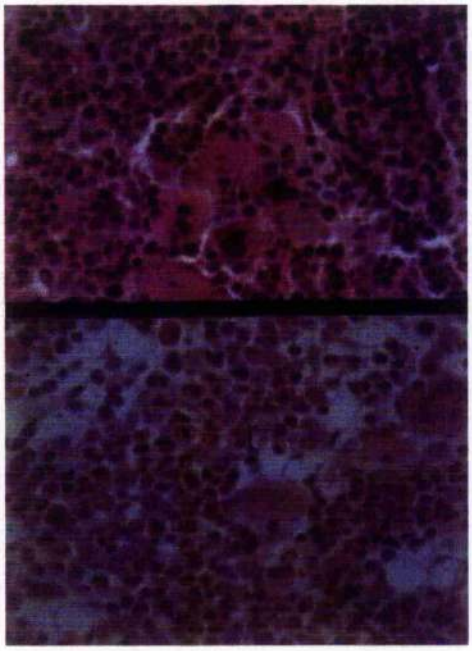


D



cell lines, the myeloid, lymphoid, erythroid, and platelet series, are susceptible to FeLV transformation (Cotter, 1990). It is still unclear exactly how FeLV contributes to the genetic dysregulation that culminates in malignancy. A number of mechanisms have been proposed including insertional mutagenesis, whereby provirus integrates adjacent to a cellular oncogene, which activates it; and transduction, where provirus incorporates cellular oncogenes in a recombination event. An investigation into spontaneous FeLV-associated thymic LSA identified changes in the cellular *myc* gene in fifty *per cent* of cases (six) (Neil *et al.*, 1984). Five genomes had *myc*-containing proviruses, and one genome demonstrated proviral integration adjacent to *c-myc*. Rearrangement of the *c-myc* locus was detected in two out of four experimentally induced tumours, associated with adjacent proviral integration. Therefore, in the field cases, oncogenesis was most often associated with recombination between FeLV provirus and *c-myc*. These findings were similar to other reports (Levy *et al.*, 1984; Mullins *et al.*, 1984), where naturally-occurring FeLV-associated tumours showed evidence of *myc*-containing FeLV provirus. However, since more than seventy-five *per cent* of naturally-occurring LSA in cats do not have these acute transforming defective FeLV-*myc* viruses, other mechanisms by which FeLV can induce LSA must exist (Hardy, 1993). In mice, polytropic mink cell focus-inducing (MCF)-MLV are generated as a result of a recombination event between ecotropic MLV and endogenous *env* sequences; the formation of which appears to be the initial step in the induction of lymphomas in AKR mice (Fan, 1993). The substituted portion of the *env* gene of a Moloney virus-derived MCF-MLV virus was reported to be remarkably similar to the analogous region of FeLV-B, prompting suggestions that the recombination event which generated FeLV-B may also precipitate oncogenesis in the cat (Hardy, 1993).

1.2.10.2 Lymphosarcoma

The most common FeLV-associated tumour is LSA, a malignant tumour of lymphocytes (Hardy, 1981b). Lymphosarcoma may originate within any organ and spread to other sites, but is classified according to the site of primary involvement. The categories are thymic (mediastinal) LSA, alimentary LSA, multicentric LSA and unclassified (Hardy, 1981b; Cotter, 1990). Thymic LSA originates from thymic lymphocytes but can extend throughout the chest cavity. Three-quarters are

associated with FeLV, and usually occur in cats aged approximately two and a half years (Hardy, 1981b). Alimentary LSA is more prevalent in older cats; however, a much lower percentage of these, approximately twenty-five *per cent*, are associated with FeLV (Pedersen, 1988). Alimentary LSA was the most commonly diagnosed form of LSA in Scotland (Crighton, 1969b; Crighton, 1969a). These tumours may arise from any source of lymphocytes along the gastrointestinal tract (GIT) but most are derived from B cells within the lamina propria (Hardy, 1981b). Multicentric LSA originates from multiple sites, such as the internal organs and lymph nodes. Ninety *per cent* are associated with FeLV and they tend to occur in cats of approximately four years of age. Multicentric LSA was the most common LSA form identified in the U.S. (Hardy, 1993). Unclassified LSA originates from a single site anywhere, apart from the GIT and thymus, such as skin, eyes, or nervous system; this is the least common form of LSA (Hardy, 1993).

1.2.10.3 Leukaemia

Leukaemia without concurrent LSA is rare (Crighton, 1969b; Hardy, 1981b). Where leukaemia occurs, it is often associated with multicentric LSA. In fact, approximately thirty *per cent* of cats with LSA have concurrent leukaemia (Hardy, 1993), the majority of which have multicentric LSA.

1.2.10.4 Feline Leukaemia Virus-Negative Tumours

Approximately thirty *per cent* of cats with LSA are aviraemic. However, FeLV proviral DNA sequences, distinct from endogenous sequences, have been detected in virus-negative LSA tissues (Koshy *et al.*, 1979; Koshy *et al.*, 1980). In addition, proviral DNA has been detected in non-LSA tissues, often bone marrow cells, in sixty *per cent* of these cats (Hardy *et al.*, 1980; Hardy, 1993). Feline leukaemia virus was reactivated from the bone marrow, but not LSA cells, in two aviraemic cats with LSA (Rojko *et al.*, 1982b). These findings suggested that FeLV was able to induce transformation but was not required to maintain the transformed state (Hardy, 1993). Francis *et al.* (1979) reported that out of one hundred and eighty-four cats diagnosed with either LSA or lymphoblastic leukaemia, sixty-one were aviraemic (thirty-three *per cent*), using the IFA test. He observed a distinct variation in the age distribution

of viraemic and aviraemic cats at the time of diagnosis; viraemic cats were diagnosed with LSA or lymphoblastic leukaemia at a significantly lower age (3.5 years), than aviraemic cats (4.9 years). Another study reported that in old cats, a higher proportion of tumours occurred in aviraemic animals (Gardner *et al.*, 1974). However, in Francis' study (1979), the actual number of tumours in young aviraemic cats was equivalent to those in older aviraemic cats, but tumours were rarely associated with viraemia in old cats.

1.2.10.5 Myeloproliferative Disease

Primary bone marrow neoplastic disorders are referred to as myeloproliferative diseases (MPD), which may involve any one or a combination of cells that originate in the bone marrow. Twenty *per cent* of FeLV-infected cats were reported to suffer from MPD, such as reticuloendotheliosis, erythemic myelosis, erythroleukaemia, myelogenous leukaemia, megakaryocytic leukaemia and myelofibrosis (Hardy, 1981b).

1.2.10.6 Anaemia

The incidence of anaemia is high among FeLV-viraemic cats (Mackey *et al.*, 1975). Secondary anaemia associated with concurrent FeLV disease, such as LSA, MPD or immunosuppressive disease, is common. Fifty-four *per cent* of LSA-positive cats with no accompanying bone marrow infiltration, suffered concurrent anaemia, which was suggested to be of haemolytic origin (Mackey *et al.*, 1975). In addition, transient regenerative anaemia was reported to occur following the experimental infection of kittens with FeLV-A and FeLV-B, prior to the development of haematopoietic neoplastic lesions (Mackey *et al.*, 1975). This form of anaemia was accompanied by increased bone marrow erythropoiesis and extramedullary haematopoiesis in the spleen, consistent with haemolytic anaemia.

However, primary non-regenerative anaemia without concurrent FeLV disease is an important syndrome in viraemic cats and is particularly associated with subgroup C. Kittens inoculated neonatally with FeLV-C died within sixteen weeks of infection, typically of congestive heart failure (Mackey *et al.*, 1975). Bone marrow was

severely depleted of erythropoietic tissue consistent with aplastic anaemia (Fig. 1.8.D). Similar results were reported by Hoover *et al.* (1974); neonatal kittens were inoculated with a KT-FeLV isolate containing subgroups A-C, which was donated by a cat suffering from progressive non-regenerative anaemia three months prior to death. Thirteen out of fifteen inoculated kittens demonstrated a severe progressive non-regenerative anaemia, presumably due to the FeLV-C component. Microscopic examinations of the bone marrow revealed a marked paucity of erythroid cell precursors. The pathogenesis of FeLV-C associated anaemia is yet unresolved. Research is currently focused on establishing the target cells for FeLV-C and identifying the pathogenic viral genetic determinants. Previous studies have indicated that FeLV-C greatly impairs the differentiation of early erythroid progenitor cells (burst-forming units, BFU) within the bone marrow, inhibiting erythrocyte production (Onions *et al.*, 1982; Hoover and Mullins, 1991).

1.2.10.7 Other FeLV-Associated Diseases

(i) Immune Complex Disease

Immune complexes form in situations of soluble antigen excess, rendering persistently viraemic cats susceptible to the development of immune complex disease. Many immune complexes deposit in the renal glomeruli (Weksler, 1975), although very few cats develop associated signs.

(ii) Reproductive Abnormalities

A variety of reproductive abnormalities has been associated with FeLV. Abortion, foetal resorption, infertility, stillbirths and neonatal deaths are reported to occur in over eighty *per cent* of FeLV-positive cats (Pedersen, 1988).

(iii) Enteritis

Feline leukaemia virus has a predilection for the rapidly dividing intestinal crypt cells. Feline leukaemia virus-associated enteritis (FAE) is a non-neoplastic condition associated with persistent FeLV infection, which is characterised by diarrhoea,

haematemesis and anaemia (Hardy, 1993). Viral proteins can be detected immunohistochemically in the small intestines of FeLV-positive cats; although only FeLV SU protein gp70 and TM protein p15E proteins are strongly expressed in regions of pathological change associated with FAE (Kipar *et al.*, 2000).

1.3 Immune Responses to Retroviral Infections

1.3.1 General Introduction

Retroviruses are obligate intracellular pathogens; therefore, to survive, they must establish a productive infection within a host cell. The immune system has evolved to resist such a scenario, and if successful, the animal recovers and is immune to subsequent exposure. However, if the host fails to mount an effective immune response, or if the retrovirus itself has evolved escape mechanisms, a persistent retroviral infection usually results. Persistent infections may be latent or productive, depending on whether the virus is actively replicating. If provirus remains in the host cell genome and fails to express viral proteins, the immune system cannot be alerted to its presence; thus, provirus persists in a latent state, such as often occurs following FeLV viraemia (Madewell and Jarrett, 1983). Alternatively, failure of the immune system to clear actively replicating virus may lead to a productive persistent infection, as transpires in most cases of HIV, FV and lymphocytic choriomeningitis virus (LCMV) infections.

Many retroviruses successfully establish either latent or productive persistent infections in host cells. Several viral factors contributing to the failure of the immune response have been identified. Some retroviruses, such as HIV-1, are specifically cytotoxic to key elements of the specific immune response, such as the $CD4^+$ T cell. In addition, HIV-1 is an effective immune escape artist: heavy glycosylation of the gp120 protein, a key VNA epitope, masks other potential immunogenic sites (Haseltine *et al.*, 1989). The HIV genome is also highly variable (Hu and Temin, 1990), so that mutations of key epitopes may arise within an individual at a sufficiently high frequency to escape VNA or cytotoxic T lymphocyte (CTL) recognition (Phillips *et al.*, 1991; Couillin *et al.*, 1994; Borrow *et al.*, 1997).

Escape mutants appear to have a survival advantage, as they persist (Borrow *et al.*, 1997). Furthermore, some retroviruses may contain immunosuppressive elements, such as the FeLV TM protein p15E, which has been reported to selectively inhibit cells within the immune system (Mathes *et al.*, 1979). The HIV-1 Nef protein causes the downregulation of major histocompatibility complex (MHC) class I molecules, effectively protecting infected cells against CTL killing (Collins *et al.*, 1998).

1.3.1.1 Humoral Immune Response

Virus neutralising antibodies have a critical role in inhibiting viral infectivity and cell-to-cell transmission. Secreted immunoglobulins bind free virus particles in mucosal secretions, serum, lymph and bone marrow. The exposed envelope and capsid proteins are bound by the immunoglobulin, sterically inhibiting viral attachment to cellular receptors; therefore, any potential viral effect on the cell is neutralised. Although most retroviruses induce VNA, in many instances they are either not protective (Bex *et al.*, 1979; Essex, 1982), or require other elements of cellular immunity in order to elicit a protective immune response (Hasenkrug and Chesebro, 1997; Dittmer *et al.*, 1999).

1.3.1.2 Cell-mediated Immune Response

(i) $CD4^+$ T cells

In humans and mice, $CD4^+$ T cells can be divided into two subtypes, T helper (Th)1 and Th2, according to their pattern of cytokine secretion (Mosmann *et al.*, 1986; Fiorentino *et al.*, 1989; Mosmann and Coffman, 1989; Abbas *et al.*, 1996). However, the distinction is not as complete as originally proposed; many cells have been identified which produce an array of cytokines that is not consistent with either Th1 or Th2 subsets. These cells are termed Th0 cells, and are more numerous in the early stages of lymphocyte activation (Kelso, 1995). However, both experimental and natural conditions have been identified, which induce clearly polarised immune responses, such as persistent infection with parasites such as *Leishmania major*, and bacteria such as *Listeria monocytogenes* and *Mycobacteria* spp. (Abbas *et al.*, 1996). In fact, chronic conditions of antigen persistence provide the most obvious examples

of $CD4^+$ T cell polarisation (Sher and Coffman, 1992).

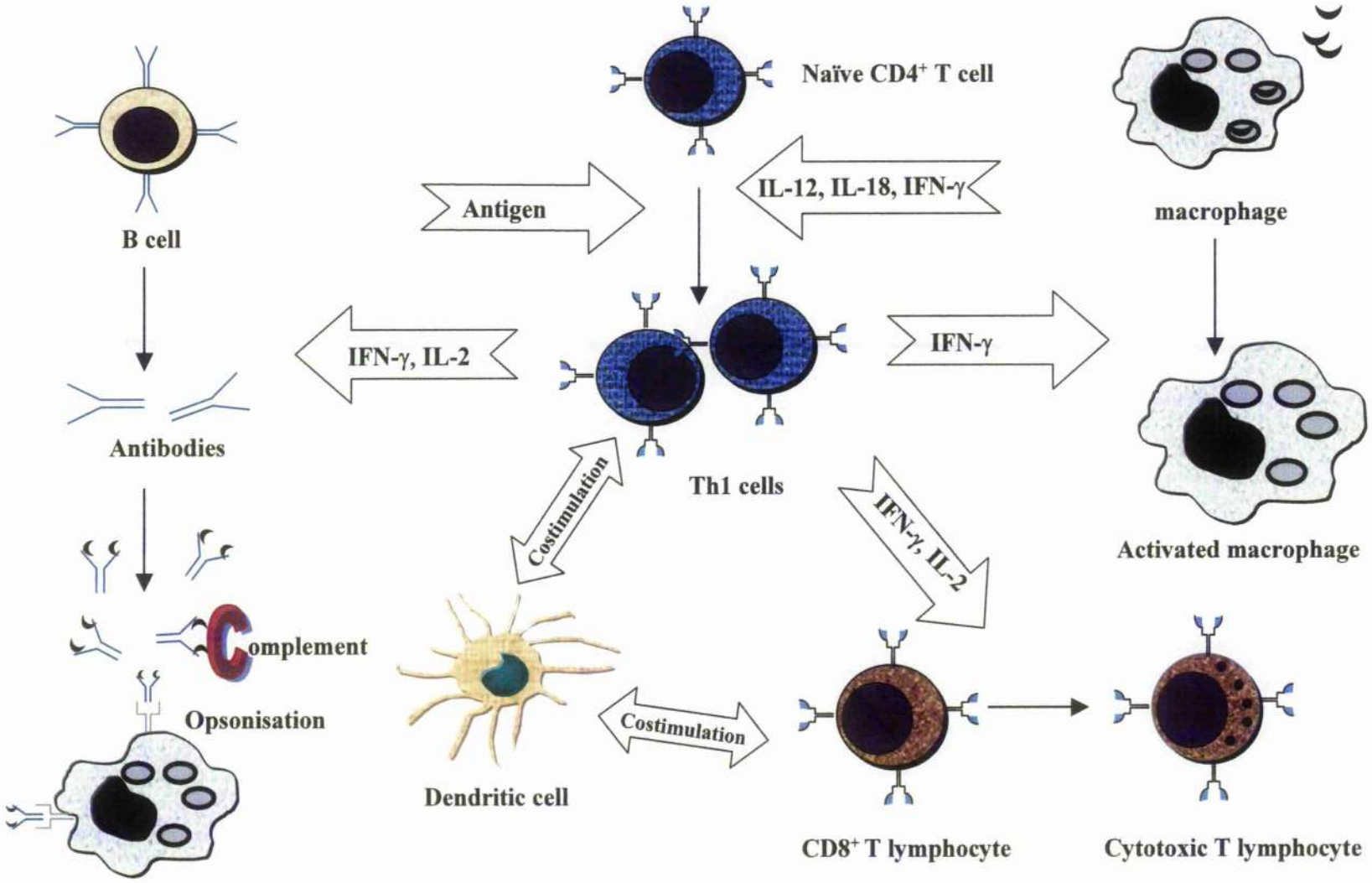
The key Th1 cytokine, interferon (IFN)-γ, is a critical element of the host immune response, activating macrophages and enhancing their microbicidal activity, promoting the production of opsonising antibodies, immunoglobulin (Ig)G2a and IgG3, and activating CTL (Farrar and Schreiber, 1993; Boehm *et al.*, 1997) (Fig. 1.9). Therefore, the principal function of Th1 cells lies in their ability to activate the phagocytic and CTL-mediated destruction of intracellular microbes (Abbas *et al.*, 1996). Th2 cells are characterised by their production of IL-4 and IL-5. Interleukin-4 elicits the isotype switching of B cells to IgE, which activates mast cells, while IL-5 activates eosinophils. Therefore, Th2 responses are the dominant responses in helminth infections and allergic phenomena, which are characterised by the activation of both mast cells and eosinophils (Abbas *et al.*, 1996).

Mature naïve $CD4^+$ T cells are non-polarised, and division into subtypes is a product of the cytokine microenviroment in which they first encounter an antigenic stimulus (Abbas *et al.*, 1996; Swain, 1999). Interleukin-12, IL-18 and IFN-γ, are all involved in eliciting Th1 responses, whereas IL-4 drives Th2 polarisation (Trinchieri, 1995; Tominaga *et al.*, 2000). Recently, it has been shown that human dendritic cell (DC) subsets can be defined according to whether they promote differentiation of Th cells into Th1 or Th2 subsets (Rissoan *et al.*, 1999). Dendritic cell subset-1 (DC1) produced IL-12 shortly after CD40L activation; conversely, DC2 produced neither IL-12 nor IL-4. The type I IFNs, IFN-α and IFN-β, are also significant products of uninfected precursor DC2 subset (pDC2) (Siegal *et al.*, 1999), and have a controversial role in directing human $CD4^+$ T cells into Th1 cells (O'Shea and Visconti, 2000).

$CD4^+$ T cells are important in the priming, maturation and maintenance of $CD8^+$ T cell responses through mechanisms as yet unexplained. However, the role of $CD4^+$ T cells in the activation of DC and secretion of key cytokines such as IFN-γ, both involved in the initiation and regulation of cellular and humoral immune responses, is likely to be relevant.

Fig. 1. 9 Effector function of $CD4^+$ T cells

The activation of naïve $CD4^+$ T cells is dependent on the recognition of cognate antigen presented in the form of an MHC class II complex, the activity of cytokines, and interactions with costimulatory molecules of the DC. Activated $CD4^+$ T cells promote the activation of macrophages and CTL, and enhance the production of opsonising antibodies by B cells through the activity of cytokines. $CD4^+$ T cells also activate DC through cell-to-cell interactions, which in turn stimulate the development of CTLs.


Naïve CD4+ T cell
B cell
Antigen
IL-12, IL-18, IFN-γ
macrophage
IFN-γ, IL-2
IFN-γ
Antibodies
Th1 cells
Costimulation
IFN-γ, IL-2
Activated macrophage
Complement
Opsonisation
Dendritic cell
Costimulation
CD8+ T lymphocyte
Cytotoxic T lymphocyte

Dendritic cells are activated through interactions between the CD40 molecule expressed on DC, and the CD40L molecule expressed on $CD4^+$ T cells (Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). Activated DC are critically important in the priming of naïve $CD8^+$ T cells (Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). Therefore, DC activation by $CD4^+$ T cells is an integral part of $CD8^+$ T cell priming (Zajac *et al.*, 1998b). $CD4^+$ T cells may also be important in maturing $CD8^+$ T cell function (Zajac *et al.*, 1998a; Appay *et al.*, 2000).

$CD4^+$ T cells are involved in the maintenance of $CD8^+$ T cell effector functions, particularly during chronic viral infections. Studies investigating chronic LCMV infection in mice have indicated that $CD8^+$ T cell responses cannot be maintained in the absence of $CD4^+$ T cell help (Battegay *et al.*, 1994; Matloubian *et al.*, 1994). In CD4-depleted mice, LCMV-specific CTL responses remain normal during the acute stage of infection, but are not maintained during the chronic phase of disease. As a result, these mice become persistently infected with the virus. Furthermore, LCMV-specific T cells in chronically infected mice are functionally inept, an effect exacerbated in a state of $CD4^+$ T cell deficiency (Zajac *et al.*, 1998b).

Virus-specific $CD4^+$ T cells are also required to maintain $CD8^+$ memory T cells in chronic human viral infections. The effectiveness of $CD8^+$ T cell clones infused into cytomegalovirus (CMV)-infected patients depended on the availability of specific $CD4^+$ T cell help (Walter *et al.*, 1995). In addition, poor $CD4^+$ T cell responses in HIV infection correlate with higher viral RNA loads (Rosenberg *et al.*, 1997).

(ii) $CD8^+$ T cells

$CD8^+$ T cells are the principal mediators of retroviral clearance. Cytotoxic T lymphocytes are activated $CD8^+$ T cells, and function as potent killers of virally infected cells via perforin-dependent or Fas-mediated mechanisms. In addition, CTL secrete inhibitory antiviral cytokines such as IFN-γ and tumour necrosis factor (TNF)-α, as well as chemokines. 'Regulated on activation, normal T cell expressed and secreted' (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β competitively inhibit binding of HIV to the CCR5 chemokine receptor *in vitro*,

effectively suppressing replication (Cocchi *et al.*, 1995). The relative importance of lytic and inhibitory functions varies with individual viruses (McMichael and Rowland-Jones, 2001).

(iii) Interferon-γ

Interferon-γ is recognised as a critical component of the innate and adaptive host immune response to viral, bacterial and parasitic pathogens of man and animals (Kemp *et al.*, 1999; Shankar *et al.*, 2000; Dittmer *et al.*, 2001; Bourreau *et al.*, 2002). Mutant mice with genetic deficiencies in IFN-γ, the receptor for IFN-γ (IFN-γR), or the Stat-1 transcription factor, were unable to generate an effective immune response against microbial infections (Shtrichman and Samuel, 2001).

Interferon-γ was, until recently, believed to be the product solely of natural killer (NK) cells (Carnaud *et al.*, 1999) and particular T cell subsets (Farrar and Schreiber, 1993), i.e. $CD4^+$ Th1 (Kohno *et al.*, 1997; Robinson *et al.*, 1997) and $CD8^+$ T cells (Cousens *et al.*, 1999). However, other sources of IFN-γ have since been identified, such as the B cell (Yoshimoto *et al.*, 1997); macrophage (Munder *et al.*, 1998; Schindler *et al.*, 2001); DC (Ohteki *et al.*, 1999; Hochrein *et al.*, 2001); the regulatory T cell (Tr) (Trinchieri, 2001), and the NKT cell (Carnaud *et al.*, 1999).

Interferon-γ interacts with the ubiquitously and almost universally expressed IFN-γR, launching a diverse series of events that directly and indirectly serve to contain microbial invasion. The induction of apoptosis and the inhibition of viral replication are antimicrobial events directly mediated by IFN-γ. Additionally, the role of IFN-γ in the upregulation of MHC class I and II, the activation of antigen presenting cells (APC) and the induction of Th1 responses, contributes indirectly but powerfully to antimicrobial resistance (Farrar and Schreiber, 1993; Young and Hardy, 1995; Boehm *et al.*, 1997). Interferon-γ acts directly to antagonise viral growth and replication through the induction of three genes encoding dsRNA activated protein kinase (PKR), 2'-5'-oligoadenylate synthestase (2-5A synthetase) and dsRNA specific adenosine deaminase (dsRAD) (Boehm *et al.*, 1997). Protein kinase is activated by binding to dsRNA intermediates produced by RNA viruses, thus

interfering with protein synthesis. Activation of 2-5A synthetase ultimately leads to activation of ribonuclease (RNAse)L that degrades single-stranded viral and cellular RNAs, inhibiting protein synthesis and viral growth. The dsRAD enzyme utilises dsRNA as a substrate in the deamination of adenosine, damaging mRNA resulting in the translation of a non-functional protein.

Interferon-γ promotes Th1 development by enhancing IL-12 secretion by macrophages, and by maintaining IL-12R expression on $CD4^+$ T cells, rendering them more responsive to IL-12 (Trinchieri, 1995). The addition of IFN-γ during Th priming *in vitro* accelerated and enhanced the effect of IL-12 on Th1 differentiation (Wenner *et al.*, 1996). Interferon-γ exerted an anti-proliferative effect on murine Th2 cells but not on murine Th1 cells *in vitro* (Maggi *et al.*, 1992), suggesting that the role for IFN-γ in Th1 differentiation lies in its ability to prevent the outgrowth of Th2 cells (Constant and Bottomly, 1997).

Major histocompatibility complex class I molecules are constitutively expressed on most nucleated cell types, with greatest expression on lymphomyeloid cells, whereas only professional APC, such as B lymphocytes, macrophages and dendritic cells, constitutively express MHC class II molecules in humans and mice (Boehm *et al.*, 1997). Interestingly, both resting and activated feline lymphocytes constitutively express MHC class II molecules (Willett and Callanan, 1995). Nonetheless, IFN-γ can induce MHC class II expression, as well as augmenting MHC class I molecule expression on virtually all cells, strongly promoting antigen presentation (Lapierre *et al.*, 1988; Boehm *et al.*, 1997). Interferon-γ promotes isotype switching from IgM by acting directly on the B cell (Snapper and Paul, 1985). Antibodies of isotype IgG2a and IgG3 are opsonising antibodies, binding to high affinity Fc receptors on macrophages and complement proteins in order to enhance phagocytosis.

Nitric oxide (NO), an unstable radical gas, is derived from L-arginine and molecular oxygen. The catalyst for this reaction comprises a series of enzymes termed nitric oxide synthase (NOS), an intermediate of which is inducible by IFN-γ acting with a second signal, such as TNF-α or lipopolysaccharide (LPS). In mice, IFN-γ-dependent NO formation appears to have a major role in the killing of intracellular

pathogens such as *Leishmania major* and *Listeria monocytogenes* (Green *et al.*, 1990). Interferon-γ also enhances the ability of macrophages to participate in other immune effector responses through the induction of high affinity Fc receptors and by boosting antibody dependent cell-mediated cytotoxicity (ADCC), and promoting complement protein synthesis (Farrar and Schreiber, 1993). Interferon-γ promotes the development of immature myeloid precursors into mature monocytes, and enhances antigen presentation through increased MHC class II expression and accelerated formation of intracellular and cells surface proteins, such as intracellular adhesion molecule (ICAM)-1 (Farrar and Schreiber, 1993).

1.3.2 Immune Response to FeLV

1.3.2.1 Introduction

The immune mechanisms that determine the outcome following exposure to FeLV have yet to be fully resolved. Both VNA and complement-dependent antibodies (CDA) have been detected in the peripheral blood from FeLV exposed cats (Charreyre and Pedersen, 1991). In some circumstances, VNA can confer protection (Jarrett *et al.*, 1977). Recently, the role of CTL in mediating both vaccinal protection and recovery has been investigated (Flynn *et al.*, 2000a; Flynn *et al.*, 2002a).

1.3.2.2 Humoral Immunity

Cats exposed to FeLV under experimental and natural conditions produce a wide range of virus-specific antibodies (Lutz *et al.*, 1980; Charreyre and Pedersen, 1991; Hawks *et al.*, 1991), regardless of whether they recover or remain persistently viraemic. Antibodies were detected to viral proteins gp70, p27, p15, p12 and p10, as well as to eight additional antigenic components of 58, 47, 36, 24, 17, 15.7, 12 and 8.5 kDa, in both recovered and persistently viraemic cats (Lutz *et al.*, 1980b) using a GEDELISA assay. This assay combines two techniques, polyacrylamide gel electrophoresis (PAGE) and ELISA; purified FeLV was electrophoresed on a polyacrylamide gel and molecular weight markers were used to detect the position of protein bands, which were cut out and used to coat polystyrene tubes. Antibodies to each of the FeLV proteins could then be detected using an ELISA.

Similarly, in a second study, both recovered and viraemic cats generated antibodies to viral proteins p15, p12, p10, p27 and gp70, detected using Western blots (Charreyre and Pedersen, 1991; Hawks *et al.*, 1991). In general, a negative Western blot pattern was consistent with persistent viraemia (Charreyre and Pedersen, 1991). Hawks *et al.* (1991) found that differences in anti-p27 antibody responses alone were statistically significant between persistently viraemic kittens and transiently viraemic or aviraemic kittens.

However, other authors reported that neither recovered nor viraemic cats produce anti-Gag antibodies, although anti-p27 antibodies can be induced in recovered cats (Jarrett, 1999). This might imply that the FeLV CA protein p27 either induces an anergic immune response, or absorbs circulating anti-p27 antibody (Neil *et al.*, 1980b; Hawks *et al.*, 1991).

(i) Virus Neutralising Antibodies

Virus neutralising antibodies may be of any isotype, although IgA antibodies may have an important role in the sites of early viral infection, such as in the oropharynx of cats infected with FeLV (Abbas *et al.*, 1997a). Virus neutralising antibodies predominately target epitopes located on the FeLV SU protein gp70 and TM protein p15E (Russell and Jarrett, 1978a; Elder *et al.*, 1987). Nick *et al.* (1990) mapped two epitopes on SU protein gp70, and one epitope on TM protein p15E, capable of inducing powerful cross-neutralising antibodies against all FeLV subgroups. Grant *et al.* (1983) demonstrated virus neutralising activity using mAbs directed against a number of FeLV Env glycoproteins. Epitope expression appeared to vary with the virus isolate under study, demonstrating antigenic polymorphism within FeLV subgroups and between isolates (Russell and Jarrett, 1978a; Grant *et al.*, 1983).

Neonatal kittens born to FeLV-immune queens may be protected by passively transferred maternal antibodies. Two queens exposed to FeLV-A/Glasgow-1 developed FeLV-specific VNA and remained clinically healthy (Jarrett *et al.*, 1973). Kittens born to each queen were allowed to suckle for two days before being challenged subcutaneously with FeLV. Kittens that received passively transferred VNA were protected from challenge, whereas kittens born to a naïve queen became

viraemic. The VNA titres from the passively protected kittens decayed exponentially, and were completely absent by sixteen weeks. In this study, maternally derived antibody protected kittens from postnatal challenge, and the authors predicted that suckling kittens are likely to be protected in the same way under natural conditions (Jarrett *et al.*, 1977).

In general, recovered cats produce higher antibody titres, which peak earlier, than persistently viraemic cats (Lutz *et al.*, 1980b; Charreyre and Pedersen, 1991). Using the data from their study, Lutz *et al.* (1980b), concluded that extreme levels of virus-specific antibody correlated with the clinical outcome of infection. Lower antibody levels were detected in cats that became persistently viraemic following experimental exposure to FeLV, compared to cats that recovered without becoming viraemic. Transiently viraemic cats produced levels of antibody that overlapped with those produced by aviraemic and persistently viraemic cats.

Although VNA appeared to be protective in Jarrett's study (1977), and are often present at higher levels in recovered cats, VNA do not necessarily mediate recovery. In a recent longitudinal FeLV immunopathogenesis study, all cats that recovered from experimental challenge developed significant VNA titres. However, the appearance of VNA followed, or was concurrent with, clearance of infectious virus (Flynn *et al.*, 2002b). Similar findings have been reported in other studies where a small proportion of cats recovering from experimental challenge with FeLV do not have detectable VNA (Hoover *et al.*, 1976; Russell and Jarrett, 1978b; Charreyre and Pedersen, 1991). Moreover, VNA were present in the serum of some cats that eventually became persistently viraemic (Charreyre and Pedersen, 1991).

Virus neutralising antibodies are unlikely to be involved in eliciting protective immunity following vaccination. Specific pathogen free kittens were vaccinated with one of three commercially available vaccines, and were exposed to FeLV viraemic cats. Significant differences in antibody responses were not detected between kittens that developed persistent viraemia, and either those that became transiently viraemic, or those that recovered without becoming viraemic (Hawks *et al.*, 1991). Furthermore, in a recent FeLV DNA experimental vaccine study (Hanlon *et al.*, 2001), protection was conferred in the absence of VNA. Moreover, infectious

virus was cleared from the blood before virus-specific VNA were generated post-challenge, indicating that VNA were not involved in recovery.

(ii) Complement-Dependent Antibodies

Complement-dependent antibody interacts with antigen, bound in an immune complex or attached to a cell surface, and mediates viral or cell lysis (Abbas *et al.*, 1997b). Complement-dependent antibodies are not present in unexposed SPF cats but appear following exposure to FeLV, and are present in both recovered and persistently viraemic cats (Grant *et al.*, 1978; Grant *et al.*, 1979). These antibodies are directed to neither Env nor Gag proteins (Grant *et al.*, 1978), and can be demonstrated *in vitro* using immune cat sera and feline complement to lyse FeLV antigen-expressing FL74 cells (Grant *et al.*, 1977). Complement-dependent antibody is typically of isotype IgG1, IgG3 or IgM. Grant *et al.* (1979) observed that in FeLV immune cats, CDA levels declined over time, whereas persistently viraemic cats experienced cyclical fluctuations in CDA levels. The author also considered whether CDA might be directed against tumour antigens rather than viral antigens as demonstrated by a significant correlation between the presence of CDA and the presence of lymphoma.

1.3.2.3 Cell-mediated Immune Response

The importance of CTL as mediators of protective immunity and effectors of recovery in retroviral infections such as FIV and HIV, has been established (Flynn *et al.*, 1996; Kaul *et al.*, 2000). The ability of CTL to control FeLV replication and mediate recovery has recently been delineated; cats that recovered following experimental exposure to FeLV developed early virus-specific CTL responses, which were maintained until infectious virus was cleared from the blood (Flynn *et al.*, 2002b). All FeLV-exposed cats demonstrated two peaks in CTL activity, at weeks four to seven, and subsequently at weeks ten to thirteen, which coincided with two peaks in proviral DNA load (at weeks four and ten). The early CTL response was Gag/Pro-specific, while the second peak was Env-specific. Cats that failed to recover from FeLV infection following experimental challenge exhibited delayed and short-lived virus-specific CTL. In addition, the adoptive transfer of a single

infusion of mixed virus-specific $CD4^+$ and $CD8^+$ T cells significantly reduced the proviral DNA burdens in persistently infected cats, indicating a direct role for virus-specific T cells in the control of FeLV viraemia (Flynn *et al.*, 2002b).

Virus-specific CTL are also important in protective vaccinal immunity. High levels of FeLV-specific CTL were detected in the blood and lymphoid tissues from FeLV DNA-vaccinated protected cats (Flynn *et al.*, 2000a; Hanlon *et al.*, 2001). Virus-specific CTL were detected at significantly higher levels in both protected and recovered cats, compared to persistently viraemic cats. Furthermore, significantly higher levels of Gag/Pro-specific CTL were detected in transiently viraemic cats that recovered, compared to persistently viraemic cats. However, differences in Env-specific CTL responses between the same groups were not significant (Flynn *et al.*, 2000a), indicating that the predominant CTL response in protected and recovered cats is Gag/Pro-specific. The authors suggested that the CTL responses of persistently viraemic cats may be directed to viral proteins other than Gag, Pro or Env, such as the Pol proteins RT and IN.

1.3.2.4 Immunosuppression

Early studies indicated that both cell-mediated and humoral immune responses were suppressed in FeLV-exposed cats (Perryman *et al.*, 1972; Cockerell *et al.*, 1976; Hebebrand *et al.*, 1977; Mathes *et al.*, 1978; Hebebrand *et al.*, 1979; Mathes *et al.*, 1979). At that time, it was not clear whether lymphopenia caused by thymic atrophy and viral infection of lymphoid tissues could account for the ensuing immunosuppression (Perryman *et al.*, 1972), or whether other, as yet undefined, immuosuppressive factors were involved. An emerging consideration was the status of the $CD4^+$ T cell, known to be important in the regulation of both cell-mediated and humoral immune responses as well as innate non-specific immune responses.

In 1976, Cockerell *et al.* examined the response of lymphocytes from FeLV-infected kittens to mitogen-induced activation using the lymphocyte blast transformation (LBT) assay. He discovered that, compared to uninfected controls, infected kittens had a greatly reduced ability to respond to Con A, and to a lesser extent, pokeweed

mitogen (PWM). Disease progression was characterised by an early decline in B cell numbers, which subsequently returned to normal levels. In humans and mice, Con A is known to be T cell selective, whereas PWM stimulates both T and B cells. If these mitogens exerted similar selective effects on feline cells, the findings from this study would indicate that FeLV exerts a greater suppressive effect on T cells, compared to B cells. The author also suggested that the early decline in B cell numbers might reflect early defective $CD4^+$ T cell function.

Eight clinically healthy but persistently viraemic cats were inoculated with ($_L$-tyrosine-$_L$-glutamic acid)-poly-$_{DL}$-alanine-poly-$_L$-lysine, ((T,G)AL) antigen, a T cell-dependent antigen in the rat. Subsequent blood sampling revealed that six out of eight cats showed a delayed and reduced (T,G)AL-specific antibody response compared to an uninfected control group (Trainin *et al.*, 1983), prompting the author to suggest that a $CD4^+$ T cell malfunction in the persistently viraemic cats might have contributed to the diminished humoral response.

The immunosuppressive effect of FeLV did not appear to be restricted to B and T cells. Persistently viraemic cats are highly susceptible to opportunistic bacterial and fungal infections, which might indicate impaired innate immunity. Indeed, polymorphonuclear (PMN) cells from FeLV-infected cats were shown to be functionally impaired *in vitro* as determined by the chemiluminescent (CL) response. Depressed PMN function first became evident during early viraemia and continued to decline throughout the course of the experiment, even though all cats had succeeded in eliminating virus and were clinically healthy (Lafrado and Olsen, 1986). In 1987, Lafrado *et al.* investigated the effect of FeLV on the functional capacity of PMN cells from healthy cats. Inactivated virus added to latex bead-stimulated PMN suppressed CL activity by fifty *per cent*, compared to controls.

The suppressive effect of UV-inactivated FeLV on lymphocyte function had previously been reported. The response of PBMC from SPF cats to the mitogen Con A in a LBT assay, was suppressed twenty to sixty-five *per cent* in the presence of inactivated FeLV (Hebebrand *et al.*, 1977). The inactivated virus was not cytotoxic, as determined using Trypan Blue exclusion to assess the viability of cultured cells. Similarly, UV-inactivated FeLV significantly suppressed cat lymphocyte recognition

of allogenic cells using the mixed leukocyte reaction (MLR) (Stiff and Olsen, 1983).

It is apparent from these studies that FeLV *in vivo,* and inactivated FeLV *in vitro,* can suppress T, and to a lesser extent B, cell function, as well as PMN function. Evidence exists to suggest that the FeLV TM protein p15E may be partly responsible for the immunosuppressive effects of FeLV. Mathes *et al.* (1979) added purified FeLV TM protein p15E to Con A-stimulated lymphocytes from SPF cats, and found a markedly reduced responsiveness in the LBT, compared to an identical incubation with purified FeLV CA protein p27. Inhibition was shown to be independent of competition for Con A binding sites and FeLV TM protein p15E toxicity. Lafrado *et al.* (1987) also observed a suppression of chemiluminescent activity when latex-stimulated PMN from healthy cats were incubated with FeLV TM protein p15E. The ability of FeLV TM protein p15E to suppress human lymphocyte mitogenic and antigen-specific responses was illustrated using the LBT (Hebebrand *et al.*, 1979); proliferative responses to the mitogens Con A and phytohaemagglutinin (PHA) were reduced by up to ninety-six *per cent,* in four out of six humans tested.

The mechanisms through which inactivated FeLV and/or p15E might exert an immunosuppressive effect have been investigated. Copelan *et al.* (1983) reported that purified FeLV TM protein p15E inhibited the proliferative responses of human lymphocytes to Con A when the cells were co-cultured with IL-1, and suggested that FeLV TM protein p15E interfered with IL-2 production by T cells. Shortly afterwards, UV-inactivated FeLV was shown to depress the accumulation of IL-2 and macrophage activating factor (MAF) in Con A-stimulated murine T cells (Orosz *et al.*, 1985). Both IL-2 and MAF production by $CD4^+$ T cell and CTL populations, respectively, were blocked. It was concluded that FeLV acted directly to exert a general but temporary impairment on the ability of the T cell to produce, and respond to, cytokines.

Further evidence that FeLV impairs cytokine production has been documented. Peripheral blood mononuclear cells and splenic lymphocytes from FeLV-infected cats could not be induced to produce significant levels of feline IFN-γ (fIFN-γ) in response to *Staphylococcus aureus* protein A (SPA) (Liu *et al.*, 1984). Similar findings were recorded *in vitro*, where PMBC from SPF cats were co-cultured with

inactivated FeLV and *Staphylococcus aureus* enterotoxin A (SEA) in LBT assays. Supernatants were collected and assayed for fIFN-γ activity using a plaque reduction assay. Levels of fIFN-γ were markedly reduced where inactivated FeLV was included in the culture system, compared to activity detected using the mitogen alone (Engelman *et al.*, 1984). The effect was dose dependent, with increased antiviral activity detected at lower concentrations of inactivated FeLV.

Nonetheless, no differences in protection afforded by a canarypox virus recombinant FeLV vaccine (ALVAC-FL) expressing either intact Env, or Env in which 'immunosuppressive' sequences were eliminated, were detected (Tartaglia *et al.*, 1993).

1.3.2.5 Vaccination

Currently, three FeLV vaccines are commercially available in the U.K. (Sparkes, 1997). The first of these is an adjuvanted form of whole inactivated FeLV ('Fevaxyn', Fort Dodge Animal Health, Wyeth, New Jersey, U.S.); the second is an adjuvanted mixed subunit from FeLV-infected tissue culture filtrate ('Leukocell 2', Pfizer Ltd, Kent, U.K.). Finally, a vaccine based on a purified, adjuvanted, recombinant non-glycosylated form of FeLV SU protein gp70 (p45), is available ('Leucogen', Virbac S.A. Carros cedex, France). Ideally, an effective vaccine should protect FeLV exposed cats from both viraemia and latent infection. However, none of these vaccines fully protect exposed cats against the development of viraemia or latent infection (Jarrett and Ganière, 1996; Sparkes, 1997).

Few independent vaccine efficacy studies are available; many efficacy studies have either been conducted or supported by the manufacturer, and results are often conflicting. The preventable fraction (PF) figures (Sparkes, 1997), which represent the proportion of cats protected by vaccination in excess of that protected by natural resistance, reveal variations between studies testing the same vaccine. For instance, the 'Leukocell 2' vaccine has a reported PF which varies between 4.8 *per cent* (Jarrett and Ganière, 1996), and one hundred *per cent* (Lafrado, 1984). However, vaccine efficacy cannot truly be compared without considering other factors such as the number of cats used in the study, the number of cats that developed persistent

viraemia, the route of challenge and the age of cats at the time of challenge. Nonetheless, however these vaccines are compared, none have successfully managed to prevent transient viraemia when challenged in a controlled study (Sparkes, 1997). In fact, none of these vaccines consistently protected against the development of persistent viraemia (Sparkes, 1997).

The search for more effective vaccines has precipitated experimentation with novel adjuvants (Osterhaus *et al.*, 1985), live viral vectors (Willemse *et al.*, 1996) and DNA vaccination (Hanlon *et al.*, 2001). An experimental FeLV DNA vaccine containing *gag*, *pol* and *env* genes, as well as IL-12 and IL-18 DNA, was shown to be an effective vaccine, in that all cats receiving this construct were protected from persistent and transient viraemia (Hanlon *et al.*, 2001). However, protection from latent infection was not complete.

The mode of protection conferred by the commercially available vaccines has not been investigated. It was believed that VNA were critical mediators of protection (Loar, 1993; Sparkes, 1997), although significant VNA titres were not observed using some vaccine preparations until after challenge (Pedersen, 1993). Indeed, inoculation with the FeLV DNA vaccine described above did not elicit any anti-FeLV antibody response before challenge. After challenge, strong VNA responses were elicited in the majority of recovered and protected cats. These antibodies did not appear any earlier, or at any greater titre, in vaccinated protected cats, compared to recovered control cats; these data suggest that the VNA were not induced by the vaccine (Hanlon *et al.*, 2001). In contrast, high levels of FeLV-specific CTL were detected in the blood and lymphoid tissues from vaccinated protected cats (Flynn *et al.*, 2000a). In both protected and recovered cats, FeLV-specific CTL were detected at significantly higher levels than in persistently viraemic cats, indicating that protection was conferred by CTL. The inclusion of DNA encoding cytokines IL-12 and IL-18 in the vaccine construct would have enhanced the generation of CTL effector cells; these cytokines are known to drive Th1 immune responses (Trinchieri, 1995; Tominaga *et al.*, 2000). Therefore, for FeLV, vaccine strategies favouring Th1 responses that generate virus-specific CTL should prove the most successful in eliciting protective immunity.

1.3.3 Immune Response to Other Retroviruses

What information regarding antiretroviral immunity can be gleaned from other systems, and applied to FeLV?

1.3.3.1 Role of $CD4^+$ T Cells in Antiretroviral Immunity

Cytotoxic T lymphocyte responses, T cell proliferative responses and VNA are all required elements in a protective immune response against FV (Hasenkrug and Chesebro, 1997). Virus-specific $CD4^+$ T cells provide help for both CTL (Hasenkrug and Chesebro, 1995) and B cells (Perry *et al.*, 1994; Super *et al.*, 1998). The rapid development of $CD4^+$ T cell proliferative responses is associated with recovery from a high dose challenge of FV (Miyazawa *et al.*, 1988). Depletion of $CD4^+$ T cells *in vivo* had little effect in the early stages of recovery but did lead to a marked reduction in recovery in the final stages, suggesting that $CD4^+$ T cells were less important in the early stages of recovery but were required to maintain the recovered status (Robertson *et al.*, 1992). The $CD4^+$ T cell response is specific for determinants in the FV Env protein, and two epitopes have been characterised within the SU protein gp70.

In HIV-1 infected humans and non-human primates, $CD8^+$ T cells are central to anti-HIV-1 immune responses (Schmitz *et al.*, 1999; McMichael and Rowland-Jones, 2001). An additional role for VNA in the suppression of primate lentiviral infections has also been postulated (Shibata *et al.*, 1999). Since $CD4^+$ T cells have a fundamental role in the promotion and regulation of these two arms of the immune response, the capacity of these cells to function normally during primate lentiviral infections is of critical importance. Human immunodeficiency virus-1 infection is characterised by a steady decline in $CD4^+$ T cell numbers, presumably lost through infection and virus-induced apoptosis (Rosenberg and Walker, 1998; Altfield and Rosenberg, 2000). The number of virus-specific $CD4^+$ T cells is also low (Pitcher *et al.*, 1999). HIV preferentially infects virus-specific $CD4^+$ T cells (Douek *et al.*, 2002), which may be particularly vulnerable to HIV infection because of their association with HIV-bearing dendritic cells within lymph nodes. However, a functional defect in $CD4^+$ T cells is apparent early in the asymptomatic phase, before

the dramatic drop in $CD4^+$ T cell numbers occurs (Miedema *et al.*, 1988; Clerici *et al.*, 1989; McMichael and Rowland-Jones, 2001). Nonetheless, in both humans and rhesus macaques, early virus-specific $CD4^+$ T cell responses can be restored using antiretroviral therapy. The application of such therapy in rhesus macaques during primary infection with simian immunodeficiency virus (SIV) replication led to the rapid suppression of viral replication in some animals, together with the development of strong SIV-specific $CD4^+$ T cell responses. Furthermore, these animals were resistant to re-challenge. Simian immunodeficiency virus-specific $CD4^+$ T cell responses inversely correlated with viral RNA load, implicating $CD4^+$ T cells in resistance to infection (Lifson *et al.*, 2000). Early antiretroviral therapy can also 'rescue' this HIV Gag-specific $CD4^+$ T cell response in humans (Rosenberg *et al.*, 1997; McMichael and Rowland-Jones, 2001).

However, in the absence of treatment, the majority of patients infected with HIV-1 experience a progressive and irreversible loss of $CD4^+$ T cell function, characterised by a deterioration of delayed type hypersensitivity (DTH) responses to common recall antigens (Dolan *et al.*, 1995); a loss of *in vitro* responsiveness to mitogenic and antigenic stimulation (Clerici *et al.*, 1989), and a switching from Th1 to Th2 cytokine profiles (Maggi *et al.*, 1994). Human immunodeficiency virus-1-specific $CD4^+$ T cell responses have been measured in patients throughout the course of infection using lymphocyte proliferation assays (LPAs). Only the long-term non-progressor (LTNP) patients consistently demonstrated significant levels of proliferation to viral antigens (Rosenberg *et al.*, 1997; Picker and Maino, 2000). This cohort of HIV-infected individuals maintain normal $CD4^+$ T cell numbers and low to undetectable viral RNA loads, exhibit no signs of HIV-1-related disease and consistently demonstrate vigorous cellular and humoral responses to HIV (Rosenberg *et al.*, 1997). However, AIDS patients with lower viral RNA loads did show some HIV-specific $CD4^+$ T cell responses (Musey *et al.*, 1999). Responses were predominately directed towards Gag proteins; occasionally, responses towards Env and Pol proteins were detected (Rosenberg *et al.*, 1997; Musey *et al.*, 1999).

Intracellular cytokine staining (ICS) using flow cytometry to detect IFN-γ expression provides a means of MHC-independent quantification of antigen-specific $CD4^+$ T cell frequencies (Pitcher *et al.*, 1999), and is more reflective of the true frequency of

antigen-specific $CD4^+$ T cells (Picker and Maino, 2000). Using this method, HIV-1-specific $CD4^+$ T cells were detected in all LTNP and recently infected (less than three years) patients, and surprisingly, in over half of the chronically infected patients with progressive disease. The response was primarily against Gag proteins, and the cytokine profile characteristic of Th1 cells (IFN-γ and IL-2). In the proliferation studies, an inverse correlation was detected between plasma viral RNA loads and $CD4^+$ proliferative responses (Rosenberg *et al.*, 1997; Musey *et al.*, 1999); however, using ICS, there was no statistically significant correlation between HIV-1-specific $CD4^+$ T cell frequencies and viral RNA load, although the median frequencies were higher in LTNPs than in the progressor group (Pitcher *et al.*, 1999). A second study using similar ICS techniques to detect IFN-γ expression in recognition of cognate antigen, concurred; the difference in Gag-specific $CD4^+$ T cell frequencies between LTNPs and the slow progressors (SPs) was not statistically significant (Boaz *et al.*, 2002). However, Gag-specific $CD4^+$ T cells that produced both IFN-γ and IL-2 were associated with a good clinical outcome in HIV infection, in that LTNPs had higher frequencies of such cells in the peripheral blood, compared to slow progressors (SPs) (Boaz *et al.*, 2002). Furthermore, the frequency of the IFN-γ^+ IL-2^+ $CD4^+$ T cell population correlated inversely with viral RNA load, but not CD4 count. Gag-specific IFN-γ^+ $CD8^+$ T cell frequency did not differ between the two groups; and only Gag-specific IFN-γ^+ IL-2^+ $CD4^+$ T cell frequency correlated with the Gag-specific IFN-γ^+ $CD8^+$ T cell frequency.

Bovine leukaemia virus-specific cellular immune responses have been investigated using the LPA. The core antigen, p24, was prepared from a cell line constitutively producing BLV antigens, and incubated with T cells isolated from the serum of asymptomatic, seropositive animals. $CD4^+$ T cells from seropositive animals proliferated in response to p24, whereas $CD8^+$ T cells did not show any significant proliferation (Mager *et al.*, 1994). These findings suggested that the p24 responder population resided in the $CD4^+$ T cell subset.

1.3.3.2 Role of CTL in Antiretroviral Immunity

The presence of FV-specific CTL correlates with recovery from splenomegaly in

FV-infected mice (Robertson *et al.*, 1992). Depletion of $CD8^+$ T cells *in vivo* dramatically reduced the incidence of recovery. Friend virus complex-specific CTL recognise viral antigens in the context of H-2D molecules (Chesebro, 1990). The major CTL epitope is present within the FV Env protein but has yet to be identified (Hasenkrug and Chesebro, 1997).

Cytotoxic T lymphocytes are important in the control of viraemia in the initial acute phase of HIV and SIV infection, and during the asymptomatic period (Koup *et al.*, 1994; Borrow *et al.*, 1997; Ogg *et al.*, 1998; Wilson *et al.*, 1998; Jin *et al.*, 1999). However, virus-specific CTL are unable to control virus spread, despite the presence of high frequencies of virus-specific $CD8^+$ T cells, (Ogg *et al.*, 1998; Wilson *et al.*, 1998; Kuroda *et al.*, 1999; Lieberman *et al.*, 2001). These data suggest that the virus-specific CTL may not be functioning optimally. In one study, less than fifteen *per cent* of HIV-specific CTL contained perforin, which resulted in the poor killing of target cells *ex vivo* (Appay *et al.*, 2000). Low levels of perforin expression may correlate with immaturity, and might reflect inadequate $CD4^+$ T cell function (Champagne *et al.*, 2001; McMichael and Rowland-Jones, 2001). The majority of HIV-specific memory T cells detected in HIV-infected individuals resided in a pre-terminally differentiated subset, whereas most CMV-specific cells were terminally differentiated (Champagne *et al.*, 2001). Assessment of the capacity of SIV and HIV-epitope-specific $CD8^+$ T cells to produce IFN-γ has been made possible through the development of tetramer technology and ICS. These highly sensitive techniques permit the enumeration of individual epitope-specific cells (Section 1.4). In rhesus monkeys chronically infected with SIV, $CD8^+$ T cells were unable to secrete IFN-γ in response to their specific peptide epitope (Vogel *et al.*, 2001), but could in response to the mitogens phorbol myristate acetate (PMA) and ionomycin. Similar findings were reported for humans infected with HIV. In most patients, less than twenty-five *per cent* of tetramer-positive cells produced IFN-γ in response to their cognate HIV peptide epitope (Shankar *et al.*, 2000). Short-term culture with IL-2 restored the capacity of $CD8^+$ T cells to produce IFN-γ and lyse HIV-infected $CD4^+$ target cells; however, these cells could not be rescued in more advanced patients, suggesting progressive $CD8^+$ T cell dysfunction with disease advancement. Where the anti-HIV CTL response is restricted to just a few immunodominant epitopes (Phillips *et al.*, 1991; Couillin *et al.*, 1994; Borrow *et al.*, 1997), virus escape mutants can develop

and persist. In fact, HIV-specific CTL responses directed against a limited number of viral epitopes will drive the selection of escape mutants (Borrow *et al.*, 1997), and may contribute to the failure of the early CTL response to control viral replication.

1.3.3.3 Role of Humoral Immunity

Virus neutralising antibodies are an important correlate of protection against FV. A non-MHC gene, *Rfv-3,* influences the production of VNA. Mortality is increased by up to ninety *per cent* in mice unable to elicit VNA (Hasenkrug and Chesebro, 1995). In addition, B cell-depleted mice have reduced tumour-specific $CD4^+$ and $CD8^+$ T cell responses, indicating a role for B cells in antigen presentation and the induction of T cell responses *in vivo* (Schultz *et al.*, 1990).

Anti-HIV antibodies are primarily directed against the Env glycoproteins gp120 and gp41, and against the Gag proteins p17 (Barker *et al.*, 1995) and p24 (Weber *et al.*, 1987). Viruses isolated from an infected individual over time have shown variable levels of sensitivity to VNA, suggesting the development of virus escape mutants (McKeating *et al.*, 1989; Bourreau *et al.*, 2002). The Env protein is subject to greater selection pressure than Gag and Pol proteins, which tend to be more conserved; anti-Gag antibodies do not neutralise virus so no effect of antibody selection occurs. Despite the fact that high anti-Env titres can be detected in the sera of most HIV-infected individuals, the key events of CD4 binding and fusion are not inhibited, indicating that humoral immune responses are not protective (Matthews *et al.*, 1986). However, recent studies in pig-tailed macaques with chimeric simian-human immunodeficiency virus (SHIV) have demonstrated passive protection conferred by anti-HIV-1 Env neutralising antibodies alone *in vivo* (Shibata *et al.*, 1999). The *env* gene was derived from $HIV\text{-}1_{DH12}$, a dual-tropic primary virus isolate.

Bovine leukaemia virus induces antibodies to the Env protein gp51 and to the Gag protein p24 (Bex *et al.*, 1979; Bruck *et al.*, 1984). However, these antibodies, which may persist throughout the life of infected animals, are not effective in eliminating virus or in preventing disease progression. Furthermore, antibody titres may even increase as disease progresses to persistent lymphocytosis or tumour development (Mager *et al.*, 1994).

1.3.3.4 Role of Cytokines in Antiretroviral Immunity

Whether Th1 or Th2 responses predominate in recovering FV-exposed mice is not yet unknown. However, the importance of CTL in recovery suggests that Th1 responses are likely to be important. The role of IFN-γ, as well as IL-4 and IL-12, in the pathogenesis and control of a primary FV infection was determined using mice with genetic inactivations in each of the cytokine genes (Dittmer *et al.*, 2001). High levels of viraemia were detected in IFN-γ-deficient mice; furthermore, one-third developed gross splenomegaly by week ten. The remaining IFN-γ-deficient mice appeared to be able to compensate for the lack of IFN-γ. Interleukin-4 and IL-12-deficient mice behaved as normal wild-type mice. Vaccinated challenged mice did not require IFN-γ to control FV infection.

1.3.3.5 Immunosuppression

Friend retrovirus complex can suppress both cellular and humoral immune responses in certain mouse strains. In H-2D$^{d/d}$ mice (mice with the lowest incidence of recovery), humoral immune responses were suppressed, including antibody responses to T cell independent antigens, indicating that defective CD4^{+} T cell function was not the cause (Morrison *et al.*, 1986). Susceptible mice also experience FV-mediated impaired NK activity (Lu *et al.*, 1992); in addition, antigen presentation by macrophages is compromised (Jones *et al.*, 1992).

The majority of sera from BLV-infected cattle inhibited mitogen-induced lymphocyte blastogenesis in a dose-dependent manner (Takamatsu *et al.*, 1988), and impaired PMN cell activity. Sera from cattle with persistent lymphocytosis were added to an IL-2-dependent T cell line, and caused growth suppression. However, cellular viability was not impaired, suggesting the existence of an immunosuppressive factor in the serum of diseased animals.

1.3.3.6 Vaccination

Protection of susceptible mouse strains against FV can be achieved using a range of

vaccine types, such as killed or attenuated viruses, viral proteins, viral peptides or recombinant vaccinia virus (rVV) vectors expressing FV genes (Hasenkrug and Chesebro, 1997). Protective responses must include $CD4^+$ T cell proliferation and CTL activity, as well as VNA. The *env* gene within the rVV vectors appears to elicit better protective responses than the *gag* gene (Miyazawa *et al.*, 1992). However, seventy *per cent* of $H\text{-}2^{a/b}$ mice that were immunised with rVV-*gag* recovered after sixty days post challenge. The protective mechanism has not been delineated; however, co-operation between Gag-specific $CD4^+$ T cells and Env-specific effector cells was considered to be involved *e.g.* class switching of VNA to IgG following challenge with FV in Gag-immune mice. One CTL, three Th and two VNA epitopes have been identified within the SU protein gp70. The number of $CD4^+$ T cell epitopes available within the vaccine appears to be a critical factor in determining the requirement for CTL (Hasenkrug *et al.*, 1996).

With an estimated fifteen thousand new infections each day, the development of an effective therapeutic and prophylactic vaccine against HIV is critical. Researchers are striving to target virus-specific $CD8^+$ T cells in order to evoke protective immune responses. Experimental studies have shown that antibodies failed to neutralise wild-type virus *in vitro*, and neutralisation, where it occurred, was largely strain-specific. Ideally, a vaccine against HIV would deliver sterilising immunity. However, realistically, an HIV-vaccine might only offer partial protection against infection. $CD8^+$ T cells cannot prevent infection of cells by HIV, but could abort an infection before it became established, or if the virus is not eliminated, contain the virus at a significantly lower level (McMichael and Rowland-Jones, 2001), which might reduce the severity of infection, create more LTNP and, in addition, reduce the transmissibility of the virus.

Various prime and boost vaccine combinations can also be used to enhance immune responses. Prime-boost HIV vaccines are currently undergoing phase I and II clinical trials; an epitope-based DNA vaccine is administered followed by a modified vaccinia virus Ankara (MVA) vaccine incorporating a CTL multi-epitope immunogen. This combination induced good virus-specific CTL responses in rhesus macaques, detected using ^{51}Cr release assays, enzyme linked immunospot (ELISpot) assays and tetramer staining (Hanke *et al.*, 1999; Allen *et al.*, 2000). However, this

regimen failed to protect two out of three challenged animals.

Since BLV is more pathogenic in sheep than in cattle, with a shorter latent period, sheep have often been used in experimental BLV studies. Sheep vaccinated with rVV expressing the BLV glycosylated Env protein generated partially protective immune responses against BLV (Ohishi *et al.*, 1991). Vaccinated animals were not completely protected from challenge but BLV proliferation was suppressed in both healthy challenged animals and in carrier animals. Antibodies were not induced before challenge, suggesting that cell-mediated immunity was important. In fact, vaccination elicited strong DTH responses, implicating $CD8^+$ T cell activity. Antibody titres post-challenge were similar in both the control infected and vaccinated protected populations, and did not correlate with the degree of BLV proliferation. Vaccination using a rVV vector containing the complete BLV *env* gene protected sheep against challenge with BLV, and protection was shown to correlate with the activity of Env-specific $CD4^+$ T cells (Gatei *et al.*, 1993). However this response was absent four months post challenge.

1.4 Technologies Detecting Antigen-Specific $CD4^+$ T Cells

1.4.1 Lymphocyte Proliferation Assay

In this assay, specialised plates are coated overnight with mitogen or specific antigen in the form of peptides or proteins. Cells isolated from peripheral blood or lymphoid tissues are seeded into the coated wells and incubated for three days at 37°C. During the final five hours of incubation, tritiated thymidine (^{3}H-thymidine) is added, which becomes incorporated into the DNA of proliferating cells. The DNA is harvested onto filter plates and radioactivity measured using a liquid scintillation counter. The amount of radioactivity detected is proportional to the number of proliferating cells, which corresponds with the number of antigen-specific lymphocytes.

The LPA is designed to measure the ability of lymphocytes to proliferate clonally *in vitro*, in response to mitogenic or specific antigenic stimulation. Antigen-specific T cell proliferation assays are used to assess the functional capacity of $CD4^+$ T cells to respond to their cognate antigen. The assay has been used in AIDS clinical trials in

order to assess improvements in $CD4^+$ T cell function following antiretroviral therapy; to measure the development of HIV-specific responses following the administration of HIV vaccines (Lederman *et al.*, 1998), and to determine the immunopathogenesis of HIV infection (Rosenberg *et al.*, 1997; Musey *et al.*, 1999).

1.4.2 Cytokine Technologies

The analysis of cytokine production patterns is important in the elucidation of the immunopathogenesis of disease processes, and in the evaluation of novel immunotherapies and the monitoring of disease progress. Cytokine expression can be detected as mRNA using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) techniques (Dean *et al.*, 1998), or as secreted soluble proteins within body fluids using ELISA (Mateu de Antonio *et al.*, 1998). However, the detection of expressed cytokines using sensitive and quantitative technologies, such as ELISpot assays and ICS using flow cytometry, is preferable. These highly sensitive assays detect cytokine expression at the single cell level and facilitate the quantification of antigen-specific cells.

1.4.2.1 Enzyme Linked Immunospot Assay

Specialised filter plates are coated overnight with cytokine-specific antibodies before being seeded with cells isolated from PBMC or lymphoid tissues. The cells are stimulated with antigen, in the form of peptide or whole protein, for up to twenty-four hours. Cytokine expression can be detected using a second specific monoclonal or polyclonal antibody labelled with biotin or directly labelled with enzyme. An additional streptavidin-enzyme conjugate step enhances sensitivity. Colour is developed using a substrate-chromogen mixture, which reveals spots of expressed cytokine on the plate. Spots can be counted manually or by using sophisticated image analysis equipment.

The ELISpot is a highly sensitive and quantitative assay designed to detect antigen-specificity at a single cell level; this technique is very useful for evaluating the quantity and quality of T cell responses induced by immunotherapies (Czerkinsky *et al.*, 1983; Favre *et al.*, 1997; Schmittel *et al.*, 1997; Yang *et al.*, 2000). It is also

possible to dual-stain; thus, the simultaneous expression of two cytokines can be detected (Okamoto *et al.*, 1998).

1.4.2.2 Intracellular Cytokine Staining

Target cells are cultured with their specific antigen in the form of peptides, proteins or MHC-tetramers, over a short time course of four to six hours. Protein transport inhibitors are present throughout the period of cell stimulation. Cells are stained with fluorochrome-labelled antibodies to cell surface molecules in order to identify specific cell populations, then fixed and permeabilised before staining with a cytokine-specific antibody labelled with a second fluorochrome. Two-colour flow cytometry is used to simultaneously detect cell surface molecules and intracellular cytokines. Antigen-specific cells can thus be identified and quantified.

The detection of intracellular cytokines has been facilitated through the identification of reagents such as brefeldin A (BFA) and monensin, which inhibit protein secretion and allow expressed cytokines to accumulate within the cell (Fujiwara *et al.*, 1988; Nylander and Kalies, 1999). Intracellular cytokines are detected using fluorochrome-labelled cytokine-specific antibodies, and visualised using flow cytometry (Jung *et al.*, 1993). This highly sensitive and antigen-specific technique permits the enumeration of individual antigen-specific cells and the characterisation of antigen-specific cell phenotype using antibodies to cell surface molecules.

1.4.2.3 Tetramer Staining

Knowledge of MHC and immunodominant epitopes is required to quantify antigen-specific cellular immune responses using tetramer technology, rendering these assays technically complex. T cells recognise antigen in the form of an MHC-peptide complex. An MHC-tetramer complex comprises four complexes of antigenic peptide molecules, β2 microglobulin and a biotinylated MHC class I molecule, bound to fluorochrome-labelled streptavidin. This technology is currently being used to investigate virus-specific T cell responses in man and mouse to HIV, SIV, LCMV and Epstein-Barr virus (EBV), as well as to investigate immune responses in parasitic infections, cancer and autoimmune conditions (Gallimore *et al.*, 1998;

Kuroda *et al.*, 1998; Wilson *et al.*, 1998). The MHC tetramer staining technique is highly sensitive, antigen-specific, and can detect rare cells (McMichael and O'Callaghan, 1998). Flow cytometry is used to detect T cell receptors (TCRs) that recognise and bind their cognate antigen. This technique can be used to identify and sort specific T cell populations, and to detect antigen-specific responses at single cell level. Alternatively, unconjugated bound tetramers can be detected using ELISpot assays (Kaul *et al.*, 2000).

1.4.2.4 Summary

These assays vary in their sensitivity and technical requirements. The quantification of antigen-specific cells using tetramer technology is technically complex; the MHC must be defined and immunodominant epitopes characterised. Furthermore, the identification of non-functional populations of antigen-specific T cells in certain chronic disease states in mice and humans (Zajac *et al.*, 1998a) imply that functional studies are also important. Lymphocyte proliferation assays are technically straightforward and reflect the functional ability of target cells. Peptides and whole proteins can be used in antigen-specific assays, and knowledge of the MHC is not required. Furthermore, specific anti-cytokine antibodies are not needed. However, these assays require a long period of *in vitro* restimulation of cells, are often subject to high background readings and are not quantitative at the single cell level. Intracellular cytokine staining using flow cytometry, and ELISpot assays, are rapid, highly sensitive antigen-specific assays that facilitate the enumeration of IFN-γ expressing cells at the single cell level, without requiring MHC definition and knowledge of immunodominant epitopes.

1.5 Aims of Thesis

Feline leukaemia virus is a naturally occurring γ-retrovirus of domestic cats worldwide. Following exposure, most FeLV-naïve cats successfully eliminate the virus and recover; however, a number of exposed cats become persistently viraemic and die within two to three years from an FeLV-associated disease. Furthermore, an unknown proportion of recovered cats retain a latent proviral infection within their bone marrow cells. The immune mechanisms that determine the fate of the exposed

cats are unknown. Early studies showed that the elicitation of VNA correlated with recovery. However, more recently, the importance of virus-specific CTL in recovery from FeLV and in the protection of vaccinates has been described. Studies of murine and human retroviral infections illustrate that CTL are important in the elimination of virus and the elicitation of protective immune responses; inadequate $CD8^+$ T cell function culminates in viral persistence. The importance of humoral immunity in the control of retroviral disease is controversial and may vary among individual viruses.

The aim of this thesis was to identify and compare FeLV-specific cell-mediated immune responses, particularly virus-specific $CD4^+$ T cells, in recovered, latently infected, and persistently infected cats following experimental exposure to FeLV. $CD4^+$ T cells have a critical role in regulating the humoral and cell-mediated arms of the immune response through the activation of DC and the elaboration of cytokines, particularly IFN-γ. Failure of the $CD4^+$ T cell response in HIV and FV infection has been shown to result in inadequate priming, maturation and maintenance of the CTL response. Sophisticated technologies identifying virus-specific $CD4^+$ T cells were combined with highly sensitive assays to quantify viral burdens, in an attempt to correlate virus-specific immune responses with viral burdens *in vivo*.

To achieve these goals, a group of sixteen-week old SPF cats were exposed oronasally to a dose of infectious FeLV expected to result in fifty *per cent* recovery, and fifty *per cent* persistent infection. In addition, an unknown proportion of recovered cats were expected to retain a latent proviral infection. Peripheral blood was sampled at frequent intervals following exposure; bone marrow and lymphoid tissues were harvested post mortem. Virus-specific immune responses were evaluated in the peripheral blood and in the lymphoid tissues, using two techniques, the LPA, and ICS analysed using flow cytometry.

It was first necessary to develop reagents to measure fIFN-γ. Anti-fIFN-γ antibodies were generated as tools to detect and quantify virus-specific cells using ICS analysed using flow cytometry. Intracellular fIFN-γ expressed by virus-specific mononuclear cells in the peripheral blood and lymphoid tissues was detected using these novel anti-fIFN-γ antibodies; fIFN-γ-expressing cells were simultaneously characterised

using cell surface staining. Proliferative responses were measured using a liquid scintillation spectrophotometer to calculate the uptake of ^{3}H-thymidine by cellular DNA. Cells in the peripheral blood, which significantly proliferate in response to specific virus-specific stimulation, were phenotyped using flow cytometry.

Second, the specificity of the immune response was determined by assessing the ability of mononuclear cells in the peripheral blood, and in the lymphoid tissues, to proliferate, and to express fIFN-γ, in response to specific stimulation with either recombinant p27or p45 proteins, or inactivated FeLV particles.

Finally, immunohistochemistry and immunofluorescence were used to identify FeLV CA protein p27 in the bone marrow and lymphoid tissues, harvested post mortem. The bone marrow and lymphoid tissues are important sites of viral replication and expression; consequently, these are important sources of FeLV-associated disease and act as foci for the antiviral immune response. The impact of active virus replication and expression in these tissues was assessed through the haematological and cytological analysis of peripheral blood and bone marrow samples, respectively, post mortem.

The contribution of humoral immunity in the immune response to FeLV was assessed by monitoring the development of VNA throughout the study, using a focus reduction assay.

CHAPTER 2

GENERATION AND CHARACTERISATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES TO FELINE INTERFERON-γ (fIFN-γ)

2.1 Introduction

The nature, specificity and efficacy of the immune response can be characterised using assays to detect IFN-γ expression and secretion. Thus, the ELISA can detect soluble IFN-γ in plasma or culture supernatants; and ELISpots, or ICS analysed using flow cytometry, can be used to enumerate cells that express and/or secrete IFN-γ in response to mitogenic or antigenic stimuli. However, these techniques require specific and sensitive anti-IFN-γ antibodies. To date, antibodies to bovine, canine, human and porcine IFN-γ, amongst others, have been generated and characterised (Novick *et al.*, 1983; Wood *et al.*, 1990; Fuller *et al.*, 1994; Mateu de Antonio *et al.*, 1998). However, at the commencement of these studies, reagents to detect fIFN-γ were not available.

It has recently been demonstrated that early FeLV Gag/Pro-specific $CD8^+$ CTL effector responses are associated with recovery from experimental FeLV infection (Flynn *et al.*, 2002b). Furthermore, high levels of FeLV Gag-specific CTL were detected in the blood and lymphoid tissues from cats protected from FeLV challenge following inoculation with an FeLV DNA vaccine containing *gag*, *pol* and *env* genes (Flynn *et al.*, 2000a; Hanlon *et al.*, 2001). Therefore, the capacity of virus-specific $CD4^+$ T cells to activate $CD8^+$ T cells through the elaboration of cytokines such as IFN-γ, could be a key factor in the control of viraemia (Rosenberg *et al.*, 1997).

This chapter describes the generation and characterisation of monoclonal and polyclonal antibodies to fIFN-γ as tools to investigate the evolution of the immune response in cats experimentally exposed to FeLV. The sensitivity of the generated antibodies was determined using an anti-fIFN-γ indirect ELISA and Western blots. In addition, these novel reagents were assessed for their ability to detect the upregulation of fIFN-γ by specific feline T cell populations *ex vivo*, following co-culture with mitogen for four hours in the presence of protein secretion inhibitor BFA (Sigma-Aldrich Company Ltd, Dorset, U.K.). Feline IFN-γ expression was evaluated using flow cytometry to simultaneously detect phycoerythrin (PE)-labelled surface markers and fluorescein isothiocyanate (FITC)-labelled intracellular fIFN-γ.

2.2. Materials and Methods

2.2.1 Production of Recombinant fIFN-γ

2.2.1.1 Expression of fIFN-γ Gene

The *fIFN-γ* gene encodes a 167 amino-acid polypeptide with a predicted molecular weight of 19.6 kDa. The gene sequence shares seventy-eight *per cent* and sixty-three *per cent* homology with the human and murine genes respectively (Argyle *et al.*, 1995). Human and murine gene sequences share only sixty *per cent* homology (Farrar and Schreiber, 1993). In humans and mice, the biologically active IFN-γ molecule has been identified as a noncovalent homodimer, 34 kDa in size, formed from two 17 kDa polypeptides (Farrar and Schreiber, 1993; Boehm *et al.*, 1997).

The gene encoding fIFN-γ was cloned and sequenced in 1995 (Argyle *et al.*, 1995). The gene sequence was inserted into a glutathione S-transferase (GST) gene fusion vector (pGEX-4T-1, Amersham Biosciences U.K. Ltd, Buckinghamshire, U.K.) and expressed in bacterial cells (BL21(DE3), Stratagene Europe, Amsterdam, The Netherlands).

2.2.1.2 Preparation of the Bacterial Lysate

Forty ml L-broth medium[1], containing ampicillin (0.2 mg/ml, Sigma-Aldrich, U.K.), was inoculated with bacterial expression vectors from glycerol stocks. The bacteria were grown overnight at 37°C in an orbital incubator (Sanyo Gallenkamp PLC, Loughborough, U.K.) rotating at approximately 200rpm. The cultured cells were subsequently diluted by a factor of ten in fresh culture medium, and incubated further as above until the optical density reading at 600 nm ($O.D._{600}$) reached 0.6-0.8 ('Spectrophotometer DU® 640', Beckman-Coulter Inc., California, U.S.). At this point, the bacterial vectors were induced to express the recombinant protein by adding 0.4 mM isopropylthio-β-D-galactoside (IPTG, Gibco, Invitrogen Corporation, California, U.S.) to the culture medium for a final four-hour incubation.

[1] L-broth, pH 7.4: 3% (w/v) tryptone; 1.5% (w/v) yeast extract; 1.5% (w/v) NaCl

Subsequently, the bacterial culture was transferred into a JA-10 rotor (Beckman-Coulter, U.S.), and centrifuged in a J2-21 centrifuge (Beckman-Coulter, U.S.) for fifteen minutes at 6000rpm. The supernatants were decanted and the cell pellets were either frozen at -20°C, or resuspended in 20 ml phosphate buffered saline[2] (PBS), containing the protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (0.2 mg/ml, AEBSF, Sigma-Aldrich, U.K.) and aprotinin (2 μg/ml, Sigma-Aldrich, U.K.). The cells were then lysed by sonicating on ice for three, thirty-second cycles separated by intervals of one minute ('Sonicator™ XL', Heat Systems-Ultrasonics Inc., New York, U.S.), followed by shaking on ice in a solution of 1% Triton X-100 (BDH Laboratory Supplies, Poole, U.K.) for thirty minutes. To remove the insoluble debris, the bacterial lysate was transferred to a JA-20 rotor (Beckman-Coulter, U.S.), and centrifuged in a J2-21 centrifuge at 5000rpm for fifteen minutes; the supernatant containing the recombinant protein was stored at -20°C until further use.

2.2.1.3 Affinity Chromatography

The recombinant fIFN-γ protein was purified from the bacterial lysate using affinity chromatography. Glutathione coupled to Sepharose 4B via a 10-carbon linker arm can be used for the purification of fusion proteins containing the carboxyl terminus of GST. Glutathione sepharose 4B slurry (1.33 ml of 75% solution, Amersham Biosciences, U.K.) was added to an empty polypropylene column (Amersham Biosciences, U.K.). The column was centrifuged at 800rpm for three minutes using a bench-top centrifuge (Beckman-Coulter GS-6 centrifuge, U.S.) to sediment the slurry, which was then washed twice using 5 ml cold PBS. One ml PBS was then added to provide a 50% slurry.

Five ml of bacterial lysate containing the GST fusion protein was added to the slurry. The mixture was agitated at room temperature (RT) for thirty minutes, before centrifugation and the addition of a further 5 ml lysate. This was continued until all the bacterial lysate had been added to the column. The column was then washed five

[2] PBS, pH 7.2: 10x concentrated: 8% (w/v) NaCl; 0.2% (w/v) KCl; 1.15% (w/v) Na_2HPO_4; 0.2% (w/v) KH_2PO_4

times with 5 ml PBS using centrifugation as described above. Subsequently, 25 units of thrombin protease (Amersham Biosciences, U.K.) in PBS was added to the column in order to cleave the recombinant protein from the glutathione sepharose gel. The column was shaken gently overnight at RT; the eluted protein within the supernatant was then decanted following sedimentation of the gel matrix. The protein was subsequently electrophoresed on a 12% polyacrylamide gel (SDS-PAGE), and stained using Coomassie Brilliant Blue R250 to assess the purity of the eluted protein (Sections 2.2.1.4 and 2.2.1.5). The recombinant protein was quantified using the BCA protein assay (Perbio Science U.K. Ltd., Cheshire, U.K.), and then frozen in aliquots at -20°C.

2.2.1.4 Detection of Recombinant Proteins using SDS-PAGE

This method is based on the original description of polyacrylamide gels containing sodium dodecyl sulphate (SDS) in gel electrophoresis (Laemmli, 1970). A 12% polyacrylamide separating gel[3] was prepared and cast in a vertical slab gel system ('Mini-protean II', Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, U.K.). A 5% stacking gel[4] was layered above the separating gel. The recombinant protein was diluted 1:1 in SDS loading dye[5], containing bromophenol blue. Ten µl of each sample was placed in the sample wells prepared in the stacking gel. Prestained standard samples comprising proteins of known molecular weights were used as size markers (Precision protein standards, Bio-Rad, U.K.). The gel was run in a tris-glycine running buffer[6] at 200 volts for approximately thirty minutes, or until the dye front reached the bottom of the gel. The gels were either stained with Coomassie Brilliant Blue R250, or transferred electrophoretically onto nitrocellulose, as described below.

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[3] 12% separating gel: 32.6% (v/v) dH_2O; 12 % (v/v) acrylamide mix; 25.3% (v/v) 1.5 M tris, pH 8.8; 0.1% (w/v) SDS; 0.1% (w/v) ammonium persulphate; 0.026% (v/v) TEMED

[4] 5% stacking gel: 67.5% (v/v) dH_20; 5% acrylamide mix (v/v); 12.5% (v/v) 1 M tris, pH 6.8; 0.1% (w/v) SDS; 0.1% (w/v) ammonium persulphate; 0.1% (v/v) TEMED

[5] SDS-loading dye (2x): 4% (w/v) SDS; 20% (v/v) glycerol; 12% (v/v) 1 M tris, pH 6.8; 28% (v/v) dH_2O; 0.01% (w/v) bromophenol blue

[6] Running buffer: 25 mM (w/v) tris; 250 mM (w/v) glycine; 0.1% (w/v) SDS in dH_2O

2.2.1.5 Coomassie Brilliant Blue Staining of SDS-PAGE Gels

The SDS-PAGE gel was immersed in a Coomassie Brilliant Blue solution[7] for a minimum of four to sixteen hours. The gel was subsequently destained by soaking in a methanol-acetic acid solution[8] on a slowly rotating platform for four to eight hours. During this time, the destaining solution was changed several times. After destaining, the gels were immersed in distilled water and dried using a gel drier ('Easy Breeze Gel Dryer', Hoefer Scientific Instruments, San Francisco, U.S.). The gel image was recorded using a scanner ('ImageScanner', Amershain Biosciences, U.K.) and the software package 'LabScan' (Amersham Biosciences, U.K.).

2.2.2 Generation of Anti-fIFN-γ Antibodies

2.2.2.1 Animal Inoculations

Immunisation of mice and sheep was carried out by Diagnostics Scotland (Edinburgh, U.K.). Three female Balb/c mice were inoculated with 20 μg of recombinant protein fIFN-γ in complete Freund's adjuvant. Each mouse received a total volume of 200 μl, injected in equal volumes at two separate intraperitoneal (IP) sites. Two booster injections, each containing 10 μg recombinant protein in 100 μl incomplete Freund's adjuvant, were given at twenty-eight day intervals. Seven days after the second booster, sera were recovered and the antibody titres measured by ELISA (Section 2.2.2.5). After a further eight days, the mice received a final intravenous (IV) booster inoculation of 10 μg fIFN-γ in 100 μl PBS; their spleens were harvested four days later.

Polyclonal antiserum to fIFN-γ was prepared by the inoculation of a sheep subcutaneously (SC) and intramuscularly (IM) with 200 μg fIFN-γ in complete Freund's adjuvant, in a total volume of 5 ml. Three booster inoculations of 200 μg fIFN-γ in 5 ml incomplete Freund's adjuvant were given SC and IM at four-week

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[7] Coomassie Brilliant Blue solution: 0.05% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol; 40% (v/v) dH_2O; 10% (v/v) glacial acetic acid

[8] Destain solution: 45% (v/v) methanol; 45% (v/v) dH_2O; 10% (v/v) glacial acetic acid

intervals; serum was harvested seven days after each booster injection. Antibody titres were determined by ELISA (Section 2.2.2.5).

2.2.2.2 Generation of Murine Anti-fIFN-γ Monoclonal Antibodies

Harvested spleen cells were fused with mouse myeloma cells according to the method originally described by Kohler and Milstein (1975). The NS0 myeloma cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS; 100 U/ml penicillin; 10 μg/ml streptomycin and 2 mM glutamine (complete RPMI 1640 medium; GibcoBRL, Paisley, U.K.), supplemented with $5x10^{5}$ M 2-mercaptoethanol (ME, Gibco, U.K.). Adherent NSO cells in the log phase of growth were harvested using 0.02% EDTA (Sigma-Aldrich, U.K.) in complete RPMI 1640 medium, and used in the production of the hybridomas.

The murine spleens were gently teased using sterile instruments in a microbiological safety cabinet ('BioMat', Medical Air Technology, Manchester, U.K.), to generate single cell suspensions. Murine splenic lymphocytes (5.3×10^{7}) were collected over Ficoll-Paque (Amersham Biosciences, U.K.), washed and resuspended in complete RPMI 1640 medium, containing ME. The murine lymphocytes were then added to NS0 cells in a 2:1 ratio, and pelleted by centrifugation in a benchtop centrifuge at 1200rpm for five minutes. One ml PEG-1500 (polyethylene glycol-1500, BDH, U.K.), heated to 37°C, was added drop-wise to the cell pellet over thirty seconds. The mixture was then gently agitated for five minutes in a water bath (Grant Instruments Ltd., Cambridge, U.K.), which had been pre-heated to 37°C. Subsequently, 5 ml complete RPMI 1640 medium containing ME, was added drop-wise to the cell pellet over ninety seconds, followed immediately by a further 20 ml. The fused cells were pelleted and resuspended in complete RPMI 1640 containing ME. Each well of 96-well U-bottom culture plates (Becton Dickinson Labware Europe, Le Pont de Claix, France) was seeded with $1x10^{5}$ fused cells in a final volume of 200 μl complete RPMI 1640 medium supplemented with ME and HAT medium (5 mM sodium hypoxanthine, 20 μM aminopterin and 0.8 mM thymidine, Gibco, U.K.). A total of nine plates were set up, placed in polythene bags and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After fourteen

days, supernatants from visible cell colonies were collected and screened for the presence of anti-fIFN-γ antibodies using ELISA, as described in Section 2.2.2.5. Hybridomas secreting the highest levels of detectable mAbs were cloned by limiting dilution in 96-well plates at dilutions of 0.3, 3 and 10 cells/well. After two further subclonings, positive clones were finally transferred into compartmentalised flasks for expansion *in vitro* ('Cl-1000', Integra Biosciences Ltd, Hertfordshire, U.K.).

2.2.2.3 Affinity Purification of Anti-fIFN-γ Antibodies

Monoclonal antibodies were affinity purified using a 1 ml protein G affinity column ('HiTrap' protein G affinity column, Amersham Biosciences, U.K.). Protein G, a cell surface protein of Group G streptococci, is a type III Fc receptor that binds to the Fc region of IgG by a non-immune mechanism.

The column was equilibrated with ten column volumes of binding buffer[9], applied using a peristaltic pump ('P-1', Amersham Biosciences, U.K.). Culture supernatant containing the mAbs was passed through a 0.45 μm filter (Sartorius Ltd., Surrey, U.K.), and pumped through the column at a rate of approximately 1 ml per minute. The column was subsequently washed with ten column volumes of binding buffer; the purified fractions were then eluted using an elution buffer[10]. The pH of the eluted fractions was adjusted immediately to pH 7.0 using 1 M tris-HCl buffer, pH 9.0. The fractions were tested by ELISA to ensure sensitivity (Section 2.2.2.5); positive fractions were then pooled into a glass universal container. To precipitate antibody from the eluted fractions, an equal volume of saturated ammonium sulphate (Sigma-Aldrich, U.K.) was added, and the solution placed at 4°C for one hour. To collect the precipitated mAbs, the solution was placed in a JA-20 rotor and centrifuged at 10,000rpm for thirty minutes. Monoclonal antibodies were then transferred to a storage buffer[11] using a PD-10 column containing sephadex G-25 medium (Amersham Biosciences, U.K.). The PD-10 column was equilibrated using

[9] Binding buffer, pH 7.0: 20 mM (w/v) sodium phosphate

[10] Elution buffer, pH 2.7: 0.1 M (w/v) glycine-HCl

[11] Storage buffer, pH 7.0: 0.05 M (w/v) Na_2HPO_4; 0.1% (w/v) NaN_3

25 ml equilibration buffer[12]. Next, 2.5 ml of purified antibody in PBS was loaded onto the column and allowed to run through. 3.5 ml of storage buffer was then added to the column. This final run-through, which contained the purified mAb in storage buffer, was collected, quantified using a spectrophotometer, and tested for sensitivity using ELISA (Section 2.2.2.5) and Western blots (Section 2.2.2.6).

Polyclonal antiserum was affinity purified using a 1 ml N-hydroxysuccinimide (NHS)-activated affinity column ('HiTrap' NHS-activated affinity column, Amersham Biosciences, U.K.), coated with recombinant fIFN-γ. To coat the column, the recombinant protein fIFN-γ was transferred into coupling buffer[13] using a PD-10 column as described above, and used at a concentration of approximately 1 mg/ml. The coupling buffer containing the protein was then injected onto the column using a syringe, and recirculated back and forth across the column for approximately thirty minutes per mg of protein. Affinity columns were coated with up to 4 mg of fIFN-γ. The column was then washed a total of six times with alternate solutions of 3 x 2 ml Buffer A[14] and 3 x 2 ml Buffer B[15] to eliminate non-specifically bound ligands, and to inactivate excess NHS active groups. The column was then equilibrated with 5 ml start buffer[16] using a peristaltic pump, after which the polyclonal antiserum was applied to the column. The column was washed with 10-15 ml start buffer and the purified fractions eluted using an elution buffer[17]. The pH of the eluted fractions was adjusted to pH 7.0 using 1N NaOH. Positive fractions were detected using an ELISA (Section 2.2.2.5), before transfer into a storage buffer using a PD-10 column, as described above.

[12] PD-10 equilibration buffer, pH 7.0: 0.1 M (w/v) NaH_2PO_4; 0.15 M (w/v) NaCl

[13] Coupling buffer, pH 8.3: 0.2 M (w/v) $NaHCO_3$; 0.5 M (w/v) NaCl

[14] Buffer A, pH 8.3: 0.5 M (w/v) ethanolamine; 0.5 M (w/v) NaCl

[15] Buffer B, pH 4.0: 0.1 M (w/v) acetate, 0.5 M (w/v) NaCl

[16] Start buffer, pH 9.4: 0.1% (v/v) tween-20 in TBS

[17] Elution buffer (pAb), pH 2.5: 0.1 M (w/v) glycine; 0.15 M (w/v) NaCl

2.2.2.4 Isotyping of Murine Anti- fIFN-γ Antibodies

Antibody isotyping was performed using a commercially available kit ('Sigma Immunotype Kit' Sigma-Aldrich, U.K.). All reagents used to isotype the mAb were provided in the kit. The precoated nitrocellulose strip captured Ig within the hybridoma supernatant following a thirty-minute incubation. The strip was washed in PBS, containing 0.05% tween-20 (BDH, U.K.), and 1% bovine serum albumin (BSA, Sigma-Aldrich, U.K.) for five minutes, before a thirty-minute incubation with biotinylated anti-mouse Ig pAb. After a single wash in PBS-T-BSA, 'ExtrAvidin-peroxidase' was added for a fifteen-minute incubation. Colour was developed after the final washing step using the chromogen 3-amino-9-ethyl-carbazole (AEC), and substrate hydrogen peroxide (H_2O_2).

2.2.2.5 Detection of Anti-fIFN-γ Antibodies using ELISA

The wells of 96-well high-binding plates (Greiner Laboratechnik Ltd., Gloucestershire, U.K.) were coated overnight at RT with 100 ng recombinant fIFN-γ in 100 µl coupling buffer[18] per well. The plates were washed three times with tris buffered saline[19] (TBS) containing 0.05% tween-20 (TBS-T); any unreacted sites were subsequently blocked by incubation for thirty minutes at RT with 2% dried skimmed milk powder ('Marvel', Premier Brands U.K. Ltd, Merseyside, U.K.) in TBS-T. After three washes in TBS-T, 100 µl of culture supernatant was added to each well and incubated for two hours at RT. After a further six washes in TBS-T, the wells were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse Ig pAb (Bio-Rad Laboratories, U.K.) diluted 1:1000 in TBS-T, for one hour at RT. Bound antibodies were visualised using p-nitrophenyl phosphate disodium tablets ('Sigma 104^{R}', Sigma-Aldrich, U.K.), dissolved in AP buffer[20]. Absorbance was read at 405 nm after incubating for one hour in the dark ('Labsystems Multiskan Ascent', Process Analysis and Automation Ltd, Hampshire, U.K.). The same protocol was used when titrating the mAb, except that doubling dilutions of purified

[18] ELISA coupling buffer: 100 mM (w/v) $NaHCO_3$; 1 mM (w/v) EGTA

[19] TBS, pH 7.8: 10x concentrated: 6.06% (w/v) tris and 9% (w/v) NaCl

[20] AP buffer, pH 9.5: 100 mM (w/v) NaCl; 100 mM (w/v) diethanolamine; 5 mM (w/v) $MgCl_2$

mAb were made in 20% normal goat serum (NGS, Diagnostics Scotland, U.K.). Normal mouse serum (NMS), diluted in the same way as the primary mAb, was used to determine the end point of the ELISA.

To detect sheep pAbs, unreacted sites on the ELISA plates were blocked for thirty minutes with 200 µl 20% normal rabbit serum (NRS, Diagnostics Scotland, U.K.) in TBS-T. Polyclonal antibodies were diluted in 20% NRS and an AP-conjugated rabbit anti-sheep Ig pAb (Southern Biotechnology Associates, Inc., Alabama, U.S.), diluted 1:2000 in 20% NRS in TBS-T, was used to detect bound sheep antibodies. Normal goat serum, diluted in the same way as the primary pAb, was used to determine the end point of the ELISA.

2.2.2.6 Detection of Anti-fIFN-γ Antibodies using Western Blots

The transfer of proteins from a polyacrylamide gel onto nitrocellulose was originally described by Towbin *et al.* (1979). The prepared gel (Section 2.2.1.4) was blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, U.K.) using a semi-dry technique ('Trans-Blot Semi-Dry Transfer Cell', Bio-Rad Laboratories, U.K.). The gel was placed over the nitrocellulose membrane and between four sheets of blotting paper (Sigma-Aldrich, U.K.), which had been pre-soaked in transfer buffer[21]. Fifteen volts of electricity was applied for forty-five minutes. The membrane was then transferred to 4% Marvel in TBS-T overnight to block any unreacted sites, or dried and stored at 4°C.

The nitrocellulose membrane was cut longitudinally into strips and inserted into the wells of a blotting trough. All washes were conducted in duplicate at five-minute intervals using TBS-T, and all incubations proceeded at RT on a slowly rotating platform. Monoclonal and polyclonal antibodies were diluted in 20% NGS and 20% NRS in TBS-T, respectively; subsequent reagents were diluted similarly. Two hundred µl of diluted antibody solution was added to each well and incubated for two hours. Alternatively, the blots were probed with NMS (mAb) or NGS (pAb), which acted as negative controls. The blots were then washed, and incubated with AP-

[21] Transfer buffer: 25 mM (w/v) tris; 0.2 M (w/v) glycine; 20% (v/v) methanol

conjugated goat anti-mouse Ig pAb, or AP-conjugated rabbit anti-sheep Ig pAb, diluted 1:1000 and 1:2000, respectively, for one hour at RT. Following three further washes, bound antibodies were visualised using Fast 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium tablets (BCIP/NBT AP substrate, Sigma-Aldrich, U.K.), dissolved in 10 ml distilled water. The blots were rinsed with distilled water and dried overnight. The images were subsequently recorded as previously described (Section 2.2.1.5).

2.2.2.7 FITC Conjugation of Anti-fIFN-γ Antibodies

Prior to coupling, mouse and sheep antibodies were first transferred into 0.5 M carbonate buffer[22] by overnight dialysis, using either conventional dialysis tubing (Sigma-Aldrich, U.K.), or dialysis cassettes (Perbio Science, U.K.). A FITC (Sigma-Aldrich, U.K.)-dimethylsulphoxide (DMSO, Sigma-Aldrich, U.K.) solution was prepared (1 mg/ml), added to antibody (45 μg/mg) and the solution stirred for one hour at RT in the dark. Unconjugated FITC was removed using a PD-10 column equilibrated using PBS (Section 2.2.2.3); FITC-conjugated antibody was collected in PBS and stored at 4°C in the dark until used in the assays.

2.2.2.8 Intracellular Cytokine Staining and Analysis using Flow Cytometry

Each well of a 24-well tissue culture plate (Costar U.K. Ltd., Buckinghamshire, U.K.) was coated with 500-1000 μl of complete RPMI 1640 medium containing Con A (Sigma-Aldrich, U.K.); Con A is a lectin, which exhibits non-specific mitogenic activity with lymphocytes (Ruscetti and Chervenick, 1975). The plates were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. This was done to ensure that the medium was at the correct pH and temperature before the addition of cells. The amount of mitogen added was calculated so a concentration of 5 μg Con A /10^6 cells would be obtained following the addition of cells. Wells that did not contain mitogen acted as negative controls.

Expression of intracellular fIFN-γ was evaluated in mononuclear cells prepared from

[22] Carbonate buffer, pH 9.5: 0.16 M (w/v) Na_2CO_3, 0.33 M (w/v) $NaHCO_3$

the peripheral blood from SPF cats. Cryopreserved PBMC and lymphoid tissue samples were also available from a previous FeLV immunopathogenesis study (Flynn *et al.*, 2002b). All cats described were housed in groups in custom-built accommodation at the University of Glasgow. The cats were fed proprietary cat food twice daily, and had access to water *ad libitum* in accordance with Home Office guidelines. Whole blood samples from SPF cats were collected in a 1:1 ratio with Alsevers' solution (Diagnostics Scotland, U.K.). The blood-Alsevers' solution was gently layered over an equivalent volume of Ficoll-Paque, and centrifuged at 1500rpm for fifteen minutes. Peripheral blood mononuclear cells were removed from the interface and washed in complete RPMI 1640 medium. Once thawed, cryopreserved cells were purified on a Ficoll-Paque gradient and washed before use in this assay.

Cells were resuspended in complete RPMI 1640 medium (2 x 10^6 cells/ml) and added to the pre-incubated plates to give a final concentration of 1 x 10^6 cells/ml in a total volume of 1-2 ml/well. The plated cells were subsequently incubated for two or four hours at 37°C in a humidified atmosphere containing 5% CO_2. Brefeldin A (10 μg/10^6 cells) was present in all wells throughout the period of stimulation. Brefeldin A is a fungal metabolite that specifically and reversibly blocks protein transport from the ER to the Golgi apparatus in many cell types and species (Fujiwara *et al.*, 1988). In this way, BFA interferes with the transport of IFN-γ from the ER, allowing any expressed protein to accumulate within the cell to levels sufficient for detection (Nylander and Kalies, 1999).

Subsequent to the *in vitro* period of stimulation, the cells were aspirated, placed into polypropylene tubes (0.5-1 x 10^6 cells/tube, Falcon 2054, Becton Dickinson, France) and pelleted by centrifugation for five minutes at 1000rpm in a bench-top centrifuge. Following a five-minute wash using PBS, the cell pellets were stained with a PE-labelled mAb to the cell surface molecule feline CD4 (2.5 μg/10^6cells, Southern Biotechnology, U.S.), at RT for thirty minutes in the dark. After two further washes in PBS, the cells were fixed with 2% formaldehyde (Sigma-Aldrich, U.K.) in PBS for twenty minutes at RT. After washing in PBS, the cells were permeabilised using

1% saponin (Sigma-Aldrich, U.K.) in fluorescence buffer[23]. The cells were subsequently incubated for thirty minutes at RT in the dark with the anti-flFN-γ-FITC mAb (1-2 μg/10^6 cells) or an isotype-matched control mAb against T-2 mycotoxin (2 μg/10^6 cells, Southern Biotechnology, U.S.). Alternatively, the cells were stained with the sheep anti-flFN-γ-FITC pAb (2 μg/10^6 cells), while control cells remained unstained. The cells were washed twice in permeabilisation buffer, and once in fluorescence buffer, before storage in fluorescence buffer at 4°C. Cells were then analysed on a flow cytometer ('EPICS XL-MCL™', Beckman-Coulter, U.S.) using 'Expo 32 ADC' software (Beckman-Coulter, U.S.). For each sample, 10,000 events were recorded. The small lymphocyte and the lymphoblast populations were gated based on cell size and granularity, and the percentage of flFN-γ-expressing $CD4^+$ T cells within the lymphoblast population was enumerated. The gates for flFN-γ expression were determined by the reactivity of the FITC-conjugated isotype control mAb.

2.2.2.9 Immunohistochemical Detection of flFN-γ Expressing Cells

Peripheral lymph node tissues from FeLV-recovered cats were harvested post mortem (Flynn *et al.*, 2002b), embedded in tissue freezing medium ('O.C.T.', Leica Instruments, Nussloch, Germany), then immediately snap frozen in liquid nitrogen and stored at -70°C. Cryostat sections of PLN tissues were prepared by the Histopathology Laboratory, Department of Veterinary Pathology, University of Glasgow. Air-dried sections were fixed in ice-cold acetone for ten minutes before being wrapped in 'Parafilm' (BDH, U.K.) and aluminium foil, and stored at -70°C.

Frozen slides were brought to RT while still wrapped in 'Parafilm'. All incubations were carried out at RT in a humidified chamber and were followed by two, five-minute washes in TBS to remove unbound reagents. Sections were encircled using a hydrophobic marker pen to minimise reagent wastage ('Pap pen', Sigma-Aldrich, U.K.), and gently agitated in TBS for five minutes. The sections were initially incubated with 0.3% H_2O_2 (BDH, U.K.), and 0.1% NaN_3 (BDH, U.K.), in TBS for twenty minutes, to block endogenous peroxidase activity. To minimise non-specific

[23] Fluorescence buffer: 1 mM (w/v) NaN_3; 0.5% (w/v) BSA in PBS

binding, sections were incubated for twenty minutes with 10% NRS. Excess serum was shaken from the sections, which were subsequently incubated with the sheep anti-fIFN-γ pAb, diluted 1:5 in 1% NRS in TBS, for one hour. The secondary reagent, biotinylated rabbit anti-sheep Ig pAb (Southern Biotechnology, U.S.), was diluted 1:1000 in TBS containing 1% NRS, and added to the sections for thirty minutes. A final thirty-minute incubation with streptavidin-peroxidase (Diagnostics Scotland, U.K.), diluted 1:500 in TBS, preceded the development of colour using a diaminobenzidine (DAB) kit (Vector Laboratories, Ltd., Peterborough, U.K.). The slides were washed in running tap water, counterstained using Gills' haematoxylin (BDH, U.K.); then dehydrated in graded alcohols, cleared ('Histoclear', National Diagnostics, Georgia, U.S.), and mounted in DPX (BDH, U.K.). Negative control slides were prepared; in these slides, the primary mAb was omitted and replaced with 10% NRS.

2.3. Results

2.3.1 Production of Recombinant fIFN-γ

Approximately 1 mg recombinant fIFN-γ was purified per 400 ml bacterial culture. The protein was electrophoresed on a 12% SDS-PAGE to assess the purity of the recombinant protein. Two distinctive bands, 15-25 kDa in size, were evident on the Coomassie Blue-stained gel, illustrating two fIFN-γ polypeptides. A considerably fainter pair of bands, approximately 25-37 kDa in size, may represent the homodimeric proteins (Fig. 2.1, lane B).

2.3.2 Generation of Anti-fIFN-γ Antibodies

2.3.2.1 Generation of Murine Anti-fIFN-γ Antibodies

Two hundred and sixty-five hybridomas were visible microscopically at twenty-one days post fusion, out of five hundred and twenty wells seeded. Subsequently, ELISA screening revealed that thirty-one were secreting anti-fIFN-γ mAbs. The five hybridoma cells secreting the highest levels of anti-fIFN-γ antibodies were selected

for cloning and subcloning. Two hybridoma clones were then selected for further expansion and affinity purification. The selected hybrids, 5B5.C5.D9 (D9) and 2C7.B7.B3 (B3), originated from different primary clones and proliferated well in tissue culture. Both clones were shown to secrete IgG1 antibodies using the commercial isotyping kit. The experiments described in this thesis are based on the D9 mAb.

2.3.2.2 Titration of Anti-fIFN-γ Antibodies using ELISA

The sensitivity of the affinity purified D9 mAb and sheep anti-fIFN-γ pAb, and their respective FITC conjugates, in the detection of recombinant fIFN-γ, were determined using fIFN-γ specific ELISA assays. The antibodies were titrated against recombinant fIFN-γ in an indirect ELISA. The affinity purified sheep anti-fIFN-γ pAb detected 100 ng fIFN-γ at a titre of 1:1638400 (Fig. 2.2.A), while its conjugate recorded a titre of 1:819200. The D9 mAb demonstrated lower titres in ELISA, 1:5120 (Fig. 2.2.B); these values were largely unaffected by conjugation with FITC.

2.3.2.3 Detection of Anti-fIFN-γ Antibodies using Western Blots

Western blots provide an additional means of assessing antibody sensitivity. The recombinant protein fIFN-γ characteristically displays polypeptide bands, 15-25 kDa in size, in Coomassie Brilliant Blue-stained SDS-PAGE gels (Fig. 2.1, lane B). These distinctive double bands were again in evidence when nitrocellulose-blotted fIFN-γ was probed with the D9 mAb and sheep anti-fIFN-γ pAb, generated in this study (Fig. 2.1, lanes C and E). The sheep anti-fIFN-γ pAb detected 14 μg fIFN-γ diluted to a factor of 1:100,000 (Fig. 2.1, lane E); the D9 mAb detected 14 μg fIFN-γ diluted 1:1000 (Fig. 2.1, lane C). No specific staining was visible on the negative control blots (Fig. 2.1, lanes D and F).

2.3.2.4 Detection of fIFN-γ using ICS and Flow Cytometry

Both the D9 mAb and the sheep anti-fIFN-γ pAb were assessed for their ability to detect the production of fIFN-γ by $CD4^+$ T cell populations using ICS, analysed by

flow cytometry.

Mononuclear cells prepared from the peripheral blood from an SPF cat and the lymphoid tissues from an FeLV recovered cat were co-cultured with the mitogen Con A for four hours in the presence of BFA. Production of flFN-γ was compared in unstimulated and stimulated cells using flow cytometry. Both the D9-FITC mAb and sheep anti-flFN-γ-FITC pAb detected a surge in flFN-γ production by Con A-stimulated lymphoblasts within the PLN tissues from an FeLV recovered cat and the peripheral blood from an SPF cat respectively, compared to unstimulated cells (Fig. 2.3).

To determine an optimal duration of mitogenic stimulation, the sheep anti-flFN-γ-FITC pAb was used to detect flFN-γ expression in $CD4^+$ lymphoblast cells from an SPF cat, following either a two or four-hour *in vitro* stimulation with Con A in the presence of BFA. Mitogenic stimulation for four hours yielded maximal flFN-γ production (1.8%), compared to two hours (0.8%) (Fig. 2.4).

The ability of the D9-FITC mAb to detect flFN-γ expression by $CD4^+$ T cells? was assessed ICS, analysed using flow cytometry. Mononuclear cells in the peripheral blood from an SPF cat were stimulated for four hours with Con A in the presence of BFA, dual-stained with anti-fCD4-PE mAb and D9-FITC mAb and analysed using flow cytometry. The D9-FITC mAb detected flFN-γ at higher levels in Con A-stimulated $CD4^+$ T cells (4.7%), compared to unstimulated cells (0.7%); no double staining was detected using a FITC-labelled isotype-matched control mAb (Fig. 2.5). The D9-FITC mAb was also used to detect flFN-γ production in the PLN tissues from an FeLV recovered cat following mitogenic stimulation. Increased flFN-γ production was detected in $CD4^+$ T cells stimulated for four hours (5.8%), compared to unstimulated cells (2.1%) (Fig. 2.6).

In each stimulation assay, feline mononuclear cells were cultured with the mitogen Con A in the presence of BFA. Brefeldin A was not observed to interfere with CD4 expression; in a separate experiment, CD4 expression was not affected by the presence of BFA during a four-hour stimulation with Con A (Fig. 2.7).

2.3.2.5 Immunohistochemical Detection of fIFN-γ Expressing Cells

The sheep anti-fIFN-γ pAb was used to detect fIFN-γ expression in the PLN tissues from an FeLV recovered cat. Cryostat sections were prepared from PLN tissues harvested post mortem, and probed for fIFN-γ in an immunoperoxidase technique. The results are presented in Fig. 2.8. Negative control slides did not demonstrate any specific staining.

Positive fIFN-γ expressing cells were distinctly concentrated in clusters at the cortico-medullary junction (Fig. 2.8.A and B). Only a few scattered individual cells were found within the lymph node cortex, and virtually no fIFN-γ expressing cells were associated with the lymph node follicles. Conversely, many positive cells were found within the medullary zone. Here, fIFN-γ expressing cells were widely distributed; some positive cells were also evident within the efferent lymphatic vessels (Fig. 2.8.C). It was not possible to characterise the fIFN-γ expressing cells based on morphological appearance alone. To achieve this, it would be necessary to conduct double immunoenzyme staining techniques.

Fig. 2.1 SDS-PAGE and Western blots demonstrating fIFN-γ

Recombinant fIFN-γ protein (4 μg) was stained with Coomassie Brilliant Blue on an SDS-PAGE gel (lane B) and probed with either D9 mAb (1.3 mg/ml) diluted 1:1000 (lane C), or sheep anti-fIFN-γ pAb (0.84 mg/ml) diluted 1:100,000 (lane E), in Western blots. Negative control blots were probed with normal mouse serum, 1:1000 (lane D), or normal goat serum, 1:100,000 (lane F). Prestained markers were used to determine protein size (lane A).

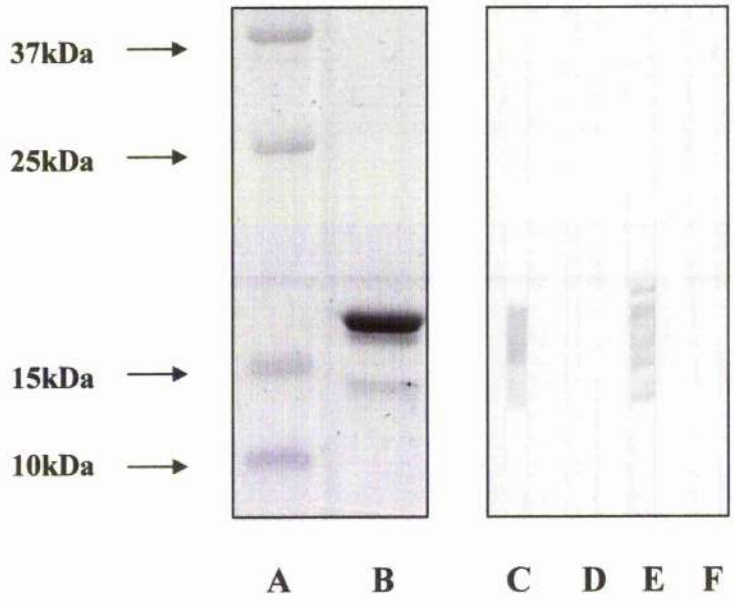
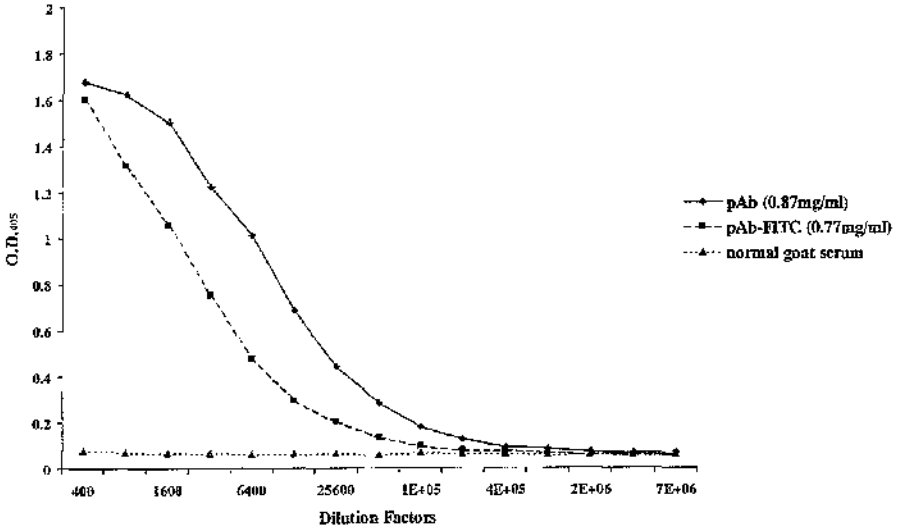

37kDa
25kDa
15kDa
10kDa
A
B
C
D
E
F

Fig. 2.2 Titration of anti-fIFN-γ antibodies using ELISA

Titration of sheep anti-fIFN-γ pAb (A), D9 mAb (B), and their respective FITC conjugates, using an indirect fIFN-γ ELISA. The antibodies were diluted in either 20% NRS (A), or 20% NGS (B), in TBS-T. Wells containing either NGS (A), or NMS (B), diluted as the primary antibodies, were used to determine the end-points of the ELISA.

A


O.D.405
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
400
1600
6400
25600
1E+05
4E+05
2E+06
7E+06
Dilution Factors
pAb (0.87mg/ml)
pAb-FITC (0.77mg/ml)
normal goat serum

B

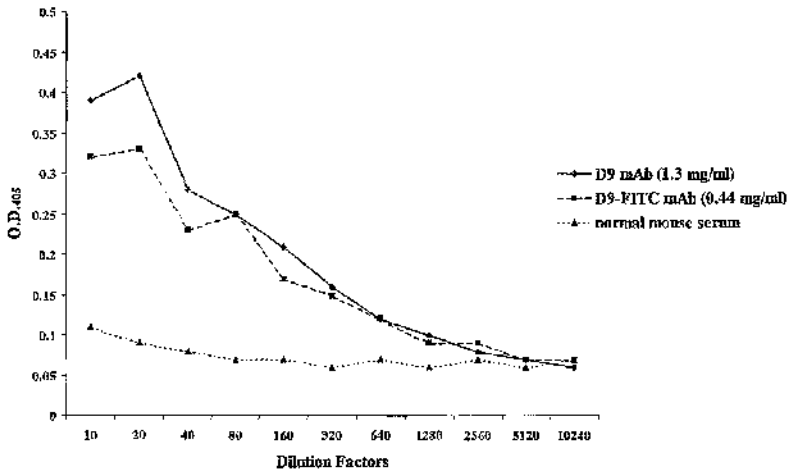

O.D.405
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
10
20
40
80
160
320
640
1280
2560
5120
10240
Dilution Factors
D9 mAb (1.3 mg/ml)
D9-FITC mAb (0.44 mg/ml)
normal mouse serum

Fig. 2.3 Overlay plots demonstrating fIFN-γ expression in response to mitogenic stimulation

Single parameter overlay plots showing intracellular fIFN-γ expression in the lymphoblast population in the peripheral blood from an SPF cat (A) and in the PLN tissues from an FeLV recovered cat (B). Feline IFN-γ expression was detected using sheep anti-fIFN-γ-FITC pAb (A), and D9-FITC mAb (B), following a 4 h stimulation with Con A *in vitro,* in the presence of BFA. Unstimulated cells were used as controls. In total, 10,000 events were counted.

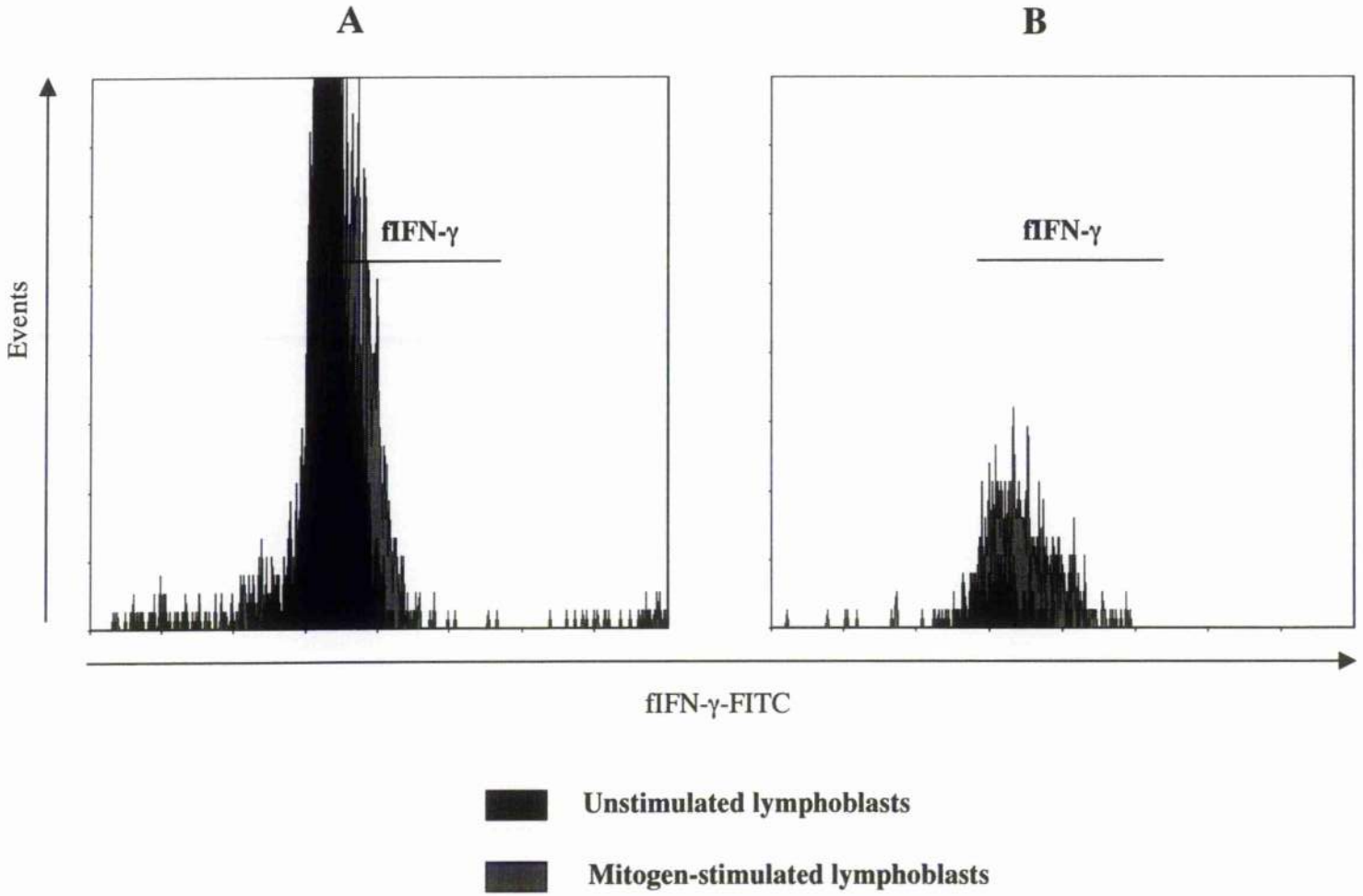

A
B
flFN-γ
flFN-γ
Events
flFN-γ-FITC
Unstimulated lymphoblasts
Mitogen-stimulated lymphoblasts

Fig. 2.4 Feline IFN-γ expression in $CD4^+$ T cells in the peripheral blood in response to either a 2 or 4 h mitogenic stimulation

Mononuclear cells in the peripheral blood from an SPF cat were stimulated with Con A for either 2 h (B) or 4 h (C) *in vitro,* in the presence of BFA. Unstimulated cells served as controls (A). CD4 expression was detected using the anti-fCD4-PE mAb (A-C); fIFN-γ expression was detected using the sheep anti-fIFN-γ-FITC pAb (A-C). Dot plots illustrating the small lymphocyte ('lymphs') and lymphoblast ('blasts') populations in the peripheral blood (i); histograms displaying CD4 expression in both the small lymphocyte population (ii) and in the lymphoblast population (iii); histograms displaying fIFN-γ expression in the lymphoblast population (iv); two-colour dot-plots demonstrating CD4 and fIFN-γ expression in the lymphoblast population (v); fIFN-γ^+ $CD4^+$ T cells were enumerated in Box 'C'. In total, 10,000 events were counted.

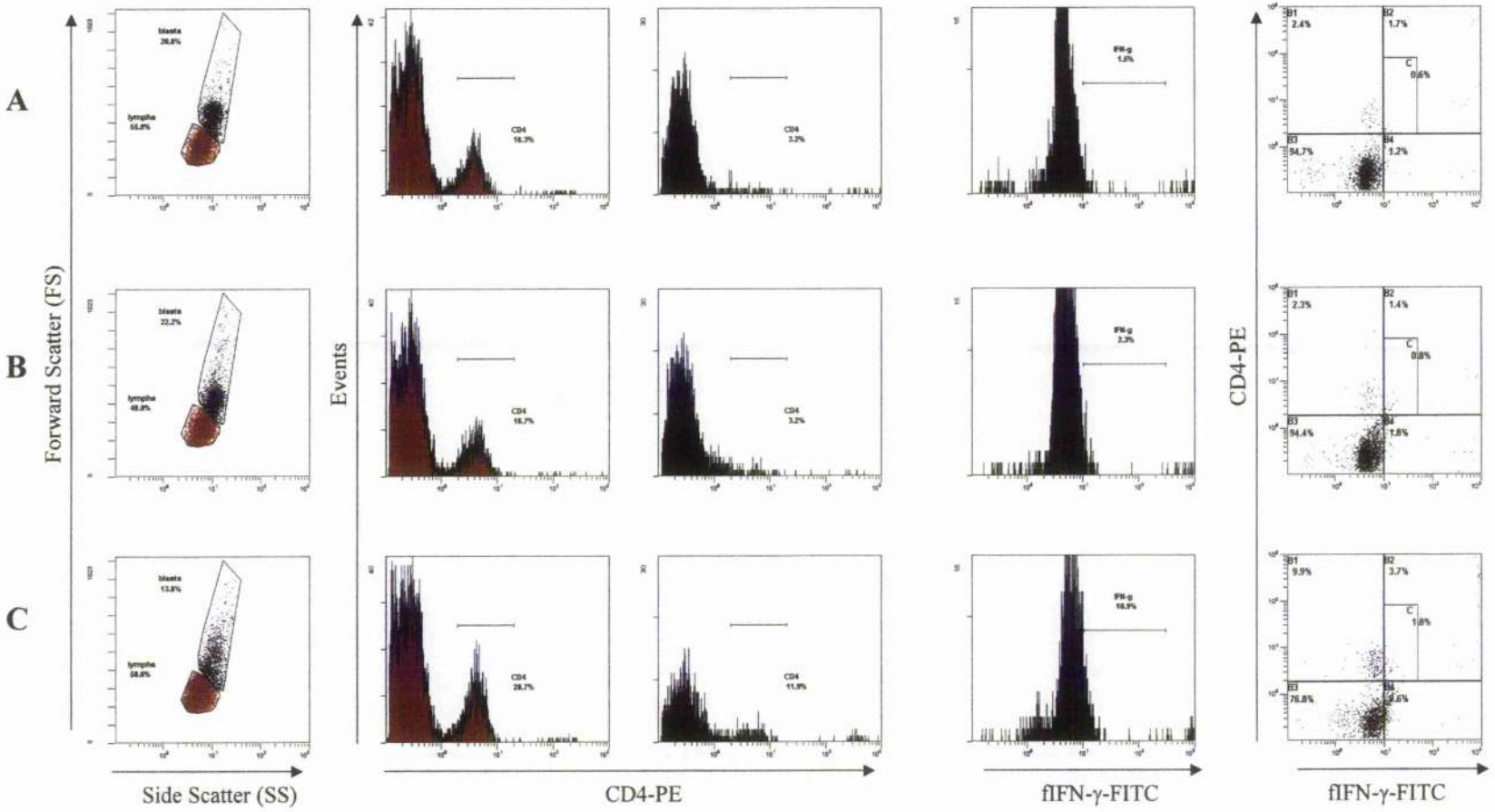

A
B
C
Forward Scatter (FS)
Side Scatter (SS)
Events
CD4-PE
flFN-γ-FITC
CD4-PE
flFN-γ-FITC
i
ii
iii
iv
v

Fig. 2.5 Feline IFN-γ expression in $CD4^+$ T cells in the peripheral blood in response to mitogenic stimulation

Mononuclear cells in the peripheral blood from an SPF cat were stimulated with Con A for 4 h *in vitro* (B-C), in the presence of BFA. Unstimulated cells served as controls (A). CD4 expression was detected using the anti-fCD4-PE mAb (A-C); fIFN-γ expression was detected using either the D9-FITC mAb (A-B), or the isotype matched control-FITC mAb (C). Dot plots illustrating small lymphocyte ('lymphs') and lymphoblast ('blasts') populations in the peripheral blood (i); histograms displaying CD4 expression in the small lymphocyte population (ii) and in the lymphoblast population (iii); histograms displaying fIFN-γ expression in the lymphoblast population (iv); two-colour dot-plots demonstrating CD4 and fIFN-γ expression in the lymphoblast population (v); fIFN-γ^+ $CD4^+$ T cells were enumerated in quadrant A2. The gates that determined fIFN-γ expression were set according to the reactivity of the isotype-matched control-FITC mAb. In total, 10,000 events were counted.

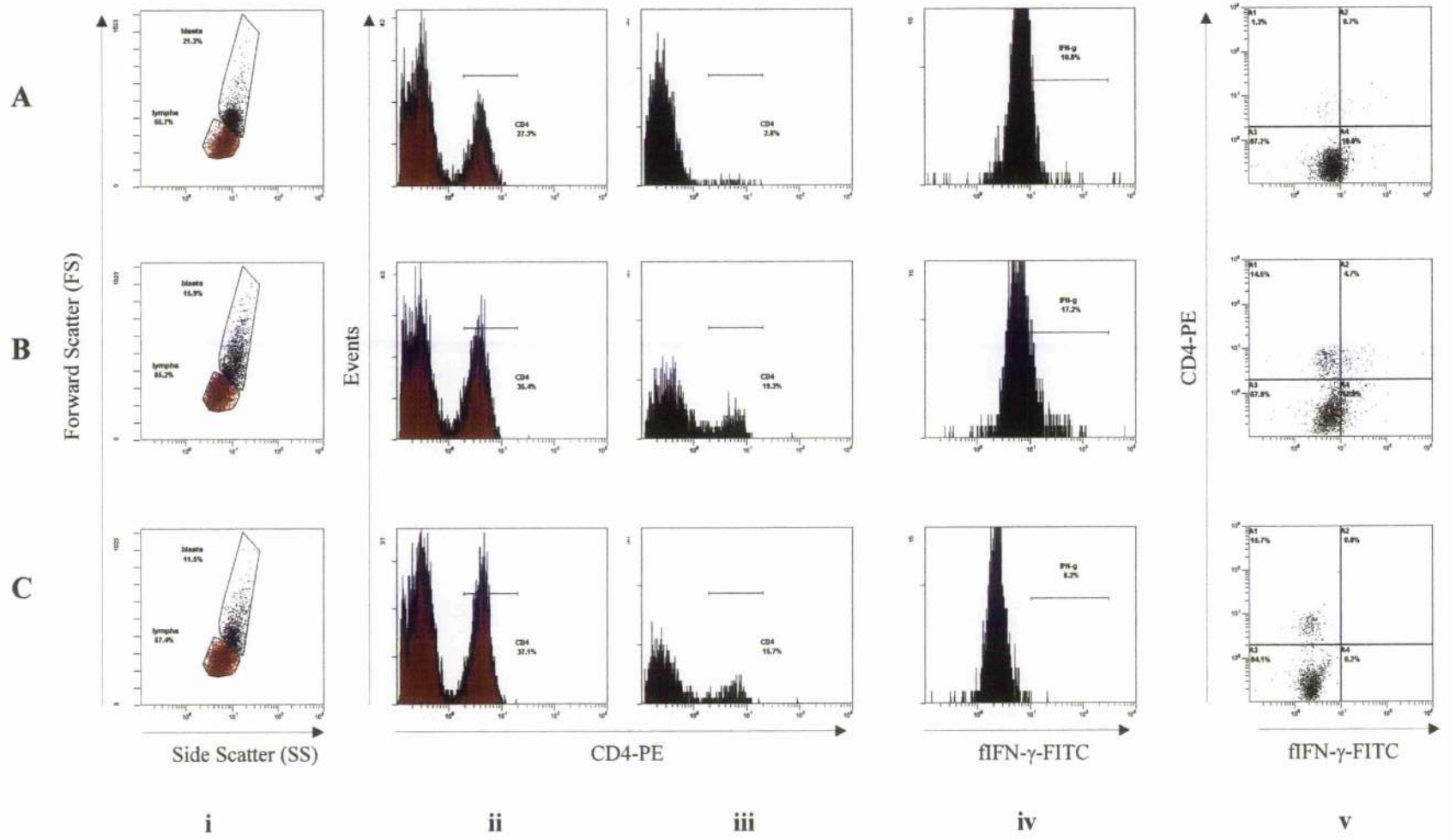

A
B
C
Forward Scatter (FS)
Side Scatter (SS)
Events
CD4-PE
fIFN-γ-FITC
i
ii
iii
iv
v

Fig. 2.6 Feline IFN-γ expression in $CD4^+$ T cells in the PLN tissues in response to mitogenic stimulation

Mononuclear cells in the PLN tissues from an FeLV recovered cat were stimulated with Con A for 4 h *in vitro* (B-C), in the presence of BFA. Unstimulated cells served as controls (A). CD4 expression was detected using the anti-fCD4-PE mAb (A-C); fIFN-γ expression was detected using either the D9-FITC mAb (A-B) or the isotype matched control-FITC mAb (C). Dot plots illustrating small lymphocyte ('lymphs') and lymphoblast ('blasts') populations from the PLN tissues (i); histograms displaying CD4 expression in the small lymphocyte population (ii) and in the lymphoblast population (iii); histograms displaying fIFN-γ expression in the lymphoblast population (iv); two-colour dot-plots demonstrating CD4 and fIFN-γ expression in the lymphoblast population (v); fIFN-γ^+ $CD4^+$ T cells were enumerated in quadrant A2. The gates that determined fIFN-γ expression were set according to the reactivity of the isotype-matched control-FITC mAb. In total, 10,000 events were counted.

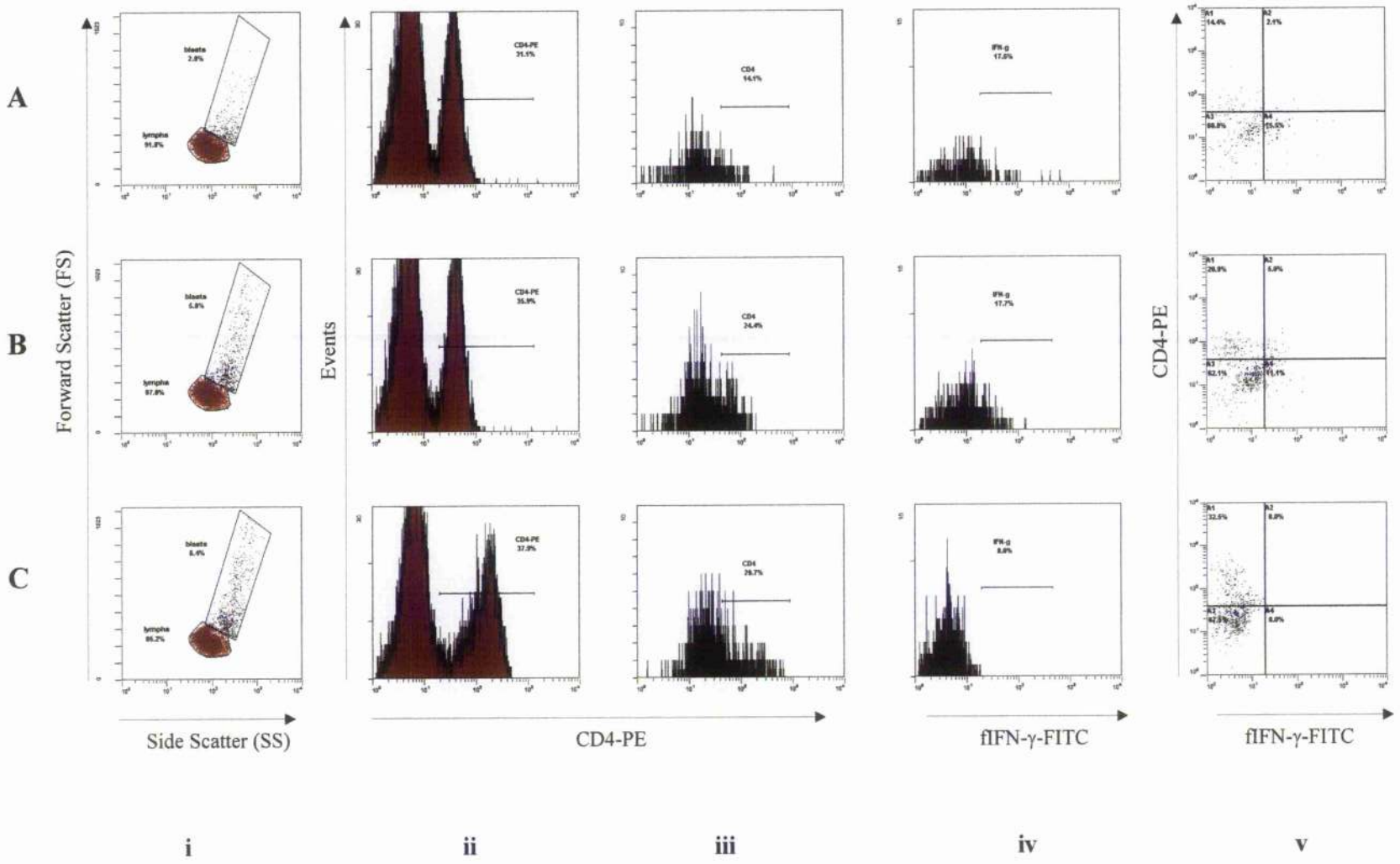

A
B
C
Forward Scatter (FS)
Side Scatter (SS)
Events
CD4-PE
CD4-PE
flFN-γ-FITC
CD4-PE
flFN-γ-FITC
i
ii
iii
iv
v

Fig. 2.7 Effect of BFA on CD4 expression following Con A stimulation

CD4 expression was detected within the small lymphocyte population in the MLN tissues from an FeLV recovered cat, following a 4 h stimulation with the mitogen Con A in the presence of BFA (B), or in the absence of BFA (C). Unstained cells are presented in (A). Dot plots illustrating the small lymphocyte ('lymphs') populations from MLN tissues (i); histograms displaying CD4 expression in the small lymphocyte population (ii); two colour dot-plot demonstrating CD4 and fIFN-γ expression in the small lymphocyte population; $CD4^+$ T cells were enumerated in quadrant A1. In total, 10,000 events were counted.

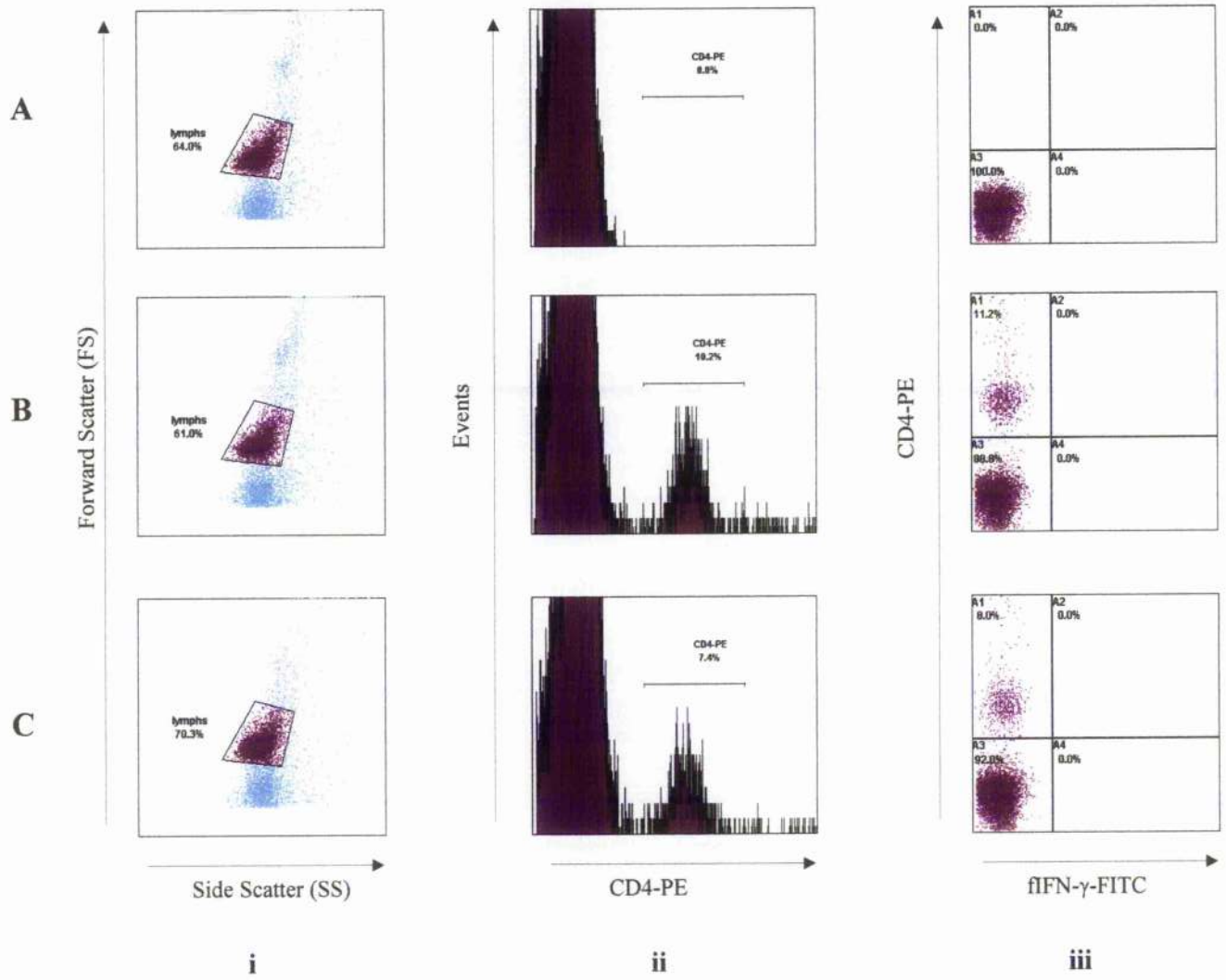
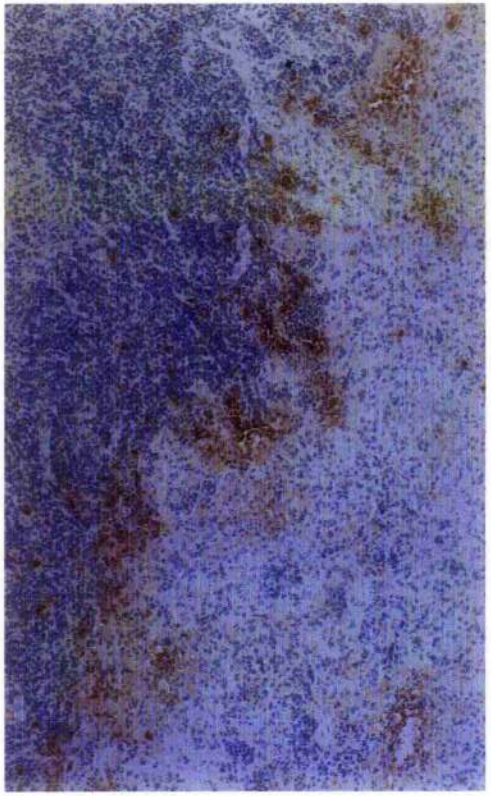

A
B
C
Forward Scatter (FS)
Side Scatter (SS)
lymphs
64.0%
lymphs
61.0%
lymphs
70.3%
i
Events
CD4-PE
CD4-PE
8.8%
CD4-PE
10.2%
CD4-PE
7.4%
CD4-PE
fIFN-γ-FITC
A1
0.0%
A2
0.0%
A3
100.0%
A4
0.0%
A1
11.2%
A2
0.0%
A3
88.8%
A4
0.0%
A1
8.0%
A2
0.0%
A3
92.0%
A4
0.0%
ii
iii

Fig. 2.8 Immunohistochemical detection of fIFN-γ in cryostat PLN sections

Cryostat PLN sections from an FeLV recovered cat were probed with the sheep anti-fIFN-γ pAb in an immunoperoxidase technique. Colour was developed using DAB; the tissue sections were counter-stained using Gills' haematoxylin. Many positive cells were visible at the cortico-medullary border (A, x10; B, x 20). Individual cells were scattered throughout the medullary cortex, and within the lymphatic vessels (C, x40).

A

B



C

2.4 Discussion

In this thesis, the evolution of the virus-specific cellular immune response was characterised following the experimental exposure of cats E1-E12 exto FeLV. Virus-specific $CD4^+$ and $CD8^+$ T cells were enumerated *ex vivo* using ICS, analysed by flow cytometry, to detect fIFN-γ expression. However, due to a lack of reagents to detect fIFN-γ at the commencement of these studies, it was necessary to raise murine monoclonal and sheep polyclonal antibodies to recombinant fIFN-γ.

Intracellular cytokine staining, analysed using flow cytometry, is a highly sensitive technique used to detect cytokine expression at the single cell level (Jung *et al.*, 1993; Pitcher *et al.*, 1999). In this chapter, this technique was used to demonstrate fIFN-γ expression by mononuclear cells following a four-hour mitogenic stimulation with Con A in the presence of BFA. In cats, the mitogenic activity of Con A is principally directed towards T, rather than B, cells (J.N. Flynn, personal communication); stimulation of feline PBMC for three days with Con A in the LPA resulted in a reduction of CD21 expression, whereas CD4 and CD8 expression were markedly upregulated (Chapter 4). Downregulation of CD4 expression has been observed to occur following stimulation of murine and rat splenic lymphocytes with the mitogens phorbol 12-myristate 13-acetate (PMA) and ionomycin (Nylander and Kalies, 1999; Caraher *et al.*, 2000), independently of BFA (Nylander and Kalies, 1999). In this thesis, Con A was observed to upregulate CD4 expression in both small lymphocyte and lymphoblast populations in the peripheral blood and PLN tissues, even after a short four-hour incubation with mitogen (Figs. 2.4-2.6). Concanavalin A mediates its activity through terminal sugar residues contained within cell surface MHC-antigen complexes (Kimura and Ersson, 1981). Brefeldin A has been reported to block the export of, and antigen presentation by, MHC class I and II molecules (Yewdell and Bennick, 1989; St.Pierre and Watts, 1990). In this thesis, Con A exhibited mitogenic activity in the presence of BFA (Figs. 2.3-2.6), suggesting that sufficient MHC complexes were present.

In human, murine and rat studies, short four-to-eight hour mitogenic and antigenic stimulations have been described for the detection of IFN-γ expression (Jung *et al.*,

1993; Nylander and Kalies, 1999; Pitcher *et al.*, 1999; Appay *et al.*, 2000; Caraher *et al.*, 2000). In this thesis, flFN-γ expression by $CD4^+$ T cells was detectable after two hours of mitogenic stimulation (Fig. 2.4), and had increased again after a further two hours. Based on these and other preliminary data, a four-hour stimulation period was used throughout this thesis. However, it may be valuable to conduct a more detailed time course to ascertain whether a four-hour period of mitogenic or antigenic stimulation was optimal for flFN-γ expression. Alternatively, assays to detect flFN-γ mRNA have been developed (Dean *et al.*, 1998; Liang *et al.*, 2000). This technique may be useful in predicting an optimal time for flFN-γ protein expression following mitogenic or antigenic stimulation. A longer duration of mitogenic stimulation may have enhanced flFN-γ expression. Some workers use protracted periods of mitogenic and antigenic stimulation to elicit maximal IFN-γ expression (Boaz *et al.*, 2002; Palmer *et al.*, 2002). However, it has been reported that prolonged periods of antigenic stimulation (>10-16 hours) may also reduce the intensity of IFN-γ expression (Nomura *et al.*, 2000).

In Figs. 2.5-2.6, a greater proportion of unstimulated lymphoblasts expressed flFN-γ when stained with the D9-FITC mAb, compared to the mitogen-stimulated lymphoblasts stained with the isotype control-FITC mAb. These findings were representative of the assays presented in this thesis, and may be attributed to any of the following factors. First, the D9-FITC mAb may have been non-specifically bound by FcR-bearing cells, such as monocytes and granulocytes. Although the analysis was conducted on the gated small lymphocyte and the large lymphoblast populations, low numbers of monocytes and granulocytes may have been unintentionally included. Activated platelets that stick to lymphocytes are also recognised as significant contributors to non-specific background staining (Nomura *et al.*, 2000). Second, unstimulated mononuclear cells were cultured *in vitro* for four hours before staining for flFN-γ expression; cells activated *in vivo* may have expressed flFN-γ *ex vivo* in the absence of antigenic or mitogenic stimulation, causing true biological background. In addition, APCs loaded with antigen *in vivo* may have activated co-cultured cells *ex vivo*. Since the unstimulated cells from an SPF cat and an FeLV recovered cat both expressed flFN-γ (Figs. 2.5 and 2.6), this explanation may not fully account for these findings. However, cells may become

non-specifically activated *in vitro* by elements within the culture medium, such as FCS, which can be mitogenic for B cells. Thus, non-specific fIFN-γ staining may be a problem when the cells are not fractionated.

In Figs. 2.5 and 2.6, the majority of mitogen-activated cells that expressed fIFN-γ were not $CD4^+$ T cells. Non-specific or biological background staining, described above, may have contributed to this result. However, other cell types such as $CD8^+$ T cells, B cells, plasmacytoid DC, NKT cells, Tr cells and NK cells are all capable of secreting IFN-γ upon activation (Section 1.3.1.2). In particular, NK cells are major producers of IFN-γ. Since these assays were conducted using unfractionated mononuclear cells, all lymphoblast cell subsets, as well as other cell types of similar size and granularity, were included in the gated lymphoblast population.

The sheep anti-fIFN-γ pAb was used to identify fIFN-γ-expressing cells in PLN tissue sections from an FeLV recovered cat using immunohistochemistry, illustrated in Fig. 2.8. The demonstration of IFN-γ within tissues *in situ* may aid in the characterisation of the immune response during disease processes (Kakazu *et al.*, 1999). Double immunoenzyme techniques could be used to characterise the fIFN-γ expressing population. In addition, fIFN-γ expression could be correlated with the presence of infectious FeLV or FeLV proteins. However, it was difficult to achieve consistency in the detection of fIFN-γ expression using immunohistochemistry. It has been reported that IFN-γ expression in cryostat tissue sections is difficult to accurately detect using immunohistochemistry; discrepancies occurred between results obtained using intact *in vitro* stimulated T cells and cryosections prepared from the same T cells (van Der Loos *et al.*, 2001). Multiple reasons were cited to explain the discrepancies; acetone fixation may result in antigen extraction; non-specific staining of various cellular constituents and plasma cells occurred frequently, and immunohistochemistry is less sensitive than flow cytometry. It has been suggested that IFN-γ expressing cells should be characterised using double immunoenzyme techniques to minimise spurious results (van Der Loos *et al.*, 2001). Furthermore, prefixation of tissues with paraformaldehyde (PFA) prior to freezing may eliminate leaching of cytokines.

The anti-fIFN-γ antibodies have additional applications in the field of feline research. In addition to FeLV, the domestic cat is the natural host for a number of significant pathogens including FIV (Pedersen *et al.*, 1987) and feline coronavirus virus (FCoV) (Pedersen *et al.*, 1987; Addie *et al.*, 1995). Currently, effective vaccines are not available for the majority of these feline infections, and curative treatment is often not possible. Although the immune mechanisms determining the disease outcome following exposure to these pathogens are becoming clearer (Pedersen, 1987; Olsen, 1993; Beatty *et al.*, 1996; Willett *et al.*, 1997; Flynn *et al.*, 1999), progress in this area is slow. This is partly due to the lack of sensitive, species-specific reagents to allow the quantitative definition of the important immune control mechanisms. The anti-fIFN-γ antibodies described in this study have the potential to facilitate the quantitative analysis of antigen-specific T cells *ex vivo* using either ICS techniques analysed using flow cytometry, or ELISpot assays. These highly sensitive assays will enable the evolution of the immune response to be monitored following infection or vaccination, or in tumour development. A second important use of these novel reagents is to detect circulating fIFN-γ in plasma using a capture ELISA. Such an approach may also be a useful prognostic indicator when evaluating novel immunotherapies. Recent studies in domestic cats have attempted to evaluate the potential of feline cytokines, for example IFN-γ, IL-12 and IL-18, as genetic vaccine adjuvants *in vivo* (Hosie *et al.*, 1998; Flynn *et al.*, 2000b; Hanlon *et al.*, 2001). An adjuvant effect has been demonstrated in certain studies as assessed by increases in virus-specific cell-mediated immune responses, or by improved vaccine efficacy following challenge.

In this thesis, these novel reagents were used to detect and quantify the evolution of virus-specific immune responses *ex vivo* following experimental exposure of cats to FeLV. The specificity of the antiviral immune response can be determined through the enumeration of fIFN-γ expressing cells following stimulation with viral antigens. To this end, recombinant p27 and p45 proteins and inactivated FeLV particles were generated and used in this thesis. Cell surface molecules, such as CD4 and CD8, were simultaneously detected, to ensure that the cytokine-positive cells were indeed the cells of interest.

2.5 Summary and Conclusions

Prior to this thesis, no antibody reagents were available to detect fIFN-γ in the cat. The sensitivity of the D9 mAb and sheep anti-fIFN-γ pAb, in the detection of fIFN-γ, was determined using an indirect IFN-γ ELISA and Western blots. In addition, these antibodies were assessed for their ability to detect the production of fIFN-γ by specific feline T cell populations *ex vivo*, following co-culture with mitogen for four hours in the presence of protein secretion inhibitor BFA. Flow cytometry was used to simultaneously detect PE-labelled cell surface molecules, and FITC-labelled intracellular fIFN-γ. Finally, expression of fIFN-γ in cryostat sections of PLN tissues was detected using the sheep anti-fIFN-γ pAb in an immunoperoxidase technique.

Using these assays, these novel reagents were shown to be highly sensitive in the detection of both recombinant feline IFN-γ and the production of intracellular fIFN-γ in physiologically stimulated cells. In this thesis, these reagents will be employed to detect fIFN-γ expression by virus-specific $CD4^+$ T cells following recognition of their cognate antigen, using ICS analysed using flow cytometry.

CHAPTER 3

LONGITUDINAL IMMUNOPATHOGENESIS STUDY

I. VIROLOGY

3.1 Introduction

A longitudinal FeLV immunopathogenesis study was conducted to investigate the role of FeLV-specific $CD4^+$ T cells in the control of FeLV infection. $CD4^+$ T cells have a critical role in regulating the humoral and cell-mediated arms of the immune response through the activation of DC and the elaboration of cytokines.

Six SPF cats, E1-E6, were exposed oronasally to FeLV-A aged sixteen weeks, in the expectation that each of three well-defined outcomes of infection would result, recovery, latency and persistence. To define the outcome of FeLV exposure in each cat, and correlate the virus-specific immune response with the viral status of exposed cats, a series of virus parameters was monitored throughout the study, and post mortem. Peripheral blood samples were taken at regular intervals following oronasal exposure to FeLV, and post mortem. In each sample, proviral DNA loads were quantified using qRT-PCR; the emergence of p27 antigenaemia was monitored using a double antibody sandwich ELISA, and the presence of infectious virus was determined using VI techniques. Samples from PLN, MLN, spleen and thymus were harvested post mortem, from which proviral DNA loads were quantified using qRT-PCR. These data indicated that all cats, E1-E6, became persistently infected with FeLV. In this thesis, it was intended to examine and compare FeLV-specific $CD4^+$ T cell responses among persistently infected, recovered and latently infected cats. Therefore, in order to enhance the probability of recovery, a second group of six SPF cats, E7-E12, was exposed to a reduced dose of FeLV-A under identical conditions as cats E1-E6. Each group of cats, E1-E6 and E7-E12, underwent the same series of assays at comparable time intervals. However, cats E1-E6 were euthanased nineteen weeks post exposure while cats E7-E12 were euthanased after thirteen weeks. Throughout this thesis, these two groups of cats are frequently referred to as cats E1-E12.

This chapter describes the experimental exposure of cats E1-E12 to FeLV, the preparation of samples and the evolution of the viral status of cats E1-E12. Virus-specific cellular immune responses will be described in Chapter 4. In Chapter 5,

expression of the FeLV CA protein p27 in the bone marrow and PLN tissues will be illustrated.

3.2 Materials and Methods

3.2.1 Experimental Animals

In total, two groups of six 8-9 week old outbred SPF domestic shorthair (DSH) kittens were procured from a commercial source and housed in a purpose built isolation unit at the University of Glasgow. The first group of six cats (E1-E6) occupied two adjoining rooms, each 9.36 m^2 and 2.36 m high; the second group (E7-E12) shared a single large room. The cats were fed proprietary dry and wet food twice daily and received water *ad libitum*. Male cats within each group were castrated at approximately eleven weeks of age under isofluorane gaseous anaesthesia (1.5% Isoflo, Schering-Plough, Uxbridge, U.K.).

3.2.2 Exposure to FeLV

Cats E1-E12 were exposed oronasally to a molecular clone of FeLV-A Glasgow-1 (Jarrett *et al.*, 1972; Jarrett *et al.*, 1973; Stewart *et al.*, 1986), when aged sixteen weeks. The virus used was taken from titrated stock prepared by the Companion Animal Diagnostic Laboratory (CADL) (Batch 53; 6 x 10^6 focus-forming units (ffu)/ml), and stored at -70°C. Immediately before use, the virus was thawed and diluted in sterile PBS to give a final volume of 1 ml/cat; 0.5 ml was administered orally and 0.25 ml applied in each nostril. Six cats (E1-E6) received 5 x 10^5 ffu FeLV-A; the remaining six (E7-E12) each received 5 x 10^4 ffu FeLV-A.

3.2.3 Blood Sampling

Blood was collected from conscious animals using jugular venepuncture. Rarely, sedation using xylazine (0.5 mg/kg, Rompun, Bayer PLC, Berkshire, U.K.) and ketamine (5 mg/kg, Ketaset, Fort Dodge Animal Health, Hampshire, U.K.) was required. The maximum volume of blood permitted by U.K. Home Office guidelines

per sampling was calculated according to the weight of the cats. The first sample was taken six weeks prior to FeLV exposure, and sampling continued at intervals of three weeks thereafter until the end of the study. An additional sample was taken one week following FeLV exposure. Whole blood was collected into lithium heparin and sodium citrate blood tubes (Dunwood Ltd., Aberdeen, U.K.), and into Alsevers' solution (1:1).

3.2.3.1 Lithium Heparin Samples

To separate the blood fractions, blood collected in lithium heparin sample tubes was centrifuged at 1500rpm for twelve minutes using a bench-top centrifuge. At least 300 µl plasma was submitted to the CADL to conduct VI, p27 ELISA and virus neutralisation assays. Virus isolation was performed by infecting QN10S cells with plasma samples from exposed cats (Jarrett and Ganière, 1996). QN10S cells were derived from a clone of feline fibroblasts, into which had been introduced the Moloney murine sarcoma virus (MoMSV) (Jarrett and Ganière, 1996). A subconfluent layer of QN10S cells, plated in wells of a 24-well tissue culture plate, was inoculated with 100 µl plasma; any virus present was absorbed for two hours, then replaced with fresh culture medium. The cells were examined for morphological changes that would indicate the presence of infectious virus. Cells were initially cultured for six days, negative cells were further subcultured for five days. The presence of VNA was determined using a focus reduction assay on QN10S cells (Jarrett and Ganière, 1996). Dilutions of plasma were incubated with an equal volume of an appropriate concentration of FeLV-A/Glasgow-1, for six hours at 37°C. Any residual infectivity was determined by VI performed on QN10S cells, described above. If virus is used at an appropriate concentration, foci of transformation are obtained and the infectious virus may be quantified. The VNA titre was calculated as the reciprocal of the plasma dilution that reduced the focus count by 75%, compared with a virus control incubated without plasma (Jarrett and Ganière, 1996). The presence of p27 antigenaemia was determined using a double antibody sandwich ELISA (Lutz *et al.*, 1983). Micro-ELISA plates were coated with a combination of two anti-p27 mAbs, which recognised distinct epitopes. Plasma samples were mixed with a biotinylated anti-p27 pAb, then added to the micro-ELISA plates, and incubated for thirty minutes at 37°C. A final thirty-minute

incubation with peroxidase-labelled strepatavidin preceeded the development of peroxidase activity. Absorbance was measured in a micro-ELISA plate reader. Positive and negative samples were included as controls; a sample was considered positive when the absorbance was equal to, or exceeded, 150% of the negative control value.

Mononuclear cells were isolated from the buffy coat as described in Section 2.2.2.8, prior to being resuspended in 200 µl freeze-mix[24] and dispensed in cryotubes (Nalge Nunc International, Assi Domän, Kolding, Denmark). The cells were stored overnight at -70°C in sealed polystyrene containers before transfer to liquid nitrogen the following day.

3.2.3.2 Sodium Citrate Samples

Blood collected in sodium citrate sample tubes was centrifuged at 1500rpm for twelve minutes using a bench-top centrifuge. The plasma was removed and centrifuged again at 3000rpm for ten minutes in a microfuge ('Micro Centaur', Sanyo Gallenkamp, U.K.) to pellet any remaining red blood cells (RBC). Two 180 µl plasma samples were aliquoted to determine plasma FeLV RNA loads using qRT-PCR analysis. Proviral DNA loads were quantified from the buffy coat layer using qRT-PCR. A volume of 200 µl buffy coat was placed in 20 µl protease (Qiagen U.K. Ltd, West Sussex, U.K.), to which 200 µl 'AL Buffer' (Qiagen, U.K.) was added. The reagents were thoroughly mixed prior to a ten-minute incubation at 56°C in a water bath. Plasma and buffy coat samples were subsequently stored at -70°C until submission to Dr Regina Hofmann, Faculty of Veterinary Medicine, University of Zurich, for qRT-PCR analysis. The primers and probes used recognised the U3 long terminal repeat (LTR) of exogenous FeLV-A. The primer sequences were 5´ ACCTGGGCCCCGGCT 3´ (15 bases; nt 2174-2188) for the sense primer, and 5´ GCGGCCTTGAAACTTCTGCT 3´ (20 bases; nt 2228-2247) for the antisense primer.

The TaqMan FeLV probe was, 5´ AGGCCAAGAACAGTTAAACCCCGGAT 3´(26 bases; nt 2191-2216) (Hofmann-Lehmann *et al.*, 2001).

[24] Freeze-mix: 10% (v/v) DMSO in FCS

3.2.3.3 Alsevers' Samples

Mononuclear cells were harvested from peripheral blood collected in Alsevers' solution, as described in Section 2.2.2.8. In most cases, fresh mononuclear cells were used in the LPA, and ICS analysed using flow cytometry (Chapter 4). Any remaining cells were frozen and stored in liquid nitrogen.

3.2.4 Post Mortem Sampling

All cats in the study were euthanased using pentobarbital sodium (120 mg/kg, Euthatal, Merial U.K., Harlow, U.K.) under sedation 13-19 weeks following FeLV exposure. Between 80 and 120 ml blood was withdrawn by cardiac puncture under terminal anaesthesia. Samples to determine proviral DNA loads using qRT-PCR analysis were collected from the PLN and MLN tissues, spleen and thymus, and snap frozen in liquid nitrogen. Samples from PLN, MLN, spleen and thymus were also collected in 'O.C.T.', and snap frozen in liquid nitrogen for immunohistochemical analysis. Remaining PLN, MLN and splenic tissue was collected into RPMI 1640 medium containing penicillin and streptomycin, for use as single cell suspensions in the LPA. Femoral bone marrow was harvested and distributed among sterile containers containing 5% BSA in PBS, 10% formalin and bone marrow transport medium[25] (BMTM), for cytological analysis and immunofluorescence, immunohistochemistry and VI, respectively.

Bone marrow cells within the BMTM were pelleted. To determine whether infectious virus was present within the bone marrow, the supernatant, or cell free suspension (CFS), was added to QN1OS cells for VI. The presence of a latent infection was established by culturing the bone marrow cells *in vitro*. The cells were cultured until confluent in MEM-alpha medium containing 10% FCS, 100 U/ml penicillin, 10 μg/ml streptomycin and 2 mM glutamine (complete MEM-alpha medium), and passaged once. To determine whether latent provirus had become reactivated, tissue culture fluid (TCF) was then added to QNIOS cells for VI.

[25] BMTM: MEM-alpha medium containing 10% (v/v) FCS, 100 U/ml (v/v) penicillin, 100 μg/ml (v/v) streptomycin, 2 mM (v/v) glutamine; 50 U (v/v) heparin/ml

3.2.4.1 Preparation of Single Cell Suspensions

Post mortem tissue samples were emptied into plastic Petri dishes (Bibby Sterilin Ltd., Staffordshire, U.K.) within a microbiological safety cabinet and washed using 20-30 ml RPMI 1640 medium containing antibiotics, as described above. Sterile instruments were used to remove excess fat from the specimens before the lymphoid tissue was gently teased out beneath the fibrous capsules. Lymphoid cells were collected into universal containers at regular intervals and aspirated when fibrous material had settled to the bottom, and fat had floated to the top. Aspirated material was centrifuged in a bench-top centrifuge at 1000rpm for ten minutes, washed in RPMI 1640 medium, frozen in freeze-mix, and stored in liquid nitrogen. Splenic cells were additionally layered over Ficoll-Paque in order to minimise RBC contamination.

3.2.5.Statistics

Statistical analysis was conducted using 'GraphPad InStat' (GraphPad Software, San Diego California U.S.). Non-parametric tests were used throughout. The Mann-Whitney test was applied to compare results from two groups, using a two-tail *P* value. Where three or more groups were compared, the Kruskall-Wallis test was used. The Spearman rank correlation test (Spearman r-Test) was conducted to analyse the association between proviral DNA loads in the peripheral blood and s:p values calculated from the p27 ELISA data. The Spearman r-Test is a non-parametric test.

3.3 Results

3.3.1 Viral Status of Cats E1-E12

Prior to experimental FeLV exposure at week 0, blood samples from cats E1-E12 were tested for the presence of FeLV p27 antigenaemia, infectious virus, buffy coat proviral DNA, and VNA, and were found to be negative for all of these parameters.

3.3.1.1 p27 ELISA

Following the experimental exposure of cats E1-E12 to FeLV at week 0, the presence of p27 antigenaemia was determined at regular intervals using a double antibody sandwich ELISA. The results are shown in Fig. 3.1 and summarised in Table 3.1. Cats were considered positive when the $O.D._{405}$ sample value was equal to, or greater than, 150% of the negative control value. The sample:positive (s:p) ratio was calculated from the $O.D._{405}$ value of each sample, using results from the positive and negative controls:

$$\frac{\text{sample } O.D._{405} - \text{negative control } O.D._{405}}{\text{positive control } O.D._{405} - \text{negative control } O.D._{405}}$$

This was done to correct for day-to-day variations in ELISA readings, allowing results to be more accurately compared over a time course (Fig. 3.1).

p27 antigenaemia was not detected in the plasma from any cat one week following FeLV exposure. By week 4, ten cats were p27-positive, with only cats E10 and E12 remaining negative. However, cat E10 became p27 antigenaemic at the next sample date (week 7), and cats E1-E11 remained p27-positive for the duration of the study. No p27 antigenaemia was detected in the plasma from cat E12 at any time (Table 3.1). While cats E1-E6 received a higher exposure dose of FeLV by a factor of one log, compared to cats E7-E12, significant differences in the s:p values between the two groups of cats were not observed until week 13, when cats E1-E6 demonstrated significantly higher s:p values (Mann-Whitney Statistic, P=0.0022).

The pattern of s:p results generally conformed to two principal peaks in values, at week 4 and/or weeks 10-13, although s:p results from three cats, E1, E2 and E3, displayed an additional low peak at week 19. Results from only three cats, E2, E7 and E8, demonstrated a biphasic pattern, peaking at both week 4 and weeks 10-13; s:p results from the remaining cats peaked at either week 4 or weeks 10-13.

3.3.1.2 Virus Isolation

The presence of infectious FeLV in the plasma was determined using VI techniques at regular intervals following experimental exposure of cats E1-E12 to FeLV at week 0. Isolation was initially attempted over a six-day culture period; any remaining negative samples were subcultured for a further five days (Jarrett and Ganière, 1996). The results are shown in Table 3.1. Virus was not isolated from the plasma of any cat one week following experimental exposure to FeLV. However, infectious virus was isolated from the plasma of cats E1-E11 at each sampling from week 4 until the end of the study, signifying that these cats became persistently viraemic following oronasal exposure to FeLV. Conversely, infectious FeLV was not isolated from the plasma of cat E12 at any time throughout the study, suggesting that cat E12 had recovered following oronasal exposure to FeLV.

Since all cats E1-E6 were persistently viraemic by week 19, it was considered not necessary to perform VI using the bone marrow from these cats; however, VI was conducted on the bone marrow samples collected from cats E7-E12 post mortem. Infectious virus was present in the bone marrow CFS from cats E7-E11, but not cat E12. However, following a short culture *in vitro*, infectious virus was isolated from the bone marrow cells from cat E12, indicating the presence of a latent bone marrow infection in this cat. Thus, while virus could not be detected in the peripheral blood from cat E12 throughout the study, which suggested that this cat had eliminated virus and spontaneously recovered, virus was still present within the bone marrow, as a latent proviral infection.

3.3.1.3 FeLV Proviral DNA Loads

The analytical sensitivity of the qRT-PCR assay was calculated by amplifying a tenfold serial dilution of the cloned standard DNA template; the qRT-PCR detected between 0.36 and 3.6 copies of the DNA template (Hofmann-Lehmann *et al.*, 2001).

(i) Buffy Coats

Feline leukaemia virus proviral DNA levels in buffy coat samples were measured

using qRT-PCR at regular intervals following experimental exposure of cats E1-E12 to FeLV at week 0 (Hofmann-Lehmann *et al.*, 2001). The results are shown in Fig. 3.2 and Table 3.1. The raw qRT-PCR data (proviral DNA per PCR reaction) comprises the number of copies of the FeLV U3 LTR in each sample. To calculate proviral copy numbers per cell, these figures were divided by two (number of LTR copies), and then by the number of buffy coat cells. These were determined from the quantity of DNA used in each PCR reaction, which was 1 μg.

The qRT-PCR data showed that proviral DNA was present in the peripheral blood from all cats E1-E12 at week 1, except cats E7 and E12 (Table 3.1). Proviral DNA was detected in the peripheral blood from cat E7 by week 4, and in cat E12 by week 7. Thereafter, proviral DNA continued to be detected in each cat until the termination of the study at week 13 (E7-E12) and week 19 (E1-E6).

Latently infected cat E12 displayed low mean levels of proviral DNA over weeks 0-13 (0.0093 ± 0.0099 copies per cell), compared to persistently viraemic cats that received an equivalent exposure dose of infectious FeLV (0.53 ± 0.46 copies per cell). The difference is statistically significant (Mann-Whitney Statistic, P=0.0173). Cats E1-E6, which received a greater exposure dose of FeLV, had greater mean levels of proviral DNA in buffy coat samples over weeks 0-19 (4.5 ± 1.2 copies per cell), compared to cats E7-E12 over weeks 0-13 (0.44 ± 0.42 copies per cell). Statistically, significantly higher proviral DNA loads were quantified in cats E1-E6 at week 1 (P=0.0260), week 4 (P=0.0411), week 10 (P=0.0022) and week 13 (P=0.0087), compared to cats E7-E12, according to the Mann-Whitney Statistic.

Fluctuations in proviral DNA copy numbers were recorded for each cat E1-E12 over the length of the study. Generally, two peaks in proviral DNA load were observed, at weeks 4-7 and/or weeks 10-16. Proviral DNA loads in cats E2, E3, E4, E5, E7 and E9 all displayed a biphasic pattern, peaking at weeks 4-7 and again at weeks 10-16. Cat E2 demonstrated a third small peak at week 19. Proviral DNA loads in cats E8, E10 and E11 peaked only at weeks 4-7, while those in cat E6 peaked at weeks 13 and 19. Cat E12 demonstrated a minor peak in proviral DNA load at week 10.

A direct correlation was detected between the proviral DNA load data measured

using qRT-PCR, and the s:p figures calculated from the p27 ELISA (Spearman r-Test, r=0.5235), over the entire study for cats E1-E16 (weeks 0-19), and cats E7-E12 (weeks 0-13) (Fig. 3.4). These results were extremely statistically significant (P=0.0001 and P<0.0001, respectively). However, no statistically significant correlation was detected between the proviral DNA data and the s:p results in either groups of cats at any single sample date throughout the study.

(ii) Lymphoid Tissues

Feline leukaemia virus proviral DNA loads in PLN, MLN, thymus and spleen from cats E1-E12 post mortem are shown in Fig. 3.3. Proviral DNA was detected in each sampled tissue from every cat. Thus, although infectious virus was not detected in the peripheral blood from cat E12 at any time throughout the study, proviral DNA was detected in the lymphoid tissues and in the bone marrow. Statistically, cats E1-E6 demonstrated significantly higher levels of proviral DNA in all tissues, compared to cats E7-E12, according to the Mann-Whitney Statistic (PLN, P=0.0043; MLN, P=0.0152; thymus, P=0.0260; spleen, P=0.0022). However, no statistically significant difference in proviral DNA loads between each tissue type was found, in either group of cats, using the Kruskall-Wallis Statistic. However, it is clear from Fig. 3.3 that the spleen contained the highest mean levels of proviral DNA (cats E1-E6, 1.035 ± 0.2 copies per cell; cats E7-E12, 0.209 ± 0.12 copies per cell), compared to all other tissues (cats E1-E6, 0.436 ± 0.31 copies per cell; cats E7-E12, 0.151 ± 0.018 copies per cell). Latently infected cat E12 displayed low mean levels of proviral copy numbers in all tissues (0.000396 ± 0.0001 copies per cell), compared to persistently viraemic cats that received a comparable dose of infectious FeLV (0.18 ± 0.07 copies per cell). The difference is statistically significant (Mann-Whitney Statistic, P=0.0159).

3.3.2. Humoral Immunity

3.3.2.1 Virus Neutralising Antibodies

The development of VNA was monitored using a focus reduction assay at three-week intervals following experimental exposure to FeLV. Throughout the course of

the study, cats E1-E11 failed to develop VNA to FeLV. However, a low VNA titre was recorded for cat E12 at week 13 (1:16).

Fig. 3.1 Detection of FeLV CA protein p27 using p27 ELISA

Comparison of sample:positive (s:p) values. These were calculated from the plasma sample $O.D._{405}$ values, determined by p27 ELISA at regular intervals from weeks 0-19 (E1-E6) and weeks 0-13 (E7-E12). The positive and negative control OD_{405} values used to calculate the s:p values, are displayed below.

	E1-E6		E7-E12	
	Pos.	**Neg.**	**Pos.**	**Neg.**
Week 0	0.21	0.06	1.62	0.26
Week 1	0.44	0.06	1.55	0.19
Week 4	1.28	0.15	0.90	0.18
Week 7	1.50	0.15	0.80	0.17
Week 10	0.78	0.13	1.03	0.19
Week 13	0.78	0.13	1.29	0.17
Week 16	0.70	0.13		
Week 19	0.75	0.13,0.14		

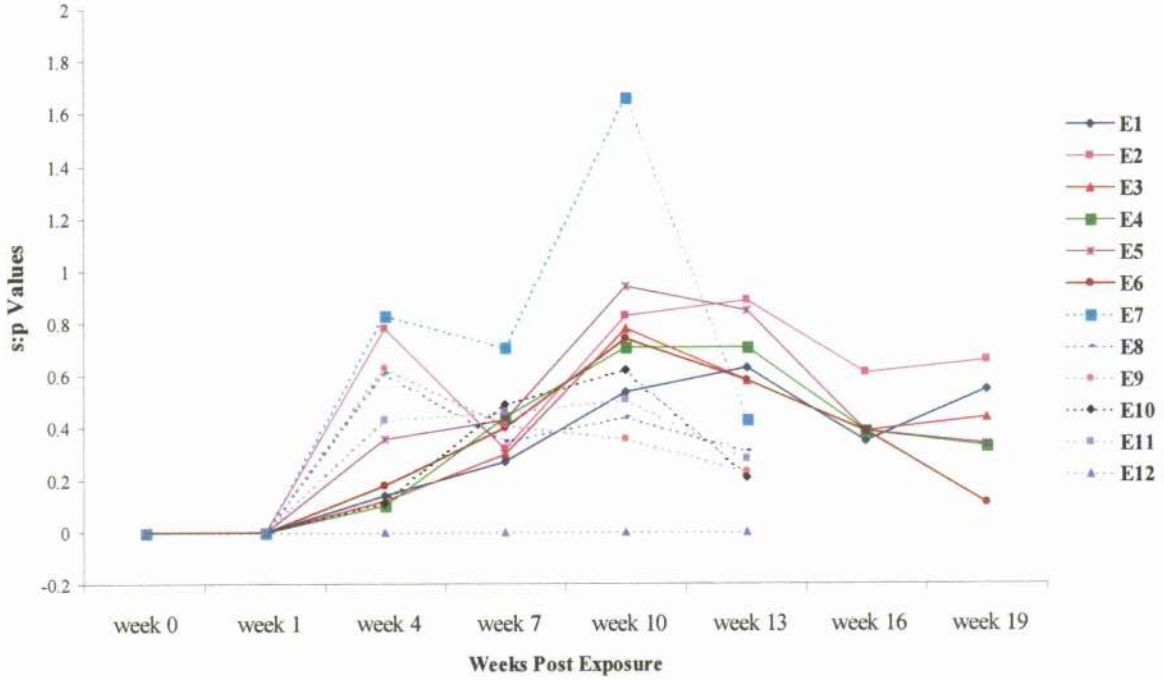

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
s:p Values
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12

Fig. 3.2 Proviral DNA copy numbers detected in the peripheral blood from cats E1-E12

FeLV proviral DNA copy numbers per cell detected in the buffy coat from cats E1-E12 over weeks 0-19 (E1-E6) and weeks 0-13 (E7-E12) using qRT-PCT, following oronasal exposure to FcLV-A.

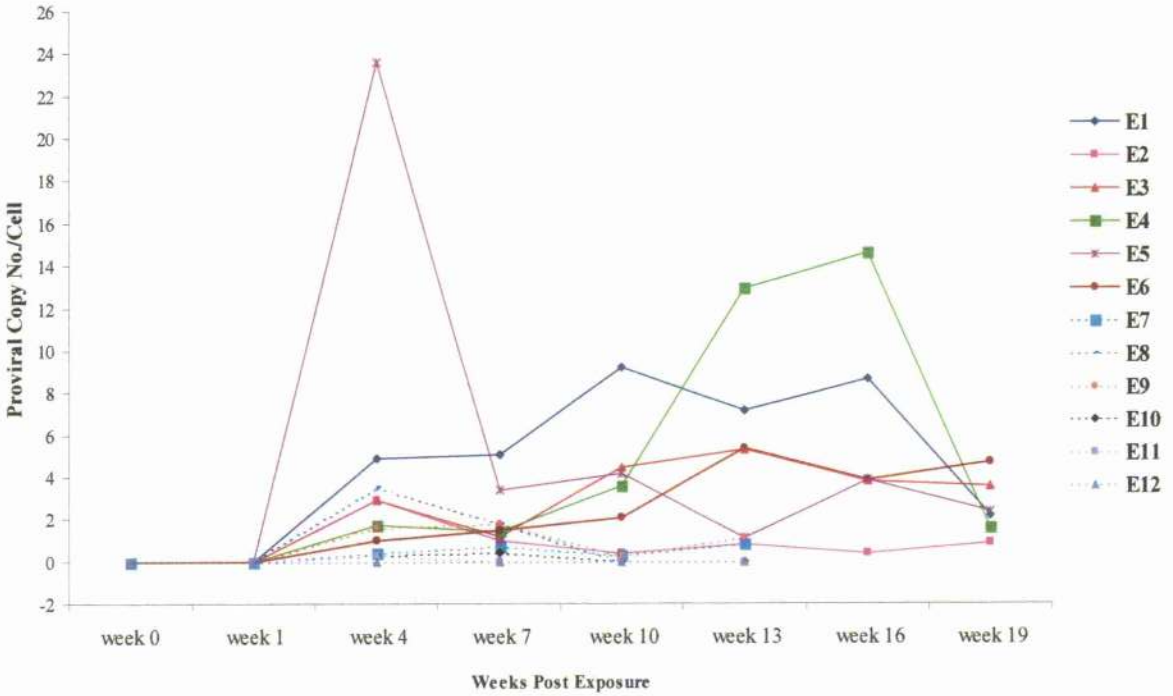

Proviral Copy No./Cell
26
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12

Fig. 3.3 Proviral DNA copy numbers detected in the lymphoid tissues from cats E1-E12

Proviral DNA copy numbers per cell quantified in PLN and MLN tissues, thymus and spleen from cats E1-E12 post mortem using qRT-PCT.

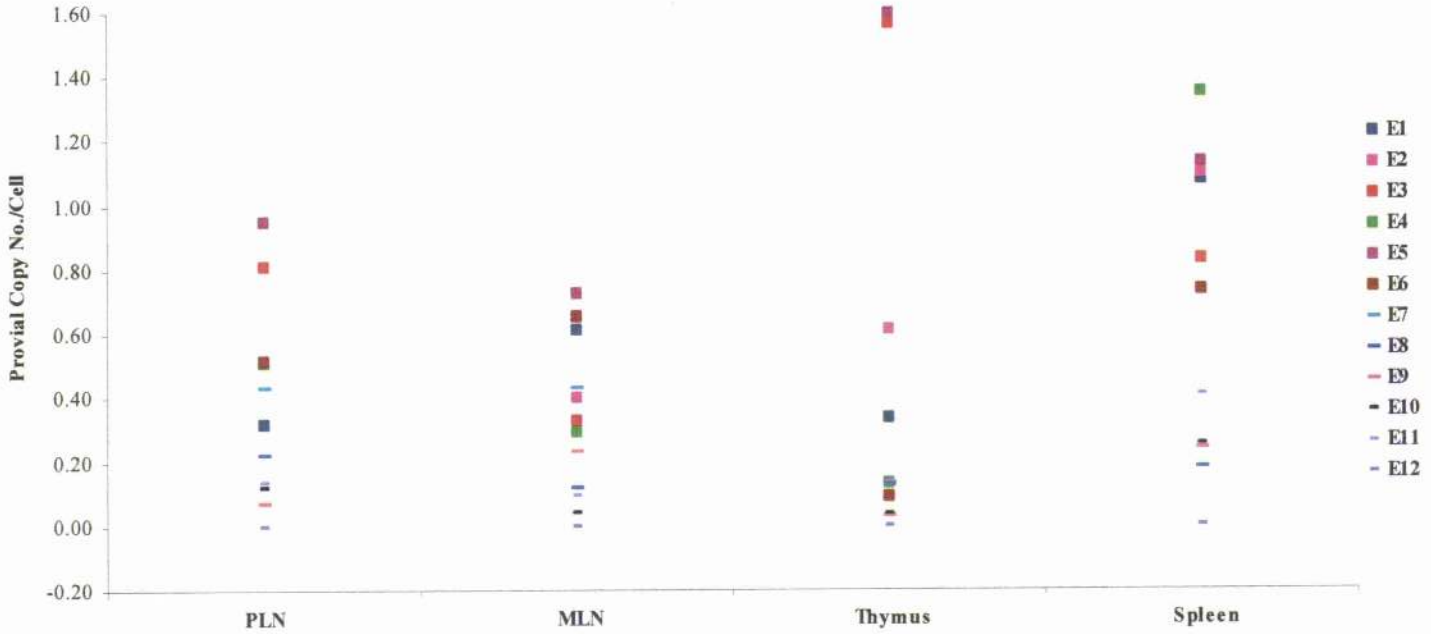
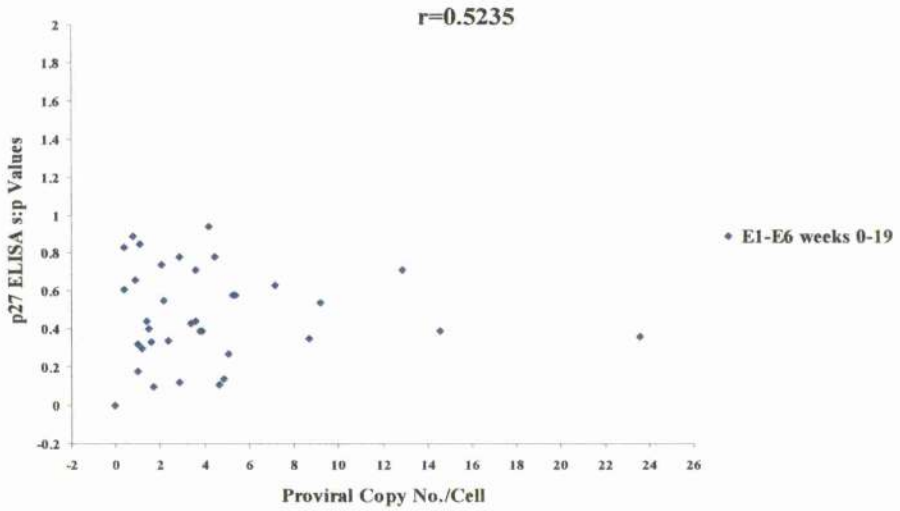

Provial Copy No./Cell
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
PLN
MLN
Thymus
Spleen
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12

Fig. 3.4 Correlation between s:p values and p27 ELISA data

Correlation of s:p values calculated from p27 ELISA data, and buffy coat proviral copy numbers measured using qRT-PCR in cats E1-E6 (A) and cats E7-E12 (B). The data was determined at regular intervals following experimental exposure of cats E1-E12 to FeLV at week 0, until the end of the study at weeks 13 (E7-E12) and 19 (E1-E6). Using the Spearman r-Test, r=0.5235 (cats E1-E6) and r=0.7666 (cats E7-E12). Both values are extremely significant (P=0.0001 and P<0.0001, respectively).

A


r=0.5235
p27 ELISA s:p Values
Proviral Copy No./Cell
E1-E6 weeks 0-19

B

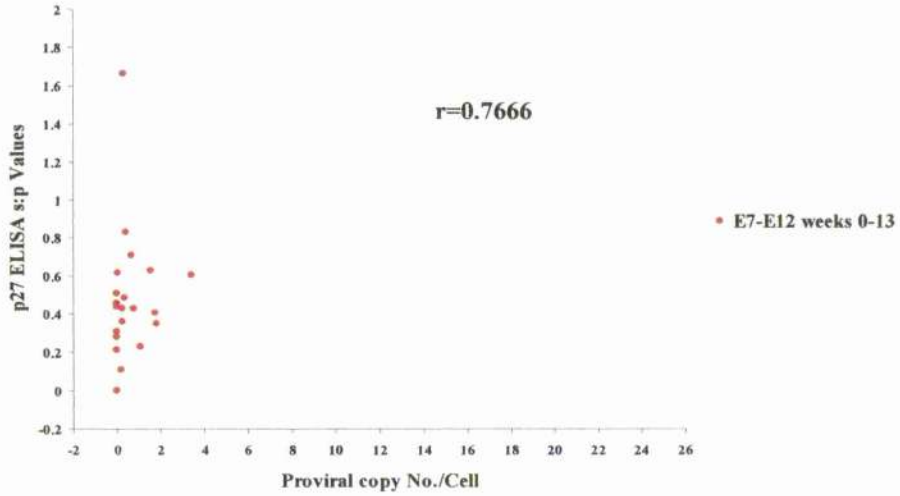
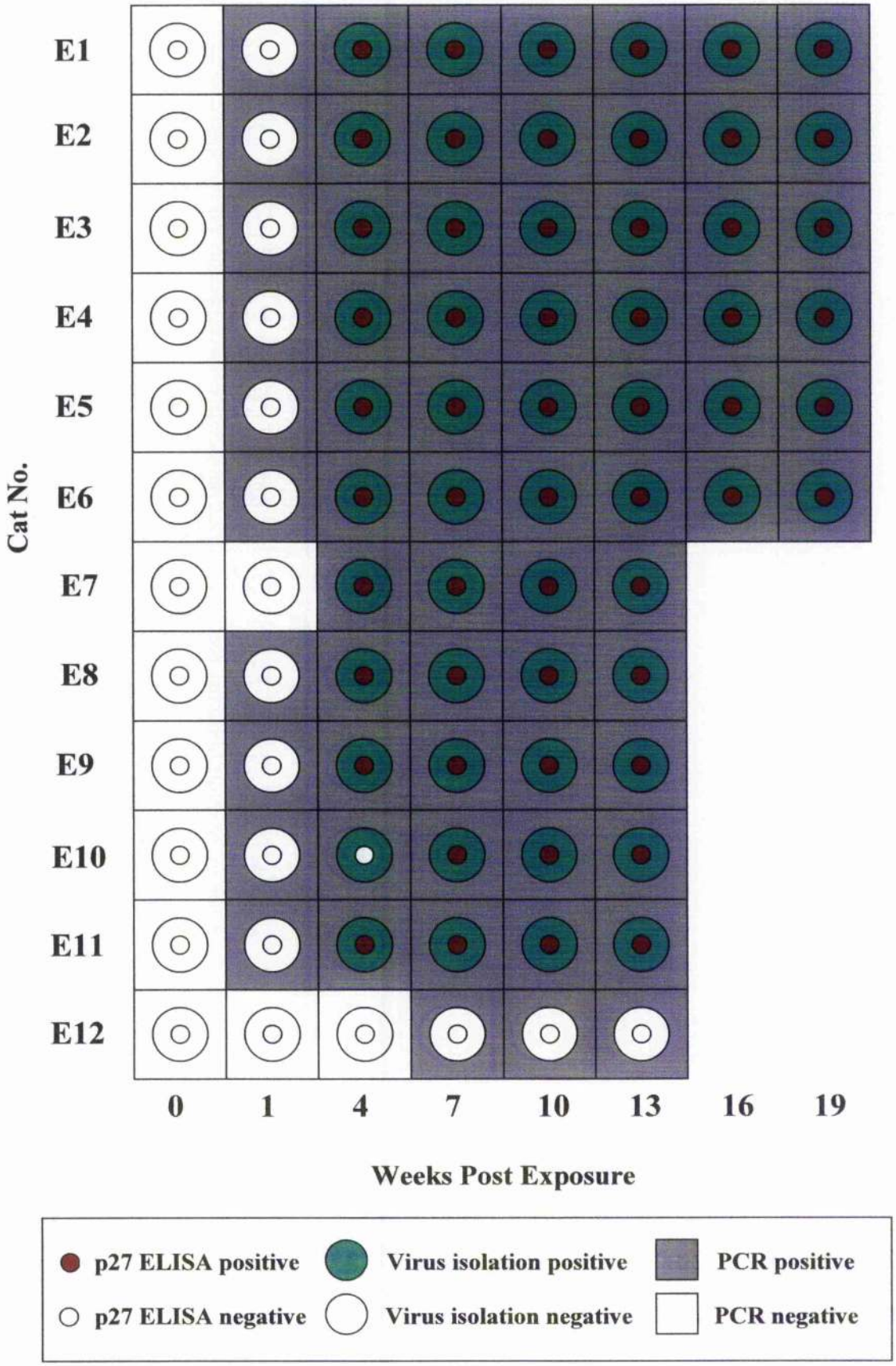

r=0.7666
p27 ELISA s:p Values
Proviral copy No./Cell
E7-E12 weeks 0-13

Table 3.1 Detection of FeLV CA protein, proviral DNA and infectious FeLV in cats E1-E12

Plot summarising detection of FeLV proviral DNA, FeLV CA protein p27 and infectious FeLV, using qRT-PCR, p27 ELISA and VI respectively, in peripheral blood samples taken at regular intervals from weeks 0-19 (E1-E6) and weeks 0-13 (E7-E12).


E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12
Cat No.
0
1
4
7
10
13
16
19
Weeks Post Exposure
p27 ELISA positive
p27 ELISA negative
Virus isolation positive
Virus isolation negative
PCR positive
PCR negative

3.4 Discussion

The aim of the FeLV immunopathogenesis study was to examine and compare FeLV-specific $CD4^+$ T cell responses in recovered, latently infected and persistently viraemic cats following exposure to FeLV. It was essential to monitor the FeLV status of exposed cats through the detection of viraemia, p27 antigenaemia and proviral burdens to define the disease outcome for each cat, and to ascertain whether the development of FeLV-specific immune responses could be correlated with the viral status of the exposed cats. To achieve these aims, cats were exposed at 16 weeks with a dose of virus previously shown to be associated with recovery in a significant proportion of animals (Hoover *et al.*, 1976; Jarrett *et al.*, 1982a; Flynn *et al.*, 2002b). However, in the present study, eleven cats became persistently viraemic and one cat retained a latent proviral infection in the bone marrow and lymphoid tissues.

Cats were dosed oronasally to mimic the natural route of exposure (Hardy *et al.*, 1973; Francis *et al.*, 1977; Hoover *et al.*, 1977a; Hoover *et al.*, 1977b; Jarrett *et al.*, 1982a; Pedersen, 1988; Hoover and Mullins, 1991; Hardy, 1993; Jarrett, 2001). Additionally, oronasal challenge avoids unsuccessful infections, where virus intended for the peritoneal cavity may instead be injected into the abdominal wall tissues. Furthermore, it was useful to compare the data collected from this study with data acquired from recent studies conducted in our laboratory, which used an identical route of exposure.

The FeLV CA protein p27 was not detected in the plasma from any cat E1-E12 at week 1; eleven of which subsequently became persistently infected. This is consistent with the findings of a previous study, where an early peak in p27 antigenaemia was absent in all persistently viraemic cats (Flynn *et al.*, 2002b). Only three persistently infected cats followed a biphasic pattern in p27 antigenaemia based on the s:p values from the p27 ELISA, peaking at weeks 4 and weeks 10-13. However, s:p values from all other persistently infected cats demonstrated single peaks in p27 antigenaemia at either week 4 or weeks 10-13.

The p27 ELISA detects FeLV CA protein p27, released free into the plasma by virally infected cells or disrupted viral particles; therefore, the presence of FeLV CA protein p27 is not necessarily indicative of an active infection. In contrast, VI from plasma samples detects the presence of infectious virus. The absence of p27 antigenaemia in the presence of viraemia in cat E10 at week 4 is a rare, discordant result. In other experimental studies, FeLV viraemia consistently occurred in the presence of p27 antigenaemia (Jarrett and Ganière, 1996; Flynn *et al.*, 2002b). However, one study describing the experimental infection of eight-week old kittens reported that although VI was successful on day 10 in two kittens, no kitten was positive in the p27 ELISA until day 14 (Jarrett *et al.*, 1982a), implying that viraemia can occur in the absence of a concurrent antigenaemia. More recently, the identification of false-negative p27 ELISA results in cats that presented with an FeLV-associated disease, and a positive IFA test, was reported (Kerr and Smith, 1995; Lutz *et al.*, 1982; Madewell and Jarrett, 1983). Nonetheless, it is considered unusual to detect viraemia in the absence of antigenaemia, as dissemination of soluble viral polypeptides by FeLV-infected cells occurs widely *in vitro* (Neil *et al.*, 1980b) and *in vivo* (Jarrett, 1999). Furthermore, viral antigens have been detected in plasma from FeLV infected cats for a short period before, and after, virus is detected in cells (Lutz *et al.*, 1980a; Pedersen, 1988). However, the presence of viraemia in cat E10 without concurrent p27 antigenaemia may not actually be a true result. The s:p values show that cat E4 (0.1) has a lower value than cat E10 (0.11) at week 4, and yet cat E4 was considered positive, and E10 negative, suggesting that the discordant result may have arisen from the interpretation of the $O.D._{405}$ ELISA figures. In this study, a cat was considered positive when the sample value is equal to, or greater than, 150% of the negative control value. In this instance, the plasma sample from cat E10 had an $O.D._{405}$ value of 0.26, whereas the negative control had an $O.D._{405}$ value of 0.18, which meant that cats with an $O.D._{405}$ value of 0.27 or greater were considered positive. Using those criteria, cat E10 was clearly only marginally negative. Others, using a similar p27 ELISA assay, have defined cats as positive when the s:p value is equal to, or greater than, 0.1 (Miyazawa and Jarrett, 1997). If the same criteria had been applied in this study, cat E10 would have been considered positive.

Quantitative RT-PCR is a sensitive and specific method of detecting and quantifying FeLV proviral DNA in blood and tissues (Hofmann-Lehmann *et al.*, 2001). The primers and probe used in this study were designed to detect exogenous FeLV-A sequences so that endogenous FeLV-related sequences were not amplified. Proviral DNA was detected in all cats one week following exposure to FeLV, except cats E7 and E12. An absence of proviral DNA in cats E7 and E12 until week 4 and week 7 respectively, suggests that the immune response may have been actively restricting FeLV integration and replication; possible immune mechanisms include IFN-γ and the type I IFNs. Interferon-γ acts directly to antagonise viral growth and replication through the induction of three genes encoding PKR, 2-5A synthetase and dsRAD (Section 1.3.1.2). Additionally, type I IFNs secreted by IFN-producing cells (IPCs) and virally-infected cells (Rissoan *et al.*, 1999; Siegal *et al.*, 1999) have an antiviral role (Levy, 2001); the loss of IPCs and IFN-α production has been associated with high HIV RNA levels and AIDS (Soumelis *et al.*, 2002). The type I IFNs also produce enzymes that collectively interfere with viral replication.

The pattern of proviral DNA loads was similar to those described in other studies, where persistently infected cats demonstrated two peaks in proviral DNA load, at weeks 4-7 and again at weeks 10-16 (Hofmann-Lehmann *et al.*, 2001; Flynn *et al.*, 2002b). These peaks in proviral DNA loads were temporally associated with the peaks in s:p values determined using the p27 ELISA. However, no statistically significant correlation between these two results was found in either group of cats at either time points (weeks 4-7 or weeks 10-16).

Although proviral DNA was detected in latently infected cat E12 from weeks 7-13, p27 antigenaemia was not detected at any sample date over the study. A group of PCR-positive, p27-negative recovered cats was identified in FeLV experimentally infected cats, that became PCR positive one week following FeLV exposure in the absence of p27 antigenaemia, and remained PCR-positive, p27-negative (Hofmann-Lehmann *et al.*, 2001). The mean proviral copy numbers for cat E12 (0.0093 ± 0.0099 copies per cell) was less than those recorded for the p27 ELISA-negative, PCR-positive cats (0.08 ± 0.07 copies per cell) (Hofmann-Lehmann *et al.*, 2001). However, neither the infection route nor the infection dose was comparable to those

used in cats E7-E12. Persistently viraemic cats E1-E6 that received an equivalent exposure dose of FeLV to cats in Hofmann-Lehmann's study, had higher mean levels of proviral DNA (4.5 ± 1.2 copies per cell), than were detected in persistently infected cats from Hofmann-Lehmann's study (1.4 ± 2.7 copies per cell). Much higher mean proviral copy numbers were detected in naturally infected viraemic cats in the same study (10 ± 47 copies per cell), although the duration of infection, age at time of exposure and challenge dose in these cats was unknown.

Quantitative RT-PCR was the most sensitive method used to detect FeLV infection in this study; proviral DNA was detected in all cats, except cats E7 and E12, one week following FeLV exposure, whereas neither the p27 ELISA nor VI detected FeLV CA protein p27 or infectious virus in those cats until at least week 4. Furthermore, although all negative qRT-PCR samples were negative by ELISA (n=28) and VI (n=28), and all positive ELISA (n=55) and VI samples (n=56) were positive by qRT-PCR; some positive qRT-PCR samples were negative by ELISA (n=13) and by VI (n=12). These results highlight incongruities between results obtained using the p27 ELISA, VI and the qRT-PCR assays in this study. Each assay detects different aspects of the FeLV life cycle: p27 antigenaemia, FeLV viraemia and FeLV integration respectively; the presence of either proviral DNA or the FeLV CA protein p27 does not signify the presence of an active infection. Discrepancies between qRT-PCR and the other assays used in this study also reflect the exquisite sensitivity of the qRT-PCR assay, which detected between 0.36 and 3.6 copies of the standard DNA template (Section 3.3.1.3) (Hofmann-Lehmann *et al.*, 2001). Despite these differences, an extremely significant correlation was identified between the p27 ELISA and PCR results obtained over the entire study in both groups of cats, E1-E6 and E7-E12.

In another study, no samples negative by both p27 ELISA and VI were positive by nested PCR (Miyazawa and Jarrett, 1997). This was clearly not the case in this study; all cats E1-E12 in the early stages of infection were PCR-positive and both VI-negative and p27-ELISA negative, as was cat E12 throughout the course of the study. Our findings concur with those reported by Hoffman-Lehmann *et al.* (2001), where 10% of naturally infected cats were found to be positive using PCR and negative using ELISA. The authors suggest that these cats either had recovered from

FeLV infection, or were in the early stages of infection.

Although neither p27 antigenaemia nor viraemia were detected in the peripheral blood from E12 at any sample date throughout the study, proviral DNA detected in buffy coat and lymphoid tissue samples suggests that E12 experienced a transient cell-associated viraemia following exposure to FeLV. Furthermore, it was possible to isolate infectious FeLV from post mortem bone marrow samples following a short *in vitro* culture on QN10S cells. These data indicate that E12 failed to wholly clear FeLV following exposure, allowing FeLV to migrate to lymphatic tissues and bone marrow, and establish a latent infection as integrated provirus. However, cell-free virus was not detected in the blood of cat E12 at any time over the study, suggesting that the immune response was sufficient to prevent widespread bone marrow replication and active infection. Virus neutralising antibodies were not detected in the plasma from persistently viraemic cats E1-E11 at any time during the study but were detected in the plasma from latently infected cat E12 at week 13. Although VNA in cat E12 appeared later than expected for recovered cats, and the titre was lower (Lutz *et al.*, 1980b; Hofmann-Lehmann *et al.*, 2001; Flynn *et al.*, 2002b), these data may be consistent with reports where transiently viraemic cats produced levels of antibody that overlapped with those produced by aviraemic and persistently viraemic cats (Lutz *et al.*, 1980b). The early delay in FeLV integration, and the persistent absence of viraemia and antigenaemia throughout the study, suggest that antiviral immune responses were actively suppressing FeLV activity in cat E12. Recovery from FeLV infection began before the appearance of plasma VNA, indicating that these phenomena were mediated by mechanisms other than VNA. Early FeLV Gag/Pro-specific $CD8^+$ CTL effector responses are known to be associated with recovery from experimental FeLV infection (Flynn *et al.*, 2002b).

FeLV isolate, dose, host susceptibility, age at time of exposure and route of exposure all interact to influence the outcome following exposure to FeLV (Hoover *et al.*, 1976). Specific pathogen free cats E1-E6 were exposed to FeLV at an age, dose and route normally expected to result in approximately fifty *per cent* recovery, fifty *per cent* persistent viraemia (Hoover *et al.*, 1976; Jarrett *et al.*, 1982a; Flynn *et al.*, 2002b). Nonetheless, all six exposed cats became persistently viraemic. With FeLV isolate and dose, age and route of exposure consistent with previous studies; host

susceptibility remains the unknown variable in this group. Cats E7-E12 were exposed to a lower dose of FeLV-A under the same conditions as cats E1-E6, and one cat, E12, cleared infectious virus from the peripheral blood. Together, these data suggest a greater inherent susceptibility of all cats to the molecular clone of FeLV-A/Glagow-1, which was somewhat improved by reducing FeLV exposure dose by a factor of one log.

3.5 Summary and Conclusions

Out of twelve cats exposed to FeLV, eleven cats became persistently infected, while cat E12, having cleared infectious virus from the peripheral blood, retained a latent proviral DNA infection in the bone marrow and lymphoid tissues.

Infectious virus was isolated from all persistently infected cats from week 4 until the end of the study. Furthermore, p27 antigenaemia was detected in these cats from weeks 4-7, until the termination of the study. Proviral DNA was detected in the peripheral blood from ten cats as early as week 1 following oronasal exposure to FeLV, and by week 7 from all cats, and continued to be detected until the termination of the study. In addition, proviral DNA was detected in the lymphoid tissues harvested from all cats post mortem.

Each of the assays used, p27 ELISA, VI and qRT-PCR, all detect different aspects of the FeLV lifecycle, which accounts for some discrepancies between results; only the VI technique detects an active infection.

CHAPTER 4

LONGITUDINAL IMMUNOPATHOGENESIS STUDY

II IMMUNOLOGY

4.1 Introduction

The previous chapter described the virological parameters measured in the peripheral blood and lymphoid tissues following the oronasal exposure of cats E1-E12 to FeLV. In this chapter, FeLV-specific cell-mediated immune responses elicited in the peripheral blood and lymphoid tissues are described, with particular emphasis on virus-specific $CD4^+$ T cell responses.

The immune mechanisms that determine the fate of FeLV-exposed cats are not fully understood. Early studies showed that the presence of VNA correlated with recovery (Hardy *et al.*, 1976; Hoover *et al.*, 1976; Russell and Jarrett, 1978a; Lutz *et al.*, 1980b), and demonstrated that VNA passively protected kittens against FeLV challenge (Jarrett *et al.*, 1977). In field situations, this passive transfer of VNA from an immune queen to suckling kittens may be critical in the protection of kittens at an age of greatest immuno-incompetence (Hoover *et al.*, 1976; Jarrett *et al.*, 1977).

However, more recent experimental studies have shown that FeLV is cleared from the blood prior to, or concurrent with, the appearance of VNA (Flynn *et al.*, 2000a; Flynn *et al.*, 2002b). Early FeLV Gag/Pro-specific $CD8^+$ CTL responses are known to be associated with recovery from experimental FeLV infection (Flynn *et al.*, 2002b), and are implicated in protective immune responses following inoculation of cats with an experimental FeLV DNA vaccine (Flynn *et al.*, 2000a; Hanlon *et al.*, 2001).

These studies illustrate that both VNA and virus-specific CTL are associated with recovery and protection following exposure to FeLV. We know that virus-specific $CD4^+$ T cells are important in the initiation and regulation of specific cellular and humoral immune responses (Battegay *et al.*, 1994; Matloubian *et al.*, 1994; Rosenberg *et al.*, 1997); therefore, the capacity of FeLV-specific $CD4^+$ T cells to activate and maintain virus-specific $CD8^+$ T cells and virus-specific B cells, may be a critical component in the immune mechanisms contributing to the control of FeLV viraemia.

Following the oronasal exposure of cats E1-E12 to FeLV, the virus-specific cell-mediated response elicited in the peripheral blood and lymphoid tissues was determined using two different techniques, the LPA, and ICS analysed using flow cytometry. Virus-specific responses in the peripheral blood were evaluated one week following exposure and at three-week intervals thereafter, while virus-specific responses in the lymphoid tissues were investigated post mortem.

The specificity of the immune response was determined using recombinant p27 and p45 proteins, and inactivated FeLV particles. The genes encoding FeLV CA protein p27, and SU protein gp70, were expressed by *Escherischia coli* (*E. coli*) as fusion proteins; the recombinant p27 and p45 proteins were then purified using affinity chromatography under denaturing conditions. Feline leukaemia virus was purified from F422 cell culture supernatants, and heat-inactivated. The purity of the recombinant p27 and p45 proteins, and inactivated FeLV particles, was assessed using SDS-PAGE and Western blots.

The LPA was used to assess the ability of mononuclear cells in the peripheral blood and lymphoid tissues to proliferate in response to mitogenic and virus-specific stimulation *ex vivo*. Mononuclear cells were stimulated for three days with Con A, and either recombinant p27 or p45 proteins, or inactivated FeLV particles. Proliferative responses were measured using a liquid scintillation spectrophotometer to calculate the uptake of ^{3}H-thymidine (methyl-^{3}H-thymidine, Amersham Biosciences, U.K.) by cellular DNA. Concurrent phenotypic analysis was conducted using flow cytometry to enumerate the proportion of $CD4^{+}$ and $CD8^{+}$ T cells, and either $CD21^{+}$ B cells or $CD45R^{+}$ white blood cells (WBC), in significant virus-specific proliferative responses in the peripheral blood.

Intracellular cytokine staining techniques evaluated the ability of mononuclear cells in the peripheral blood and lymphoid tissues to upregulate fIFN-γ in response to virus-specific stimulation *ex vivo*. Mononuclear cells were stimulated with either recombinant p27 or p45 proteins, or inactivated FeLV particles, for four hours; intracellular fIFN-γ expressed in recognition of cognate antigen was detected using an anti-fIFN-γ mAb, generated in this thesis, and analysed using flow cytometry.

Concurrent phenotypic analysis was conducted to enumerate the proportion of $CD4^+$ and $CD8^+$ T cells that expressed fIFN-γ.

4.2 Materials and Methods

4.2.1 Preparation of Recombinant p27 and p45 Proteins

4.2.1.1 Expression of FeLV Genes

The genes encoding FeLV CA protein p27, and SU protein gp70, were cloned by Drs Norman Spibey and Julie McDonald within the Department of Veterinary Pathology at the University of Glasgow, then inserted into gene fusion vectors containing six tandem histidine (6 x his) residues in the N-terminal peptide (Invitrogen Ltd., U.K.), and expressed in *E. coli* bacterial cells. The FeLV SU protein gp70 is glycosylated *in vivo*, with a molecular weight (mw) of 70 kDa; the recombinant protein expressed by *E. coli* is non-glycosylated, and has a mw of 45 kDa (p45). The polyhistidine tag has a strong affinity for the divalent nickel chloride ($NiCl_2$) cations immobilised on 'ProBond™' resin (Invitrogen Ltd., U.K.) used to purify the recombinant p27 and p45 proteins. Denaturing buffers containing 6 M urea were used in all stages of the purification process. The purification method used was based on the manufacturer's protocol (Invitrogen Ltd., U.K.) with some minor amendments: additional astringent washes were required to enhance the purity of the recombinant p45 protein.

4.2.1.2 Preparation of Denatured E. coli Lysate

The bacterial expression vectors were prepared in a similar way to the vectors described in Section 2.2.1.2. Following centrifugation of the induced cultures, the cell pellets were resuspended in 10 ml guanidinium lysis buffer[26], preheated to 37°C. The cells were subsequently agitated for ten minutes at RT to ensure thorough bacterial cell lysis, and then sonicated on ice using three five-second pulses. To remove the insoluble debris, the bacterial lysate was transferred to a JA-21 rotor and

[26] Guanidinium lysis buffer, pH 7.8: 6 M (w/v) guanidine hydrochloride; 20 mM (w/v) sodium phosphate; 500 mM (w/v) sodium chloride

centrifuged at 5000rpm for fifteen minutes; the supernatant containing the sheared lysate was stored at -20°C until further use.

4.2.1.3 Purification of Recombinant Proteins using Affinity Chromatography

Four ml 50% 'ProBond™' resin was added to an empty polypropylene column (Gibco, Invitrogen Ltd., U.K.). The column was placed in a centrifuge tube (Falcon 2070, Becton Dickinson, France) and centrifuged in a swinging bucket rotor within a bench-top centrifuge for two minutes at 1000rpm to sediment the resin. The ethanol preservative was decanted from the column and the resin resuspended by gentle agitation. The resin was washed twice with 7 ml sterile water using centrifugation as described above, followed by two washes with denaturing binding buffer[27] to equilibrate the resin. Once equilibrated, 5 ml denatured bacterial lysate was added to the resin. The column was then agitated at RT for ten minutes to allow the his-tagged proteins to bind the nickel cations. The resin was sedimented as described above and a further 5 ml denatured bacterial lysate applied to the column.

Non-specific bacterial proteins were stripped from the column using progressively astringent washes. For each wash, 4 ml denaturing wash buffer[28] was added to the resuspended resin, which was agitated at RT for two minutes before sedimentation by centrifugation at 800rpm for two minutes. Initially, the column was washed with denaturing binding buffer, followed by denaturing wash buffer pH 6.0, and finally denaturing wash buffer pH 5.3. Each wash was repeated once. For purification of the recombinant p45 protein, two additional washes using denaturing wash buffer pH 4.5 were conducted in order to remove contaminant bacterial cell proteins. The proteins were eluted from the column by applying 5 ml elution buffer[29] to the top of the column, and allowing the eluted proteins to run through the column where they were collected.

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[27] Denaturing binding buffer, pH 7.8: 8 M (w/v) urea; 20 mM (w/v) sodium phosphate; 500 mM (w/v) sodium chloride

[28] Denaturing wash buffer, pH 4.5-6.0: 8 M (w/v) urea; 20 mM (w/v) sodium phosphate; 500 mM (w/v) sodium chloride

[29] Denaturing elution buffer, pH 4.0: 8 M (w/v) urea; 20 mM (w/v) sodium phosphate; 500 mM (w/v) sodium chloride

To remove the urea used in the denaturing buffers, the proteins were placed into dialysis tubing and dialysed overnight at 4°C into 0.01 M tris buffer, pH 8.0. Removal of the urea rendered the proteins insoluble; therefore, 2-5 drops 5 M sodium hydroxide (NaOH, Fisher Scientific U.K. Ltd, Loughborough, Leicestershire, U.K.) were added using a 22 guage needle to resolubilise the proteins. The purified recombinant p27 and p45 proteins were quantified using a spectrophotometer; a proportion was electrophoresed on a 12% polyacrylamide gel (Section 2.2.1.4), then blotted onto nitrocellulose (Section 2.2.2.6). The remainder was aliquoted and stored at -20°C.

The basic protocol used for Western blots was similar to that described in Section 2.2.2.6. The blotted recombinant p27 protein and FeLV particles was probed for two hours with a sheep anti-p27 pAb (Dr A. Pacitti, University of Glasgow), diluted in 20% NRS in TBS-T, followed by a one-hour incubation using a biotinylated rabbit anti-sheep Ig pAb, diluted 1:2000 in 20% NRS in TBS-T. A further one-hour incubation with strepatavidin-peroxidase, diluted 1:500 in TBS-T, preceded the development of colour using DAB. Blots were probed with either 20% NRS or 20% NGS in TBS-T, diluted as the primary antibody, as negative controls. In addition, recombinant p27 and p45 proteins, and FeLV particles, prior to heat inactivation, were probed with serum from FeLV recovered cats, diluted 1:4 in 20% NGS, followed by a one-hour incubation with biotinylated protein A (ICN Biomedicals, Hampshire, U.K.), diluted 1:500 in TBS-T. A second one-hour incubation with peroxidase-conjugated streptavidin preceded the development of colour using DAB. Blots were probed with either 20% NGS, or 20% NRS, diluted as the primary antibody, as negative controls.

4.2.2 Preparation of Inactivated FeLV Particles

4.2.2.1 Culture of F422 Cells

F422 cells are derived from a naturally occurring feline lymphosarcoma (Rickard *et al.*, 1969), and express FeLV proteins. F422 cells were cultured in a compartmentalised flask, 'CL-1000', at 37°C with 5% CO_2. Two x 10^7 cells were seeded into the 15 ml cell compartment, and nourished through a semi-permeable

membrane by 1 litre of complete RPMI 1640 medium. At three to four day intervals, the cells were removed from the cell compartment and pelleted by centrifugation in a bench-top centrifuge at 1000rpm for ten minutes. The supernatant was decanted, and stored at 4°C in 0.1% sodium azide.

4.2.2.2 Sucrose Gradient Purification of FeLV

In 1967, Kawakami *et al.* isolated mature virus particles from the plasma of cats suffering from feline leukaemia, using linear sucrose-density gradients. A modified method of this original work was used to purify FeLV from plasma and tissue culture supernatants. A gradient comprising 5 ml 20% sucrose (Fisher Scientific, U.K.) layered over 5 ml 50% sucrose, was made in SW28 inner tubes (Beckman-Coulter, U.S.). The F422 cell culture supernatants were gently applied over the sucrose layers to fill the tubes, which were balanced using a Sartorius balance (European Instruments, Oxford, U.K.). The tubes were inserted into a SW28 rotor (Beckman-Coulter, U.S.), and centrifuged at 4°C in an ultracentrifuge (Beckman L8-70M, Beckman-Coulter, U.S.) for two hours at 25,000rpm. Purified virus was removed from the interface using a 23 guage needle and syringe and subsequently layered over a second gradient comprising 1.5 ml 50% sucrose, 1.5 ml 30% sucrose and 1.5 ml 20% sucrose, in chilled SW41 inner tubes (Beckman-Coulter, U.S.). The tubes were balanced and spun using a SW41 rotor (Beckman-Coulter, U.S.) at 4°C for one hour at 40,000rpm in an ultracentrifuge. The purified virus was removed from the interface between 50% and 30% sucrose layers as before, and dialysed overnight at 4°C in 0.01 M tris, pH 8.0.

4.2.2.3 Inactivation of Purified FeLV

Purified FeLV virus was inactivated by boiling for 2.5 minutes in a boiling bath. The inactivated virus was passed through a 0.22 μm filter ('Minisart', Sartorius Ltd., U.K.) and quantified using a spectrophotometer. A proportion was electrophoresed on a 12% polyacrylamide gel (Section 2.2.1.4), and then blotted onto nitrocellulose (Section 2.2.2.6). The remainder was aliquoted and stored at -20°C. Inactivated FeLV particles did not contain any infectious virus as determined using VI techniques on QN10S cells (Section 3.2.3.1).

4.2.3 FeLV-Specific Cell-Mediated Immune Responses

4.2.3.1 Lymphocyte Proliferation Assay

The ability of mononuclear cells in the peripheral blood and lymphoid tissues to proliferate in response to mitogenic and virus-specific stimulation was determined in the LPA.

Serial dilutions of recombinant p27 and p45 proteins, and inactivated FeLV particles, were prepared in complete RPMI 1640 medium. One hundred µl of each dilution was added in triplicate to the wells of a 96-well U-bottom tissue-culture plate (Falcon 3077, Becton Dickinson, France). To detect background proliferation, control wells were coated in 100 µl complete RPMI 1640 medium, devoid of viral antigen and mitogen. The plate was incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2 to ensure that the medium was at the correct pH and temperature before the addition of cells. Mononuclear cells were prepared from the peripheral blood by centrifugation of whole blood over Ficoll-Paque. Additionally, mononuclear cells were prepared from post mortem PLN, MLN and spleen tissues as described in Section 3.2.4.1, and layered over Ficoll-Paque. Peripheral blood and tissue mononuclear cells were resuspended in complete RPMI 1640 medium (2×10^6 cells/ml); 100 µl of which was added to each well, to give final viral protein concentrations of 25 $\mu g/10^6$ cells; 12.5 $\mu g/10^6$ cells; 6.25 $\mu g/10^6$ cells and 3.125 $\mu g/10^6$ cells. The mitogen Con A was added in triplicate at 5 $\mu g/10^6$ cells, 0.5 $\mu g/10^6$ cells and 0.1 $\mu g/10^6$ cells, and used as a positive control. The plates were sealed in plastic bags and incubated for three days, as before. On the third day of culture, methyl-^{3}H-thymidine was added to each well (0.5 µCi/well) for the final five hours of incubation. The cells were subsequently harvested ('Filtermate 196 Cell Harvester', Packard, Netherlands) onto filter plates ('UniFilter-96 GF/C', Packard Bioscience BV, Groningen, Netherlands). The filter plates were dried at 37°C; 25 µl scintillation fluid ('MicroScint', Packard, Netherlands) was then added to each well, and the plates were sealed. Radioactivity was read on a liquid scintillation counter as counts per minute (cpm) ('TopCount Microplate Scintillation Counter', Packard, Netherlands). The stimulation index (S.I.) for virus-specific and mitogenic

proliferative responses was calculated as follows:

$$\frac{\text{Mean test wells (cpm)}}{\text{Mean background wells (cpm)}}$$

4.2.3.2 Immunophenotyping of Proliferating Cells

Additional 96-well plates were prepared to determine the phenotype of the proliferating cells. This was achieved using flow cytometry to detect surface CD4 and CD8, and either CD45R or CD21, molecules, stained using anti-feline CD4-PE; anti-feline CD8-PE (10 µl/10^6 cells, Serotec, U.K.), anti-rat CD45R-PE (10 µl/10^6 cells, Serotec, U.K.) and anti-canine CD21-PE (2.5 µl/10^6 cells, Dr. P.F. Moore, University of Davis, California) mAbs.

The CD45R molecule comprises high and low molecular weight isoforms of the leukocyte common antigen CD45: $CD45R^{hi}$ and $CD45R^{lo}$. The anti-rat CD45R mAb reacts predominately with mature and precursor B cells in the rat, in addition to marginal zone B cells and a small subset of T cells. In cats, this mAb is routinely used to enumerate B cells in fresh peripheral blood samples (Flynn, J.N., personal communication). However, after three days of culture in the LPA, it became apparent that other cell types upregulated expression on the CD45R molecule; therefore, this mAb was not a suitable marker for the enumeration of B cells in the LPA.

Single concentrations of either recombinant p27 or p45 proteins, or inactivated FeLV particles, were used as virus-specific stimulation (25 µg/10^6 cells). The mitogen Con A (5 µg/10^6 cells) was present as a positive control; wells containing neither viral antigen nor mitogen were included as negative controls. After three days, the cells were harvested into polypropylene tubes (2 x 10^5 cells/tube), pelleted and washed in PBS by centrifugation for five minutes at 1000rpm using a bench-top centrifuge. The cells were then incubated with the mAbs described above for thirty minutes at RT. The cells were subsequently washed in PBS as before, fixed in 2% formaldehyde and resuspended in fluorescence buffer as previously described

(Section 2.2.2.8).

4.2.3.3 Intracellular Cytokine Staining Analysed Using Flow Cytometry

The production of intracellular fIFN-γ by mononuclear cells in the peripheral blood and lymphoid tissues in response to virus-specific stimulation was determined following a short four-hour culture *ex vivo*. The protocol followed was similar to that described in Section 2.2.2.8, except that virus-specific responses were now also measured. Tissue culture plates were pre-incubated with inactivated FeLV particles, as well as recombinant p27 and p45 proteins; mononuclear cells were then added to give a final viral protein concentration of 25 $\mu g/10^6$ cells.

For each sample, 10,000 events were recorded. The small lymphocyte and the lymphoblast populations were gated based on cell size and granularity, and the percentage of fIFN-γ-expressing $CD4^+$ T cells within the lymphoblast population was enumerated. The gates for fIFN-γ expression were determined by the reactivity of the FITC-conjugated isotype matched control mAb.

4.2.4 Statistics

Statistical analysis was conducted using 'GraphPad InStat'. Non-parametric tests were used throughout. The Mann-Whitney test was applied to compare results from two groups, using a two-tail *P* value. The Spearman r-Test was performed to correlate FeLV proviral DNA loads and cell-mediated immune responses in peripheral blood and lymphoid tissues, following virus-specific and mitogenic stimulation.

4.3 Results

4.3.1 Production of Recombinant p27 and p45 Proteins, and Inactivated FeLV Particles

The genes encoding FeLV CA protein p27, and SU protein p45, were expressed by *E. coli* as fusion proteins containing polyhistidine N-terminal tags. The recombinant

p27 and p45 proteins were released following guanidinium bacterial cell lysis and sonication, then purified over $NiCl_2$ resin under denaturing conditions. Feline leukaemia virus particles were purified from F422 cell culture supernatants using sucrose density gradients, and heat-inactivated. The yield of recombinant p27 and p45 proteins following purification and dialysis was approximately 2.5-5 mg and 3-5 mg respectively, from 400 ml bacterial cultures. Aliquots of each recombinant protein, and inactivated FeLV particles, were electrophoresed on a 12% polyacrylamide gel using SDS-PAGE, and probed in Western blots, to ensure purity of the reagents. Recombinant p27 and p45 proteins and inactivated FeLV particles were stored in aliquots at -70°C. The same batch of recombinant p27 protein was used throughout the study to ensure consistent results. Two batches of recombinant p45 protein were produced, using the original glycerol preparation, and used for cats E1-E6 or cats E7-E12, respectively. Each batch of recombinant p45 protein was consistently pure, according to Coomasssie-stained SDS-PAGE, and reactivity in Western blots.

Prior to heat-inactivation, purified FeLV displayed three prominent bands in the Coomassie Brilliant Blue-stained gel, approximately 70 kDa, 27 kDa and 15 kDa in size, which corresponded to the FeLV SU protein gp70, CA protein p27 and TM protein p15E, respectively (Fig. 4.1.I). However, these proteins became disrupted following heat-inactivation, and an additional two bands, between 37 kDa and 75 kDa in size, were evident on the Coomassie Brilliant Blue-stained gel (Fig. 4.1.I). Feline leukaemia virus CA protein p27 has a molecular weight of approximately 27 kDa. A single dense band of this approximate size was evident on the Coomassie Brilliant Blue-stained gel, confirming the successful purification of the recombinant p27 protein (Fig. 4.1.I). The FeLV SU protein gp70 expressed by *E. coli* is non-glycosylated and only 45 kDa in size. A single band of this size was evident on the Coomassie Brilliant Blue-stained gel (Fig. 4.1.IV), which corresponded to the recombinant p45 protein.

Western blots were used to verify that the purified recombinant p27 and p45 proteins were the reagents of interest, and that each contained antigenic determinants that were immunologically relevant in FeLV-exposed cats; antibodies in sera from FeLV recovered cats recognised and bound to proteins of approximately 27 kDa (Fig.

4.1.II) and 45 kDa (Fig. 4.1.V) in size, which corresponded to the recombinant p27 and p45 proteins, respectively. Furthermore, the sheep anti-p27 pAb recognised antigenic determinants shared by the recombinant p27 protein and the FeLV CA protein p27 (Figs. 4.1.II and 4.1.III).

4.3.2 FeLV-Specific Cell-Mediated Immune Responses

Following oronasal exposure of cats E1-E12 to FeLV, virus-specific cell-mediated immune responses elicited in the blood and lymphoid tissues were compared using two different techniques, the LPA, and ICS analysed using flow cytometry. These assays were conducted using mononuclear cells prepared from peripheral blood sampled throughout the study, and from lymphoid tissues harvested post mortem. The specificity of the immune response was determined by measuring the ability of these mononuclear cells to proliferate, and to upregulate fIFN-γ, in response to stimulation with recombinant p27 or p45 proteins, or inactivated FeLV particles (Section 4.2.3). Mononuclear cells in the peripheral blood and lymphoid tissues were also assessed for their ability to respond to mitogenic stimulation in the LPA. Concurrent phenotypic analysis was conducted using flow cytometry to identify the virus-specific cells in each assay. Statistical analysis of the results was performed using the methods described in Section 4.2.4.

4.3.2.1 Lymphocyte Proliferation Assay

The virus-specific and mitogenic proliferative responses of mononuclear cells in the peripheral blood and lymphoid tissues are shown in Figs. 4.2-4.7. The net counts (cpm) recorded in the unstimulated wells are included in the figure legends. These background counts were used to calculate the S.I. Virus-specific proliferation was considered significant when the S.I. was equal to, or greater than, 2. Prior to FeLV exposure, no significant virus-specific proliferation was observed in the peripheral blood from any cat (Figs. 4.2-4.3). Unless otherwise stated, S.I. or statistical analyses are recorded as (cats that received an exposure dose of 5×10^5 ffu FeLV-A; cats that received an exposure dose of 5×10^4 ffu FeLV-A, respectively).

4.3.2.1.1 Proliferative Responses in Peripheral Blood

(i) Proliferative Responses to Inactivated FeLV Particles

Over the course of the study, S.I. equal to, or greater than, 2 were not detected in any cat in response to stimulation with inactivated FeLV particles (Figs. 4.2.A and 4.3.A). Overall, mean proliferative responses to inactivated FeLV particles were similar in cats that were exposed to a higher dose of infectious FeLV by a factor of one log, compared to cats that received a lower exposure dose (S.I. 1.05 ± 0.10; S.I. 1.04 ± 0.07, respectively).

(ii) p27-Specific Proliferative Responses

Two distinct patterns of p27-specific proliferative responses were identified in the peripheral blood over the study, which appeared to be influenced by the FeLV exposure dose (Figs. 4.2.B and 4.3.B). Thus, cats that were exposed to a higher dose of FeLV-A by a factor of one log, displayed peaks in mean p27-specific proliferative responses at weeks 4 and 10 (S.I. 2.09 ± 1.11; S.I. 2.6 ± 0.9, respectively); whereas cats that received a lower exposure dose peaked in mean p27-specific proliferative responses at weeks 7 and 13 (S.I. 2.2 ± 1.2; S.I. 1.76 ± 0.32, respectively). Statistical analysis revealed that the peaks in proliferative responses at weeks 7, 10 and 13 were significant (Mann-Whitney Statistic, P=0.0260; P=0.0022; P=0.0411, respectively), compared to week 0. Although the temporal shift in peaks of p27-specific proliferative responses appeared to be related to the exposure dose, mean p27-specific proliferative responses recorded over the study were similar in both groups of cats (S.I. 1.56 ± 0.46; S.I. 1.48 ± 0.36, respectively).

Stimulation indices equal to, or greater than, 2, were detected in the peripheral blood from seven cats in response to p27-specific stimulation (cats E1; E3; E4; E6; E9; E10 and E11), on at least one sample date during the study. In cats E4 and E9, the mean S.I. over the study, exceeded 2 (S.I. 2.27 ± 1.05; S.I. 2.15 ± 1.23, respectively). Statistical analysis showed that mean p27-specific proliferative responses in the peripheral blood from cats E4 and E9 were significantly greater than such responses in the peripheral blood from cats E2, E3, E5 and E12, when compared with cats that

received a comparable exposure dose of FeLV (Mann-Whitney Statistic, P=0.0104; P=0.0499; P=0.0070; P=0.0411, respectively).

For each peripheral blood sample that showed a significant level of proliferation, i.e. S.I. equal to, or greater than, 2, the proportion of $CD4^+$and $CD8^+$ T cells, and either $CD21^+$ B cells or $CD45R^+$ WBC, was calculated (Figs. 4.4.A and 4.5.A). An increased proportion of $CD4^+$ T cells proliferating in response to stimulation with the recombinant p27 protein, compared to medium alone, was detected in the peripheral blood from five cats (E4; E6; E9; E10 and E11), signifying the presence of p27-specific $CD4^+$ T cells in the peripheral blood from these cats. In three cats (E4, E9 and E10), p27-specific $CD4^+$ T cells were not detected during the earliest significant proliferative response, but were detected in each sample thereafter. p27-specific $CD4^+$ T cells were detected early in two cats, E4 and E6; cat E4 maintained these cells over weeks 4-10. Cat E11 developed p27-specific $CD4^+$ T cells by week 7, while cats E9 and E11 failed to generate p27-specific $CD4^+$ T cells until week 13.

The presence of p27-specific $CD4^+$ T cells among the proliferating cell population was not always accompanied by an increased proportion of either $CD8^+$ T cells or $CD21^+$ B cells. A concomitant increase in the percentage of $CD8^+$ T cells was detected in the peripheral blood from cat E4 only (weeks 4, 7 and 19), while concurrent increases in the percentage of $CD21^+$ B cells were detected in cats E4 (weeks 13 and 19), E6, E9 (week 13) and E11 (week 7). Where p27-specific proliferative responses were not associated with detectable p27-specific $CD4^+$ T cells, an increased proportion of $CD8^+$ T cells, $CD21^+$ B cells or $CD45R^+$ WBC, were enumerated among the proliferating cells. Thus, in cats E1 and E3, p27-specific proliferative responses were associated with an increased proportion of $CD45R^+$ WBC (weeks 10 and 4, respectively), while in cats E3, E4 and E9, such responses were associated with increased numbers of $CD8^+$ T cells, and either $CD45R^+$ WBC or $CD21^+$ B cells (weeks 10, 4 and 7, respectively).

(iii) p45-Specific Proliferative Responses

The mean peaks and patterns in p45-specific proliferative responses in the peripheral blood occurred at time points similar to those previously described for p27-specific

proliferative responses (Figs. 4.2.C and 4.3.C). Thus, p45-specific proliferative responses in cats that received the highest FeLV exposure dose peaked at weeks 4 and 10, with an additional peak at week 19 (S.I. 1.67 ± 0.85; S.I. 1.71 ± 0.52; S.I. 1.67 ± 0.39, respectively). Such responses peaked later in cats that received a lower exposure dose, at weeks 7 and 13 (S.I. 1.61 ± 0.63; S.I. 1.89 ± 0.56, respectively).

Despite the disparate patterns of p45-specific proliferative responses in cats that received different exposure doses, mean responses recorded in each group over the study were similar (S.I. 1.38 ± 0.25; S.I. 1.39 ± 0.26, respectively). The mean S.I. recorded in response to stimulation with the recombinant p45 protein were lower in both groups of cats, than those recorded in response to stimulation with the recombinant p27 protein. The difference was not statistically significant (Mann-Whitney Statistic, P=0.5512).

At various sample dates over the study, eight cats recorded S.I in excess of 2 in response stimulation with the recombinant p45 protein (E1; E4; E6; E7-E11). An increased proportion of $CD4^+$ T cells proliferating to stimulation with the recombinant p45 protein, compared to medium alone, was detected in the peripheral blood from five of these cats (E1; E8; E9; E10 and E11), indicating the presence of p45-specific $CD4^+$ T cells (Figs. 4.4.B and 4.5.B). p45-specific $CD4^+$ T cells were not detected in any cat until week 7 (cats E9 and E11). Cats E1, E8 and E10 developed p45-specific $CD4^+$ T cells by week 13. The presence of such cells was accompanied by a corresponding increase in $CD21^+$ B cells in cat E9 at week 13, while a concomitant increase in $CD8^+$ T cells, and either $CD45R^+$ WBC or $CD21^+$ B cells, was detected in each of the remaining samples. In the absence of detectable p45-specific $CD4^+$ T cells, increased proportions of $CD8^+$ T cells, and either $CD45R^+$ or $CD21^+$ B cells, were enumerated among the proliferating cells (cats E1, E4 and E6 at weeks 19, 4 and 10, respectively).

(iv) Mitogenic Proliferative Responses

At each sample date, marked differences in the ability of mononuclear cells in the peripheral blood from cats E1-E12 to respond to mitogenic stimulation were observed (Fig. 4.6). The highest mean mitogenic proliferative responses over the

study were recorded in the peripheral blood from cats E9 and E10 (S.I. 53.5 ± 23.3 and S.I. 44.1 ± 31.4, respectively), while cats E2 and E12 recorded the lowest mean values (S.I. 10.9 ± 8.5; S.I. 12.2 ± 6.5, respectively).

Over the study, greater mean mitogenic proliferative responses were detected in the peripheral blood from cats that were exposed to a lower dose of infectious FeLV by a factor of one log (S. I. 37.37 ± 13.81), compared to cats that received the higher exposure dose (S.I. 22.62 ± 11.2); these differences were not quite statistically significant (Mann-Whitney Statistic, P=0.0931).

4.3.2.1.2 Proliferative Responses in the Lymphoid Tissues

(i) Proliferative Responses to Inactivated FeLV Particles

Over the course of the study, S.I. equal to, or greater than, 2 were not detected in the PLN tissues from any cat in response to stimulation with inactivated FeLV particles (Fig. 4.7.A). Nonetheless, using statistical analysis, significantly greater proliferative responses to inactivated FeLV particles were demonstrated in the PLN tissues from cats that received a lower exposure dose of FeLV (Mann-Whitney Statistic, P=0.0152), compared to cats that received a higher exposure dose. Significantly lower proviral DNA levels were also recorded in the PLN tissues from cats that received a lower exposure dose of FeLV (Section 3.3.1.3).

Stimulation indices equal to, or greater than, 2 were not detected in the MLN or splenic tissues from any cat tested in response to stimulation with inactivated FeLV particles (Figs. 4.7.B and 4.7.C).

(ii) p27-Specific Proliferative Responses

No S.I. greater than, or equal to, 2 was recorded in the PLN tissues from any cat in response to stimulation with the recombinant p27 protein (Fig. 4.7.A). Nonetheless, statistical analysis showed that significantly greater p27-specific proliferative responses were detected in the PLN tissues from cats that received a lower FeLV exposure dose (Mann-Whitney Statistic, P=0.0043), compared to cats that received a higher exposure dose. Cats exposed to a lower dose of infectious FeLV also

recorded significantly lower proviral DNA levels in the PLN tissues (Section 3.3.1.3).

Out of ten cats tested, p27-specific proliferative responses were detected in the MLN tissues from only one cat, E12 (S.I. 2.1) (Fig. 4.7.B). Viral protein concentrations of 25 μg/10^6 cells were generally optimal; occasionally, lower concentrations resulted in S.I in excess of 2, e.g. mononuclear cells in the MLN tissues from cat E7 proliferated optimally at 12.5 μg p27/10^6 cells.

Mononuclear cells in the splenic tissues from six cats (E2; E4; E7; E9; E10 and E12) were tested for their ability to respond to specific stimulation with the recombinant p27 protein; however, no S.I. greater than, or equal to, 2 were detected (Fig. 4.7.C).

(iii) p45-Specific Proliferative Responses

Mononuclear cells in the PLN tissues from all cats were examined for p45-specific proliferative responses; however, only cat E9 recorded an S.I. equal to, or greater than, 2 (S.I. 2.38) (Fig. 4.7.A).

The MLN tissues from cats E7 and E12 alone recorded an S.I. equal to, or greater than, 2 in response to stimulation with the recombinant p45 protein (S.I. 2.03; S.I. 2.6, respectively) (Fig. 4.7.B). p45-specific proliferative responses were also detected in the PLN tissues from cat E8 when the viral protein concentration was reduced to 12.5 μg /10^6 cells. Significantly greater p45-specific proliferative responses were recorded in the MLN tissues from cats that received a lower exposure dose of FeLV (Mann-Whitney Statistic, P=0.0381), compared to cats that received a higher exposure dose. Lower proviral DNA levels were also quantified in the MLN tissues from cats exposed to a lower dose of infectious FeLV (Section 3.3.1.3).

Mononuclear cells in the splenic tissues from six cats (E2; E4; E7; E9; E10 and E12) were tested for their ability to respond to specific stimulation with the recombinant p45 protein; however, no S.I. greater than, or equal to, 2 were detected (Fig. 4.7.C).

(iv) Mitogenic Proliferative Responses

In each lymphoid tissue examined, marked differences were detected in the ability of mononuclear cells to respond to mitogenic stimulation (Fig. 4.7.D). Thus, the S.I. recorded in response to the mitogenic stimulation of mononuclear cells in the MLN tissues ranged from 6.3-63.0 (cats E3 and E12, respectively), while in the PLN tissues, the S.I. ranged from 6.7-48.0 (cats E4 and E6, respectively). Finally, in the splenic tissues, the S.I varied between 17.62-87.28, (cats E2 and E9, respectively).

Greater mean proliferative responses to Con A were detected in the MLN tissues from cats that received a lower exposure dose of FeLV (S.I. 37.37 ± 21.18), compared to cats that received a higher exposure dose (S.I. 13.39 ± 7.64); the difference was statistically significant (Mann-Whitney Statistic, P=0.0381). Differences in the mean proliferative responses in the PLN tissues were less affected by the exposure dose (S.I. 25.3 ± 6.17; S.I. 21.57 ± 15.1, respectively); this also applied to the splenic tissues (S.I. 50.37 ± 26.65; S.I. 44.22 ± 37.62, respectively).

4.3.2.2 Intracellular Cytokine Staining Analysed using Flow Cytometry

Intracellular cytokine staining analysed using flow cytometry, was used to evaluate the ability of mononuclear cells in the peripheral blood and the PLN tissues from cats E1-E12 to upregulate intracellular fIFN-γ in response to a four-hour virus-specific stimulation. The results are shown in Figs. 4.8-4.11.

The percentage of fIFN-γ-expressing $CD4^+$ and $CD8^+$ T cells within the gated lymphoblast population was recorded. These data were corrected by deducting the percentage of any $CD4^+$ and $CD8^+$ T cells that expressed fIFN-γ in the absence of virus-specific stimulation, in order to calculate the percentage of virus-specific $CD4^+$ and $CD8^+$ T cells (Figs. 4.8, 4.9 and 4.11). The percentage of lymphoblast cells that expressed fIFN-γ in the absence of virus-specific stimulation is tabulated in the figure legends (Figs. 4.8, 4.9 and 4.11).

Since a larger area of cells, which included some non-lymphoblast cells, was gated

for cats that received a higher exposure dose of FeLV by a factor of one log, compared to cats that received the lower exposure dose; the percentage of $CD4^+$ T cells and $CD8^+$ T cells that co-expressed fIFN-γ was automatically lower in each sample from those cats. Therefore, direct comparisons between the results from these two groups of cats could not be made.

4.3.2.2.1 Virus-specific Responses in the Peripheral Blood

(i) $CD4^+$ T cell Responses to Inactivated FeLV Particles

Specific $CD4^+$ T cell responses to inactivated FeLV particles were detected in the peripheral blood from all cats, except E7 (Figs. 4.8.A and 4.9.A). However, the timing of the appearance of such cells varied between individual animals; thus, in five cats, E2, E3, E6, E11 and E12, a specific $CD4^+$ T cell response to inactivated FeLV particles was detected as early as one week following exposure to infectious virus. In three of these cats, such responses were transient; however, a second wave of specific $CD4^+$ T cells was observed in the peripheral blood from each of these cats later in the study. Cat E3 maintained specific $CD4^+$ T cells to inactivated FeLV particles until at least week 10, while latently infected cat E12 maintained such cells throughout the study (weeks 0-13).

At week 4, specific $CD4^+$ T cell responses to inactivated FeLV particles were detected in the peripheral blood from two cats, E1 and E8. Cat E8 maintained these responses until the termination of the study at week 13, while additional specific $CD4^+$ T cells were not detected in the peripheral blood from cat E1 until week 19. By week 7, specific $CD4^+$ T cell responses to inactivated FeLV particles were detected in the peripheral blood from a further three cats, E5, E9 and E10. The frequencies of these cells were particularly high in cat E10 (2.15%). Specific $CD4^+$ T cell responses to inactivated FeLV particles did not appear in the peripheral blood from cat E4 until week 16.

It was difficult to discern a consistent pattern in the development of $CD4^+$ T cell responses to inactivated FeLV particles over the study. Nonetheless, if the mean responses were compared among cats that received a comparable exposure dose, two

distinct patterns could be identified. Thus, in cats that received a higher exposure dose of FeLV by a factor of one log, an early peak of $CD4^+$ T cell responses to inactivated FeLV particles was observed at week 4 (0.20% ± 0.23%) followed by a lower peak at week 16 (0.14% ± 0.16%) (Fig. 4.8.A). In cats that received a lower exposure dose of FeLV, specific $CD4^+$ T cells to inactivated FeLV particles peaked only at week 10 (1.01% ± 1.7%) (Fig. 4.9.A). Over the entire study, the mean percentage of specific $CD4^+$ T cells to inactivated FeLV particles from this latter group of cats was 0.42% ± 0.36%; while specific responses from cats that received a higher FeLV exposure dose averaged 0.075% ± 0.075%.

Statistical analysis showed that the correlation between $CD4^+$ T cell responses to inactivated FeLV, and proviral DNA loads, was not significant, irrespective of the exposure dose (Spearman r-Test, r=-0.6, P=0.2417; r=-0.7143, P=0.1361, respectively).

(ii) *$CD8^+$ T cell Responses to Inactivated FeLV Particles*

Specific $CD8^+$ T cell responses to inactivated FeLV particles were detected in the peripheral blood from all twelve cats (Figs. 4.8.D and 4.9.D). Six cats, E3, E4, E7, E8, E11 and E12, developed specific $CD8^+$ T cell responses to inactivated FeLV particles one week following oronasal exposure to FeLV; these were particularly numerous in cats E4 and E12 (0.49%; 2.22%, respectively). Cat E4 maintained these specific responses over weeks 1-7, with an additional high peak at week 16. Although these early responses were transient in the remaining cats, each developed a second wave of specific $CD8^+$ T cell responses to inactivated FeLV particles; such responses were particularly high in cat E12 over weeks 7-10, and in cat E7 at week 10. Moderately high frequencies of specific $CD8^+$ T cells to inactivated FeLV particles were detected in the peripheral blood from cat E10 over weeks 4-10, and at lower levels in cat E2 over weeks 7-10. The remaining cats failed to develop any specific $CD8^+$ T cell responses to inactivated FeLV particles until late in the study, at weeks 10 (E5 and E9), 13 (E1) and 16 (E6).

The mean specific $CD8^+$ T cell responses to inactivated FeLV particles recorded in the peripheral blood from cats E1-E6 peaked at weeks 1 (0.11% ± 0.12%) and 16

(0.18% ± 0.12%), while the mean responses from cats E7-E12 peaked at weeks 1 (0.61% ± 0.86%) and 10 (1.35% ± 1.03%). The mean values of specific $CD8^+$ T cell responses to inactivated FeLV particles were comparable to those recorded for specific $CD4^+$ T cell responses. Thus, cats that received a higher exposure dose demonstrated mean specific $CD8^+$ T cell responses of 0.075% ± 0.043%; cats that received a lower exposure dose of FeLV demonstrated mean specific $CD8^+$ T cell responses 0.45% ± 0.38%.

Using statistical analysis, a significant correlation was not detected between $CD8^+$ T cell responses to inactivated FeLV, and proviral DNA loads, irrespective of the exposure dose (Spearman r-Test, r=-0.5429, P=0.2972; r=-0.6, P=0.2417, respectively).

(iii) p27-Specific $CD4^+$ T cell Responses

Eleven cats developed p27-specific $CD4^+$ T cell responses in the peripheral blood following oronasal exposure to FeLV (Figs. 4.8.B and 4.9.B). Two cats, E6 and E8, developed such responses by week 1; however, p27-specific $CD4^+$ T cells were not detected in the peripheral blood from cat E8 at any further time point. Fluctuating frequencies of p27-specific $CD4^+$ T cells were apparent in the peripheral blood from cat E6, reappearing over weeks 7-10 and at week 16.

p27-specific $CD4^+$ T cells were detected in the peripheral blood from cats E3 and E12 over weeks 4-10. At week 4, the proportion of p27-specific $CD4^+$ T cells in the peripheral blood from cat E12 was initially very high (0.88%), and had increased further by week 10 (2.13%). However, such responses were undetectable by week 13 (Figs. 4.9.A and 4.10). Cats E2, E9 and E10 developed p27-specific $CD4^+$ T cell responses by week 10; these were particularly high in cat E10 (3.59%). Three cats, E1, E5 and E7 developed p27-specific $CD4^+$ T cell responses in the peripheral blood by week 13. Cat E4 developed moderately high frequencies of p27-specific $CD4^+$ T cells in the peripheral blood by week 16 (0.35%).

In cats that received a higher exposure dose of FeLV, mean p27-specific $CD4^+$ T cell responses did not vary greatly over the course of this study. However, an early low

peak in p27-specific $CD4^+$ T cell frequency could be discerned at week 4 (0.06% ± 0.14%), which was followed by a slightly higher peak at week 16 (0.08 ± 0.14). Cats that were exposed to a lower dose of FeLV peaked in mean p27-specific $CD4^+$ T cell responses at weeks 4 (0.15% ± 0.36%) and 10 (0.96% ± 1.54%). These latter cats recorded 0.23% ± 0.3% in mean p27-specific $CD4^+$ T cells over the course of the study, while cats that received a higher exposure dose recorded a mean value of 0.055% ± 0.028%. Thus, the mean levels of p27-specific $CD4^+$ T cell responses were lower than recorded to specific $CD4^+$ T cell responses to inactivated FeLV particles, irrespective of the exposure dose.

Statistical analysis showed that the correlation between p27-specific $CD4^+$ T cell responses, and proviral DNA loads, was not significant, irrespective of the exposure dose (Spearman r-Test, r=-0.3143, P=0.5639; r=-0.3143, P=0.5639 respectively).

(iv) p27-Specific $CD8^+$ T cell Responses

All twelve cats developed p27-specific $CD8^+$ T cell responses in the peripheral blood following oronasal exposure to FeLV (Figs. 4.8.E and 4.9.E). Eight cats, E2, E4, E5, E6, E7, E8, E11 and E12, developed p27-specific $CD8^+$ T cell responses in the peripheral blood as early as week 1 post exposure to FeLV. In two of these cats, E4 and E8, such responses were maintained over weeks 1-4, before disappearing. Cat E11 maintained these levels for a further three weeks; in addition, a large peak in p27-specific $CD8^+$ T cell numbers was observed in this cat at week 13 (3.23%). Cats E1, E3 and E9 had all developed transient levels of p27-specific $CD8^+$ T cells by week 7, while cat E10 generated a high peak of p27-specific $CD8^+$ T cell responses at week 10 (3.92%).

The patterns of mean p27-specific $CD8^+$ T cell responses in the peripheral blood over the study were similar to those observed for p27-specific $CD4^+$ T cell responses and $CD4^+$ and $CD8^+$ T cell responses to inactivated FeLV particles. Cats that received a higher dose of infectious virus peaked in mean p27-specific $CD8^+$ T cell responses at weeks 4 (0.23% ± 0.49%) and 16 (0.12% ± 0.14%), while in cats that received a lower exposure dose of FeLV, mean responses peaked at weeks 1 (0.44% ± 0.47%) and 10 (1.02% ± 1.7%). In the latter group of cats, mean responses were comparable

to specific $CD4^+$ and $CD8^+$ T cell responses to inactivated FeLV particles (0.41% ± 0.32%). However, in cats that were exposed to a higher infectious dose of FeLV, p27-specific $CD8^+$ T cell responses were much higher than p27-specific $CD4^+$ responses and both specific $CD4^+$ and $CD8^+$ T cell responses to inactivated FeLV (0.12% ± 0.088%).

The correlation between p27-specific $CD8^+$ T cell responses and proviral DNA loads was not statistically significant, irrespective of the exposure dose (Spearman r-Test, r=0.7827, P=0.1028; r=-0.5429, P=0.2417, respectively).

(v) p45-Specific $CD4^+$ T cell Responses

Ten cats developed p45-specific $CD4^+$ T cells in the peripheral blood following oronasal exposure to FeLV; such responses were not detected in the peripheral blood from cats E7 and E10 (Figs. 4.8.C and 4.9.C). Five cats, E6, E8, E9, E11 and E12 developed p45-specific $CD4^+$ T cell responses in the peripheral blood one week following exposure to FeLV. All such responses had disappeared by week 4; however, additional p45-specific $CD4^+$ T cell responses in the peripheral blood were detected later in the study for all of these cats except E6; a particularly high peak was recorded in the peripheral blood from cat E12 at week 10. By week 4, a further two cats, E1 and E3, had developed transient low levels of p45-specific $CD4^+$ T cell responses. Low numbers of p45-specific $CD4^+$ T cells were detected in the peripheral blood from cats E4 and E5 for the first time at week 7. Cat E2 failed to develop p45-specific $CD4^+$ T cell responses in the peripheral blood until week 13.

In cats exposed to a higher infectious dose of FeLV, the patterns of mean p45-specific $CD4^+$ T cell responses observed over the course of the study were dissimilar from those detected in response to both the recombinant p27 protein and inactivated FeLV particles, peaking only at week 7 (0.07% ± 0.08%). The mean p45-specific $CD4^+$ T cell responses in the peripheral blood from cats exposed to a lower exposure dose of FeLV peaked at weeks 1 (0.17% ± 0.21%) and 10 (0.25% ± 0.61%). The values of the mean responses were much lower than previously recorded for specific $CD4^+$ T cell responses to p27 and inactivated FeLV particles, irrespective of the exposure dose. Thus, cats that were exposed to a higher infectious dose averaged

0.033% ± 0.017% p27-specific $CD4^+$ T cells, much lower than the specific $CD4^+$ T cell responses to either the recombinant p27 protein or inactivated FeLV particles. Cats that received a lower exposure dose by a factor of one log, demonstrated 0.14% ± 0.16% mean p27-specific $CD4^+$ T cells over the course of the study, which was also much lower than the mean specific $CD4^+$ T cell responses to either the recombinant p27 protein or inactivated FeLV particles.

Using statistical analysis, a significant correlation was not observed between p45-specific $CD4^+$ T cell responses, and proviral DNA loads, irrespective of the exposure dose (Spearman r-Test, r=-0.02857, P>0.9999; r=-0.2319, P=0.6583, respectively).

(vi) p45-Specific $CD8^+$ T cell Responses

Ten cats generated p45-specific $CD8^+$ T cell responses in the peripheral blood following oronasal exposure to FeLV; such responses were not detected in the peripheral blood from cats E8 and E11 (Figs. 4.8.F and 4.9.F). p45-specific $CD8^+$ T cell responses were detected in the peripheral blood from one cat, E7, at week 1 post exposure. However, four cats, E4, E5, E6 and E10, all developed p45-specific $CD8^+$ T cell responses by week 4, which were particularly high in cat E4 (0.7%). Each of these early peaks in p45-specific $CD8^+$ T cell responses was followed by a second, at weeks 10 (E5 and E10) and 13 (E4 and E6), which was particularly high in cat E10 (1.74%). By week 7, three cats, E2, E3 and E9 had developed low levels of p45-specific $CD8^+$ T cell responses in the peripheral blood; these were transient in cats E2 and E9. However, cat E3 maintained these cells until at least week 16. Cats E12 and E1 did not generate p45-specific $CD8^+$ T cells until weeks 10 and 13, respectively.

The pattern of p45-specific $CD8^+$ T cell responses recorded over the course of the study differed among cats exposed to a different dose of infectious FeLV. Thus, the mean responses from cats that received a higher exposure dose peaked at weeks 4 (0.15 ± 0.27) and 13 (0.12 ± 0.16) while p45-specific $CD8^+$ T cell responses from cats that received a lower exposure dose peaked at weeks 4 (0.06 ± 0.14) and 10 (0.64% ± 0.76%). These latter cats generated mean p45-specific $CD8^+$ T cell responses of 0.126% ± 0.146% over the course of the study, while cats that received

a higher exposure dose averaged 0.05% ± 0.051%. Thus, mean specific $CD8^+$ T cell responses to the recombinant p45 protein in the peripheral blood from cats E7-E12 over the course of the study were much lower than to either the recombinant p27 protein or inactivated FeLV particles. However, mean p45-specific $CD8^+$ T cell responses in the peripheral blood from cats E7-E12 were greater than p27-specific $CD8^+$ T cell responses and p45-specific $CD4^+$ T cell responses.

Using statistical analysis, a significant correlation was not detected between p45-specific $CD8^+$ T cell responses, and proviral DNA loads, irrespective of the exposure dose (Spearman r-Test, r=0.1429, P=0.8028; r=-0.4348, P=0.4194, respectively).

4.3.2.2.2 Virus-specific Responses in the PLN Tissues

Specific $CD4^+$ T cell responses to either the recombinant p27 or p45 proteins, or inactivated FeLV particles, were examined in the PLN tissues from cats E4-E12 post mortem. Similarly, virus-specific $CD8^+$ T cell responses were investigated in the PLN tissues from cats E4-E7, and cat E12 (Fig. 4.11).

(i) Specific T cell Responses to Inactivated FeLV Particles

Moderate numbers of specific $CD4^+$ T cells to inactivated FeLV particles were enumerated in the PLN tissues from cats E4, E5, E6 and E9 (Fig. 4.11). A low percentage of concurrent specific $CD8^+$ T cells were detected in the PLN tissues from cat E5 (0.06%). Corresponding data was not available for cats E8-E11.

(ii) p27-Specific T cell Responses

Moderate numbers of p27-specific $CD4^+$ T cells were recorded in the PLN tissues from cats E4, E5, and E9, while the PLN tissues from cat E6 demonstrated strong p27-specific $CD4^+$ T cell responses (1.81%) (Fig. 4.11). Low numbers of p27-specific $CD8^+$ T cells were detected in the PLN tissues from cats E5 and E12 (0.12%; 0.3%, respectively). No data was available for cats E8-E11.

(iii) p45-Specific T cell Responses

The PLN tissues from cats E5, E8, E9 and E10 all contained moderately low numbers of p45-specific $CD4^+$ T cells, while the PLN tissues from cat E6 contained much higher numbers of p45-specific $CD4^+$ T cells (2.05%) (Fig. 4.11). Peripheral lymph node tissues from cats E4 and E5 all contained high frequencies of p45-specific $CD8^+$ T cells, whereas lower frequencies were detected in cat E12 (0.74%, 1.18% and 0.26%, respectively); similar data was unavailable for cats E8-E11.

Fig 4.1 SDS-PAGE and Western Blots demonstrating FeLV proteins

Recombinant p27 and p45 proteins, and FeLV particles, were electrophoresed on a 12% SDS-PAGE gel, and either stained with Coomassie Brilliant Blue (I, IV), or transferred to nitrocellulose and probed in Western blots (II, III, V). Prestained protein standards were used throughout to determine protein size (lane A).

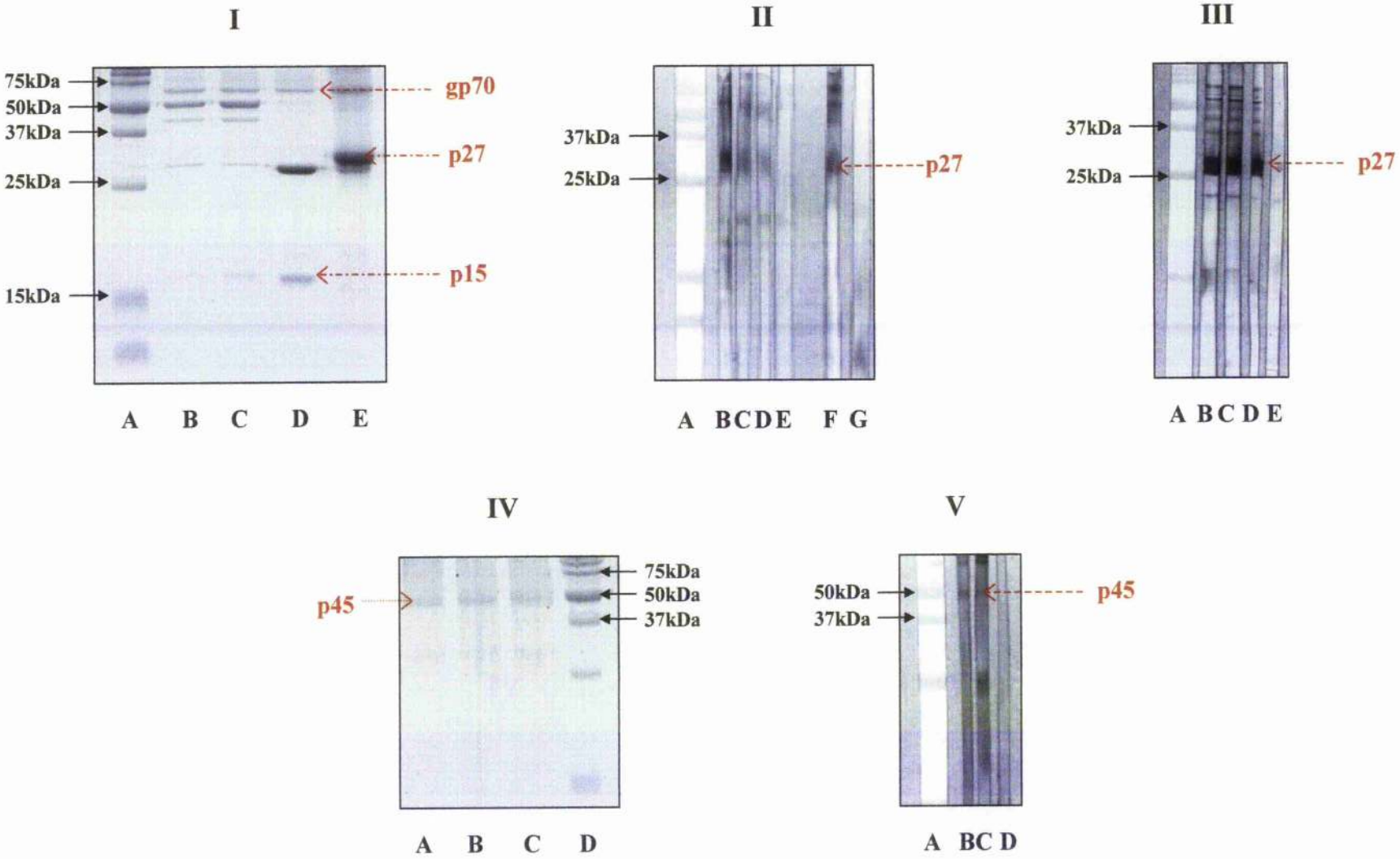
I. Heat-inactivated FeLV particles (7-14 μg, lanes B and C, respectively), FeLV particles (7 μg, lane D) and recombinant p27 protein (6.4 μg, lane E).

II. Recombinant p27 protein (4.3 μg) probed with sheep anti-p27 pAb 1:50,000 (lane B); 1:100,000 (lane C) and 1:250,000 (lane D), and serum from a FeLV recovered cat 1:4 (lane F), in Western blots. Blots probed with 20% NRS or 20% NGS in place of primary antibody, or recovered serum, respectively, were used as negative controls (lanes E and G, respectively).

III. FeLV particles (8 μg) probed with sheep anti-p27 pAb 1:25,000 (lane B); 1:50,000 (lane C); 1:100,000 (lane D) in Western blot. Blot probed with 20% NRS in place of primary antibody was used as negative control (lane E).

IV. Recombinant p45 protein (3.1-4.4 μg, lanes A-C, respectively).

V. Recombinant p45 protein (3.5 μg) probed with sera from two FeLV-recovered cats, 1:4 (lanes B and C) in Western blot. Blot probed with 20% NGS in place of recovered serum, was used as negative control (lane D).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37kDa
25kDa
15kDa
gp70
p27
p15
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25kDa
p27
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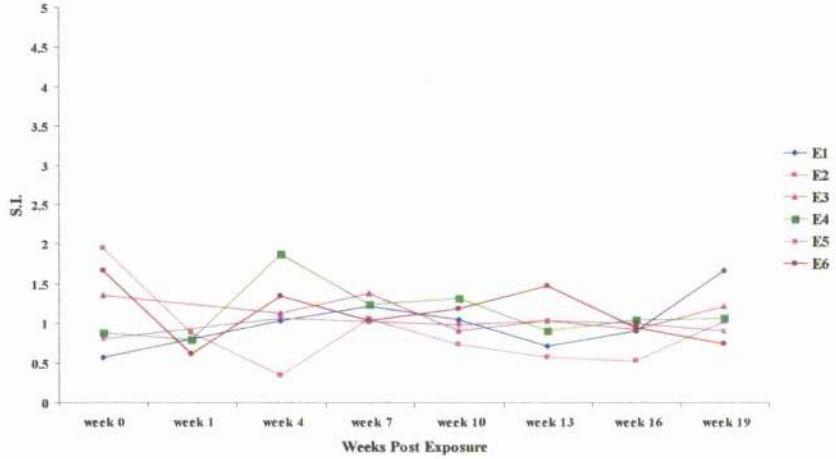
III
37kDa
25kDa
p27
A B C D E
IV
p45
75kDa
50kDa
37kDa
A B C D
V
50kDa
37kDa
p45
A BC D

Fig. 4.2 Virus-specific proliferative responses in the peripheral blood from cats E1-E6

Proliferative responses of mononuclear cells in the peripheral blood from cats E1-E6 to stimulation with either inactivated FeLV particles (25 $\mu g/10^6$ cells) (A), or recombinant p27 (25 $\mu g/10^6$ cells) (B), or p45 proteins (25 $\mu g/10^6$ cells) (C). Mononuclear cells were prepared from peripheral blood sampled at regular intervals from weeks 0-19 following oronasal exposure to FeLV. Cells were cultured for 3 days *ex vivo*; ^{3}H-thymidine was added for the final 5 h of incubation. The net counts (cpm) recorded by unstimulated cells are displayed in the table below; these data were used to calculate the S.I. In addition, the range of the lowest and highest net counts (cpm) recorded by antigen-stimulated cells is displayed in bold.

Cat No.	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13	Wk 16	Wk 19
E1	388.40	492.33	416.12	368.11	383.90	173.03	482.61	362.42
E2	169.53	594.83	588.47	630.83	403.60	863.50	475.80	437.77
E3	217.47	498.57	291.31	460.41	395.78	510.49	496.84	476.00
E4	335.13	359.50	168.73	318.73	367.74	431.64	428.21	326.10
E5	473.47	535.87	582.46	436.20	446.14	493.37	653.26	482.44
E6	519.20	399.47	384.66	449.70	527.52	405.85	620.92	386.81
Range	**145.00-520.60**	**219.67-750.80**	**208.50-1046.20**	**329.80-1023.03**	**294.57-2069.43**	**122.17-1100.30**	**252.10-1170.07**	**273.50-972.30**

A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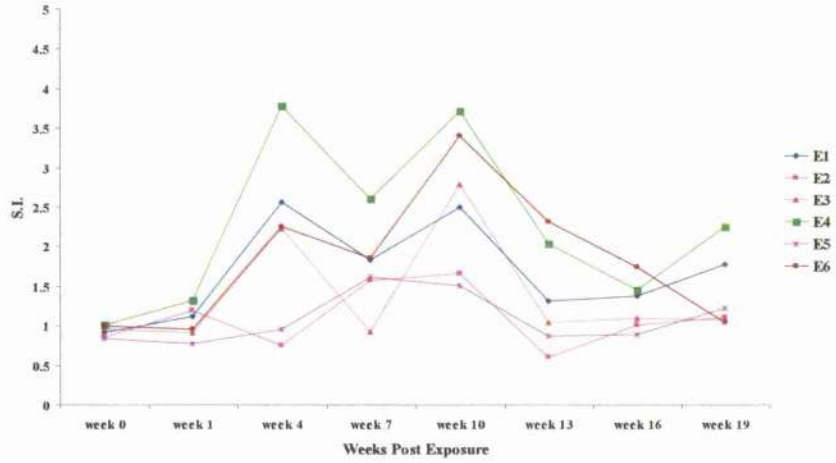
4
3.5
3
2.5
2
1.5
1
0.5
0
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6

B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1
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0
week 0
week 1
week 4
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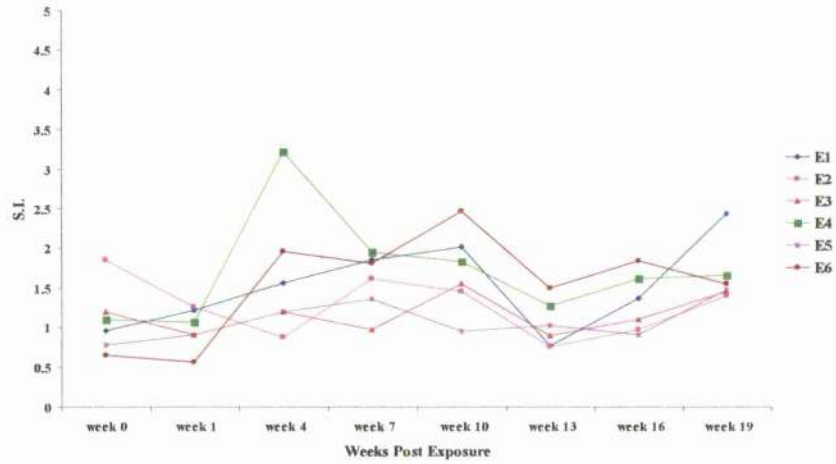
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6

C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3
2.5
2
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0
week 0
week 1
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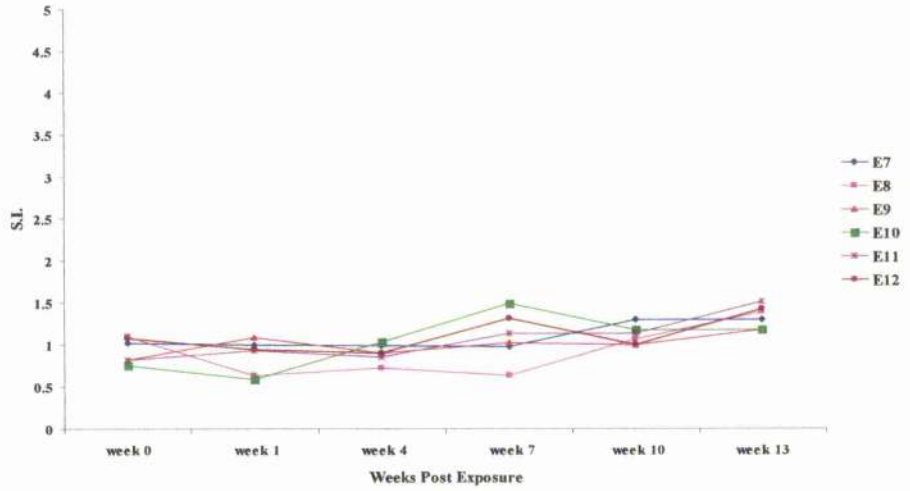
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6

Fig. 4.3 Virus-specific proliferative responses in the peripheral blood from cats E7-E12

Proliferative responses of mononuclear cells in the peripheral blood from cats E7-E12 to stimulation with either inactivated FeLV particles (25 $\mu g/10^6$ cells) (A), or recombinant p27 (25 $\mu g/10^6$ cells) (B) or p45 proteins (25 $\mu g/10^6$ cells) (C). Mononuclear cells were prepared from peripheral blood sampled at regular intervals from weeks 0-13 following oronasal exposure to FeLV. Cells were cultured for 3 days *ex vivo*; ^{3}H-thymidine was added to the cultures for the final 5 h of incubation. The background net counts (cpm) recorded in the unstimulated wells from each cat E7-E12 are displayed below; these data were used to calculate the S.I. In addition, the range of the lowest and highest net counts (cpm) recorded by antigen-stimulated cells is displayed in bold.

Cat No.	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13
E7	463.41	208.70	165.83	482.53	602.44	271.42
E8	398.37	203.67	278.07	404.82	473.94	504.99
E9	499.57	271.67	385.71	424.97	590.41	622.36
E10	388.43	276.73	309.99	646.86	825.21	194.77
E11	995.33	596.73	332.80	414.78	673.56	456.35
E12	558.46	626.03	199.39	412.57	993.49	638.70
Range	**253.73-1171.73**	**131.03-764.17**	**144.70-672.20**	**211.33-1687.43**	**439.40-1036.20**	**227.63-1291.90**

A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4
3.5
3
2.5
2
1.5
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0.5
0
week 0
week 1
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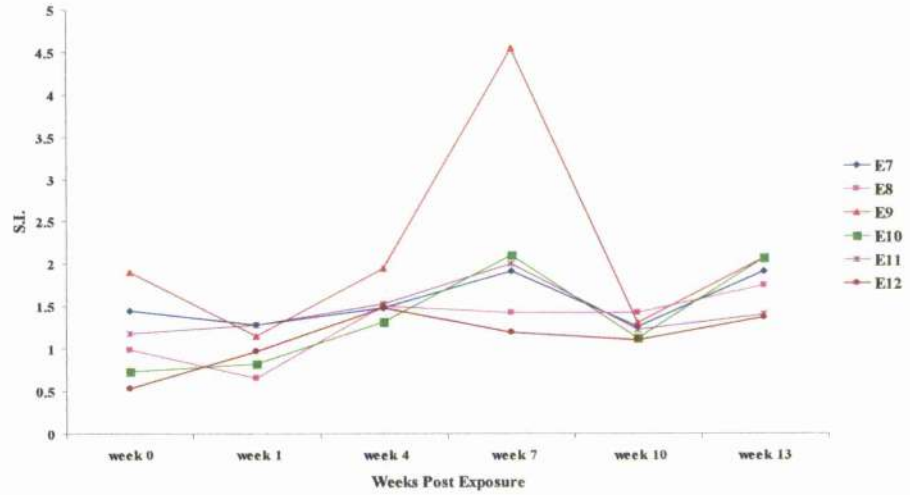
Weeks Post Exposure
E7
E8
E9
E10
E11
E12

B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3
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week 0
week 1
week 4
week 7
week 10
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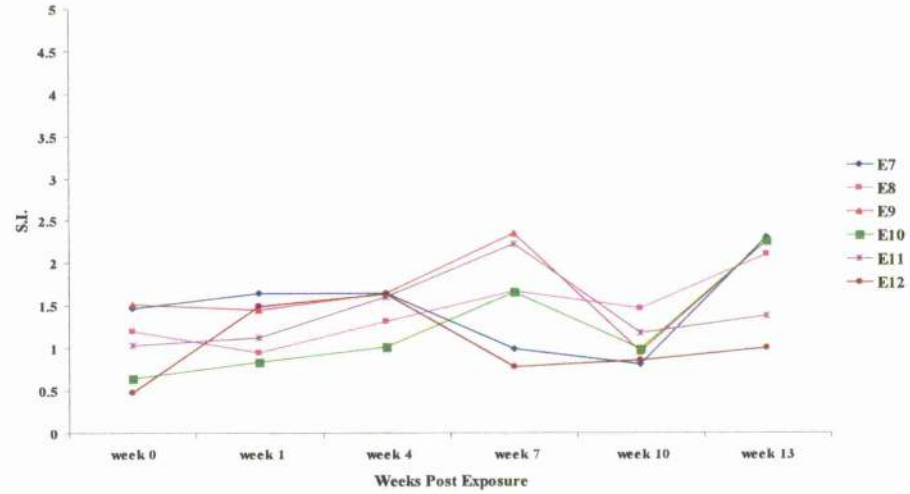
E7
E8
E9
E10
E11
E12

C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3.5
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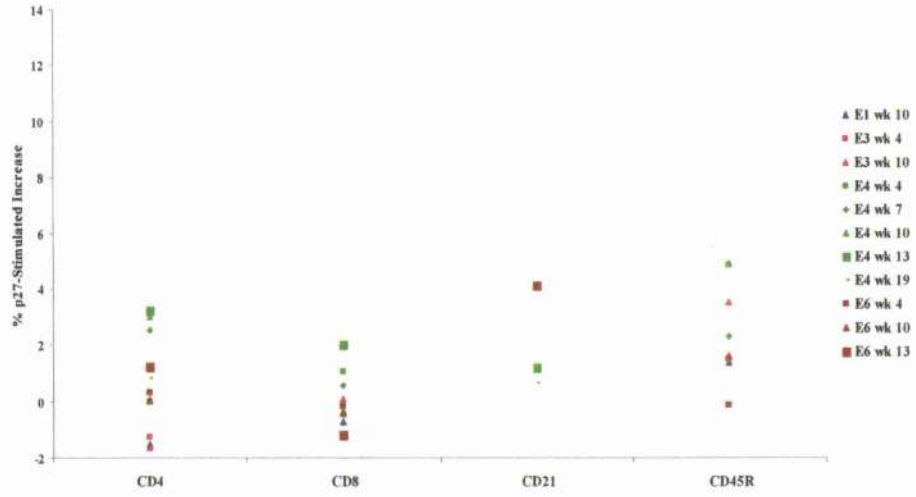
1.5
1
0.5
0
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12

Fig. 4.4 Phenotypic analysis of virus-specific T and B cells in the peripheral blood from cats E1-E6

Percentage increase in $CD4^+$ and $CD8^+$ T cells, and either $CD45R^+$ WBC or $CD21^+$ B cells, in the peripheral blood from cats E1-E6 following virus-specific stimulation with recombinant p27 (25 $\mu g/10^6$ cells) (A) or p45 proteins (25 $\mu g/10^6$ cells) (B), or mitogenic stimulation with Con A (5 $\mu g/10^6$ cells) (C) in the LPA, compared to unstimulated cells. Only data from cats that recorded an S.I. greater than, or equal to, 2 at any time point is shown.

A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14
12
10
8
6
4
2
0
-2
CD4
CD8
CD21
CD45R
E1 wk 10
E3 wk 4
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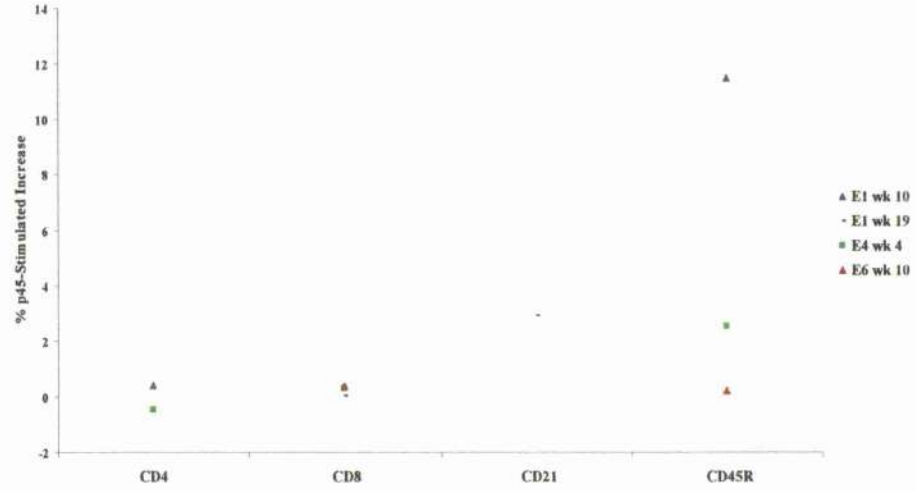
E4 wk 19
E6 wk 4
E6 wk 10
E6 wk 13

B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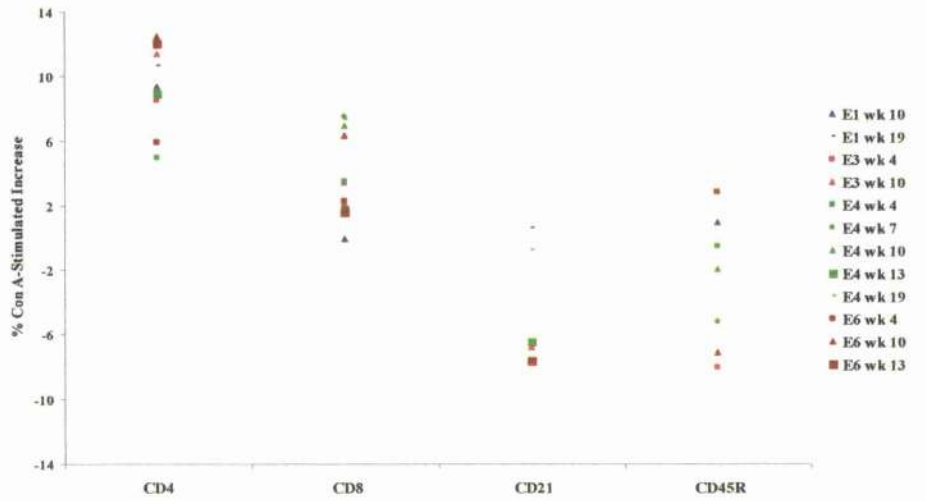
12
10
8
6
4
2
0
-2
CD4
CD8
CD21
CD45R
E1 wk 10
E1 wk 19
E4 wk 4
E6 wk 10

C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10
6
2
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CD4
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CD21
CD45R
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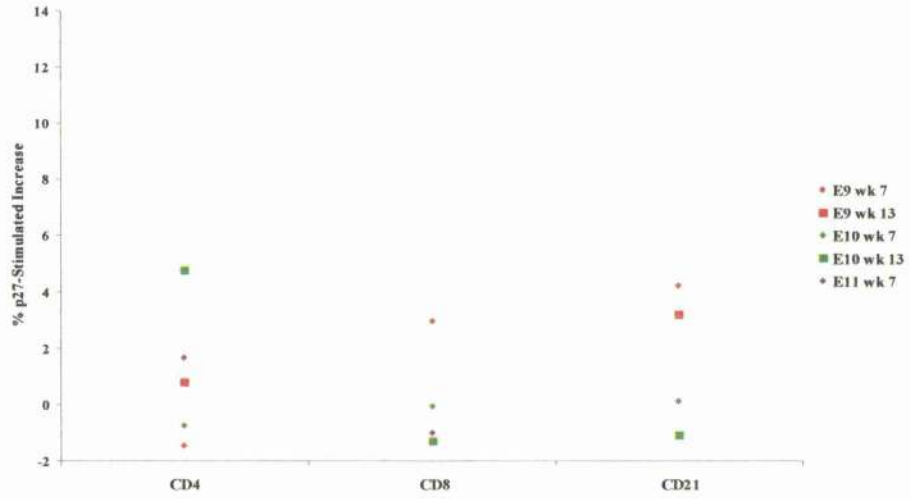
E1 wk 19
E3 wk 4
E3 wk 10
E4 wk 4
E4 wk 7
E4 wk 10
E4 wk 13
E4 wk 19
E6 wk 4
E6 wk 10
E6 wk 13

Fig. 4.5 Phenotypic analysis of virus-specific T and B cells in the peripheral blood from cats E7-E12

Percentage increase in $CD4^+$ or $CD8^+$ T cells, or $CD21^+$ B cells, in the peripheral blood from cats E7-E12 following virus-specific stimulation with recombinant p27 (25 $\mu g/10^6$ cells) (A) or p45 proteins (25 $\mu g/10^6$ cells) (B), or mitogenic stimulation with Con A (5 $\mu g/10^6$ cells) (C) in the LPA, compared to unstimulated cells. Only data from cats that recorded an S.I. greater than, or equal to, 2 at any time point is shown.

A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10
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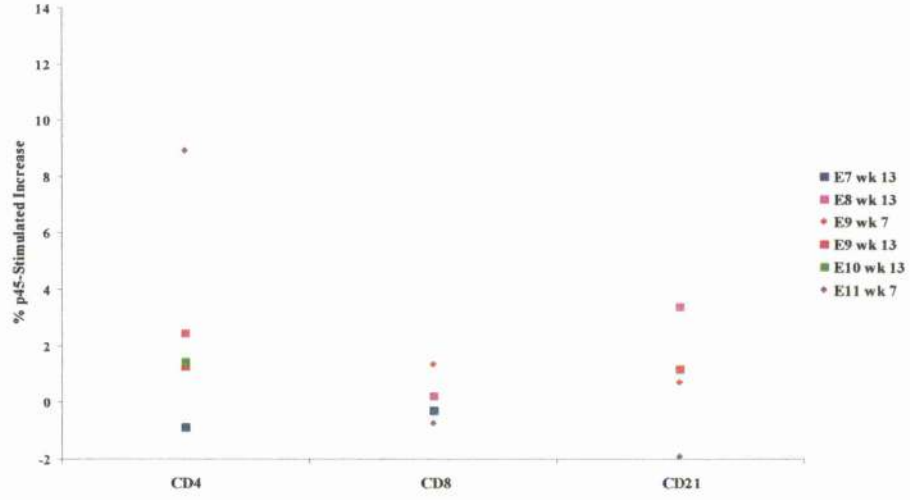
CD4
CD8
CD21
E9 wk 7
E9 wk 13
E10 wk 7
E10 wk 13
E11 wk 7

B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8
6
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0
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CD4
CD8
CD21
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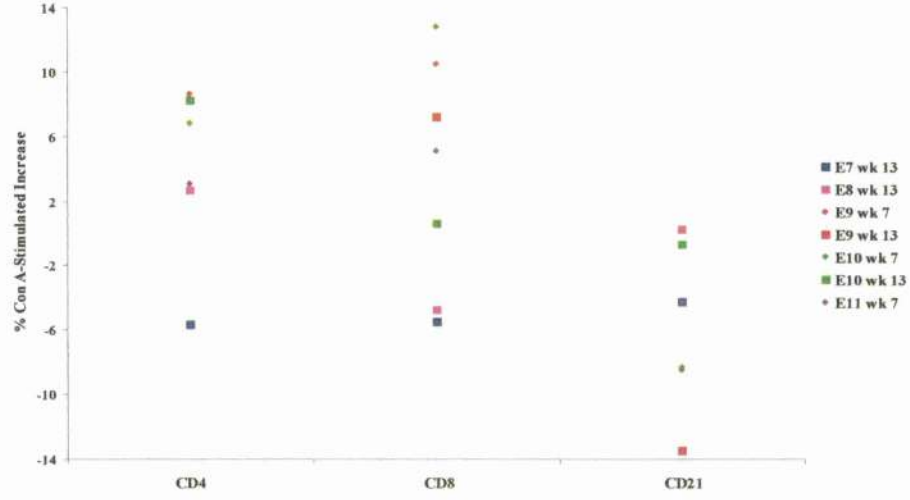
E9 wk 7
E9 wk 13
E10 wk 13
E11 wk 7

C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CD4
CD8
CD21
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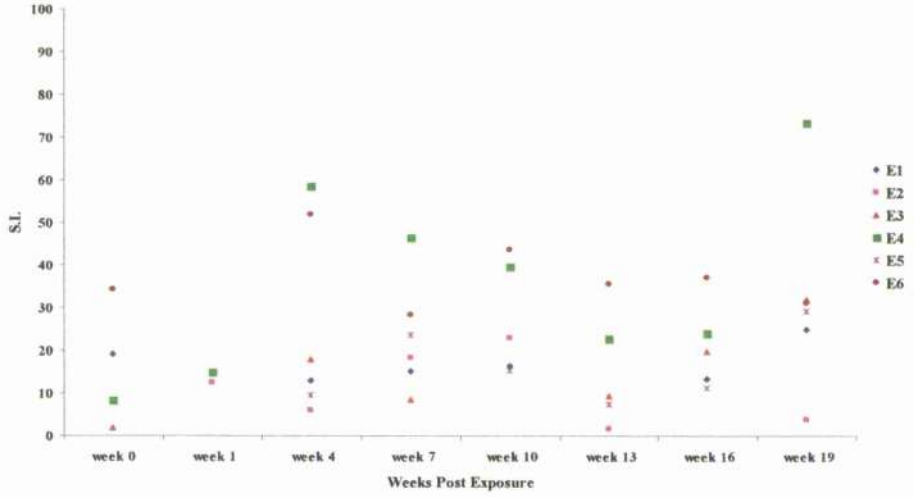
E9 wk 13
E10 wk 7
E10 wk 13
E11 wk 7

Fig. 4.6 Mitogenic proliferative responses in the peripheral blood from cats E1-E12

The ability of mononuclear cells in the peripheral blood from cats E1-E6 (A) and cats E7-E12 (B) to proliferate to the mitogen Con A (5 $\mu g/10^6$ cells) over weeks 0-19 (E1-E6) and weeks 0-13 (E7-E12) is illustrated. Mononuclear cells were prepared from the peripheral blood at regular intervals following oronasal exposure to FeLV. Cells were cultured for three days *ex vivo*; ^{3}H-thymidine was added to the cultures for the final 5 h incubation. The background net counts recorded by the unstimulated cells were used to calculate the S.I. These are the same data used to calculate the virus-specific proliferative responses for cats E1-E6 over weeks 0-19 (Fig. 4.2), and cats E7-E12 over weeks 0-13 (Fig. 4.3).

A


100
90
80
70
60
50
40
30
20
10
0
S.I.
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6

B

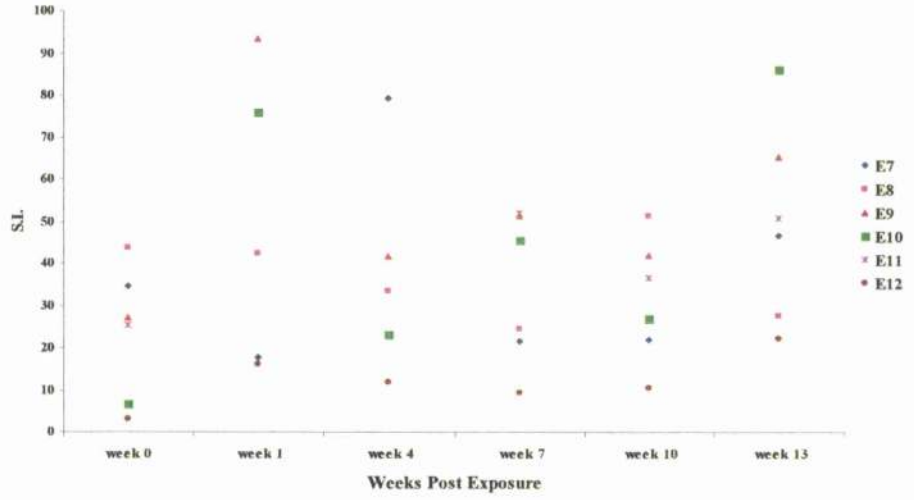
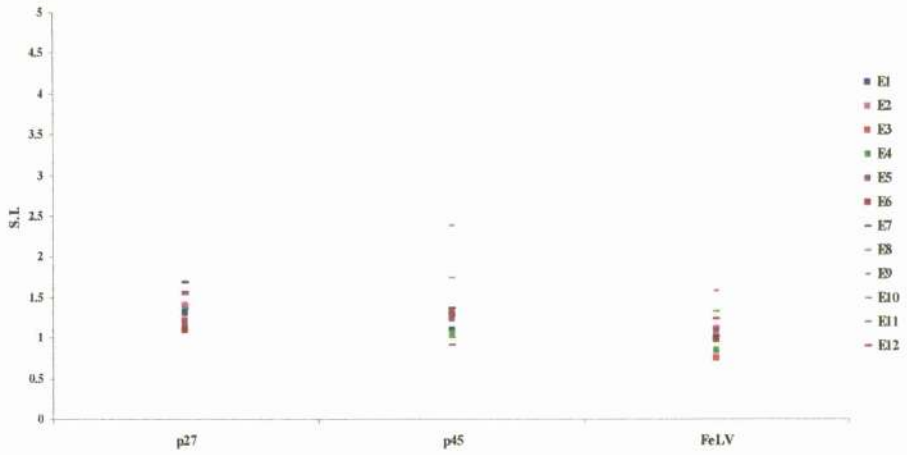

100
90
80
70
60
50
40
30
20
10
0
S.I.
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12

Fig. 4.7 Virus-specific and mitogenic proliferative responses in the lymphoid tissues from cats E1-E12

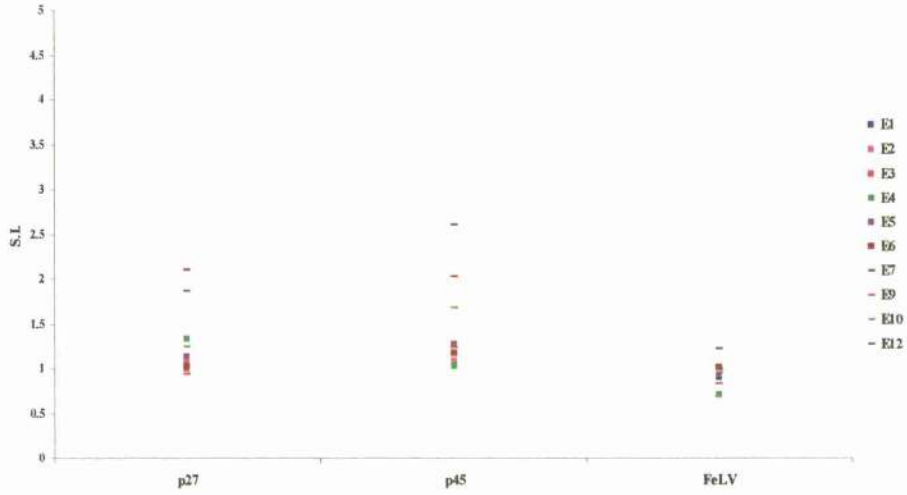
Proliferative responses in the PLN tissues from cats E1-E12 (A), MLN tissues from cats E1-E7, E9, E10 and E12 (B), and splenic tissues from cats E2; E4; E7; E9; E10 and E12 (C), were recorded following stimulation with either recombinant p27 (25 μg /10^6 cells) or p45 proteins (25 μg /10^6 cells), or inactivated FeLV particles (25 μg /10^6 cells). Proliferative responses to Con A (5 μg/10^6 cells) in the MLN, PLN and splenic tissues from cats E1-E12 are illustrated in chart D. Mononuclear cells were prepared from the lymphoid tissues post mortem and stimulated *ex vivo* for 3 days; ^{3}H-thymidine was added for the final 5 h of culture. The background net counts (cpm) recorded by the unstimulated cells from each tissue are displayed below; these data were used to calculate the S.I. in response to both virus specific and mitogenic stimulation. The data are presented as 'not done' (n.d.) when samples were not analysed.

Cat No.	PLN	MLN	Spleen
E1	363.00	375.01	n.d
E2	623.02	420.24	446.33
E3	342.13	358.65	n.d
E4	509.47	285.77	186.02
E5	404.63	346.03	n.d
E6	280.60	302.86	n.d
E7	731.11	176.66	536.89
E8	967.22	n.d	n.d
E9	828.32	319.87	530.52
E10	700.26	236.81	365.11
E11	527.75	n.d	n.d
E12	807.53	265.97	270.62

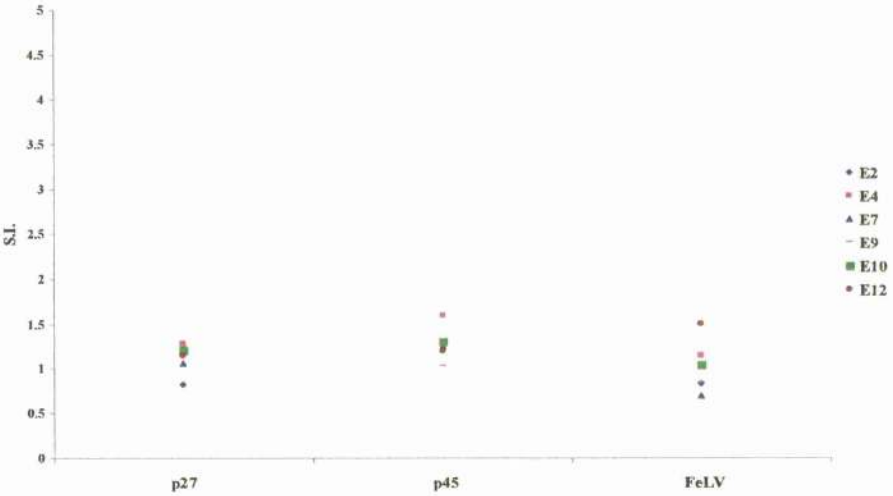
A


S.I.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
p27
p45
FeLV
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12

B


S.I.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
p27
p45
FeLV
E1
E2
E3
E4
E5
E6
E7
E9
E10
E12

C


S.I.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
p27
p45
FeLV
E2
E4
E7
E9
E10
E12

D

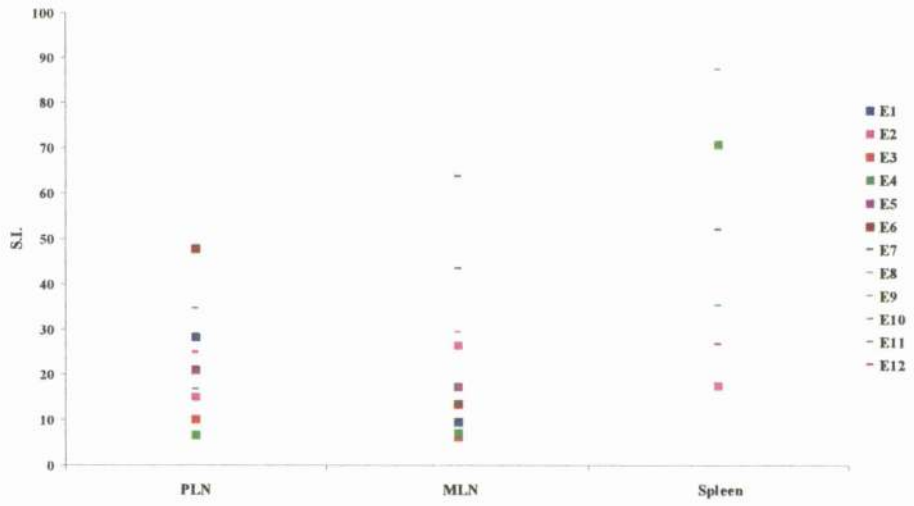

100
90
80
70
60
50
40
30
20
10
0
S.I.
PLN
MLN
Spleen
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12

Fig. 4.8 Virus-specific $CD4^+$ and $CD8^+$ T cells in the peripheral blood from cats E1-E6, detected using ICS

Graphs depicting the expression of fIFN-γ by $CD4^+$ T cells (A-C) and $CD8^+$ T cells (D-F) in the peripheral blood from cats E1-E6 following a 4 h stimulation with either inactivated FeLV particles (A, D), or recombinant p27 (B, E) or p45 proteins (C, F), in the presence of BFA. The gates that determined fIFN-γ expression were set according to the reactivity of the isotype-matched control-FITC mAb. In total, 10,000 events were counted. Feline IFN-γ expressing $CD4^+$ and $CD8^+$ T cells within the lymphoblast population were enumerated at regular intervals following experimental exposure to FeLV at week 0. These data were corrected by deducting the percentage of any $CD4^+$ and $CD8^+$ T cells that expressed fIFN-γ in the absence of virus-specific stimulation, in order to calculate the percentage of virus-specific $CD4^+$ and $CD8^+$ T cells. The data recorded for unstimulated $CD4^+$ and $CD8^+$ T cells are shown in tables (i) and (ii), respectively. On each sample date, the mean percentage of virus-specific $CD4^+$ and $CD8^+$ T cells was calculated (Mean E1-E6).

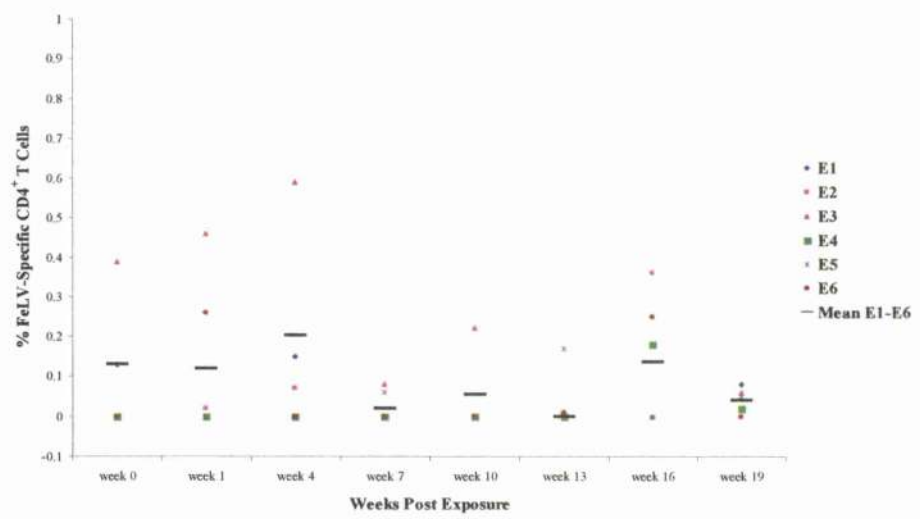
(i)

Cat No.	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13	Wk 16	Wk 19
E1	0.68	0.07	0.15	0.00	0.16	0.28	0.16	0.13
E2	0.17	0.03	0.00	0.13	0.00	0.00	0.14	0.02
E3	0.14	0.06	0.00	0.00	0.07	0.35	0.32	0.08
E4	0.28	0.51	0.53	0.21	0.25	0.45	0.39	0.20
E5	2.53	0.05	0.41	0.00	0.17	0.00	0.24	0.01
E6	0.61	0.00	0.54	0.06	0.08	0.00	0.06	0.03

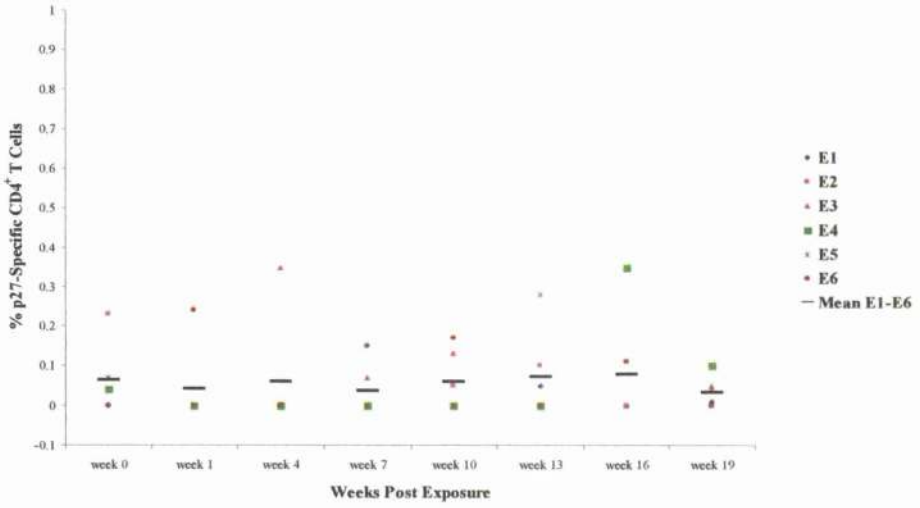
(ii)

Cat No.	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13	Wk 16	Wk 19
E1	0.72	0.07	0.58	0.00	0.18	0.10	0.16	0.04
E2	0.69	0.04	0.22	0.00	0.00	0.00	0.10	0.00
E3	0.57	0.00	0.53	0.09	0.07	0.18	0.22	0.22
E4	0.43	0.00	0.00	0.00	0.34	0.34	0.09	0.33
E5	1.33	0.05	0.34	0.00	0.00	0.10	0.17	0.05
E6	0.50	0.00	0.50	0.16	0.29	0.00	0.06	0.02

A


% FeLV-Specific CD4+ T Cells
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
Mean E1-E6

B


% p27-Specific CD4+ T Cells
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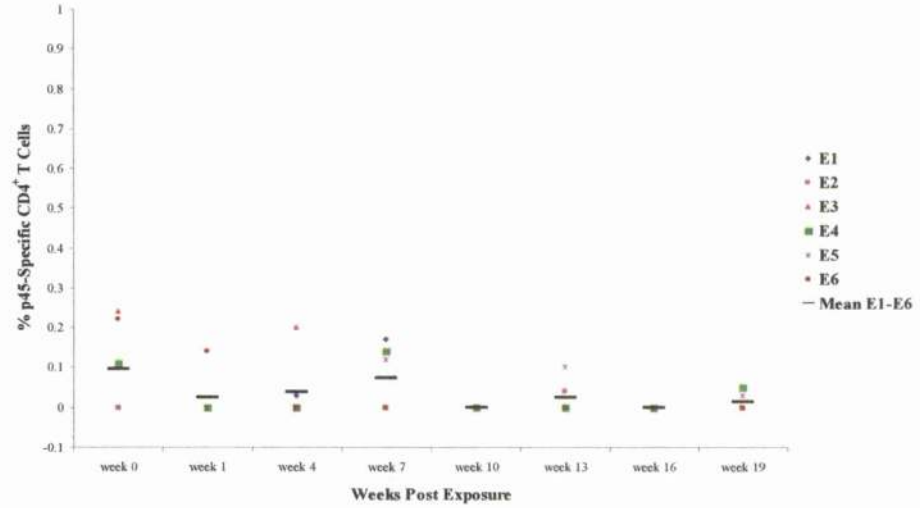
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
Mean E1-E6

C


% p45-Specific CD4+ T Cells
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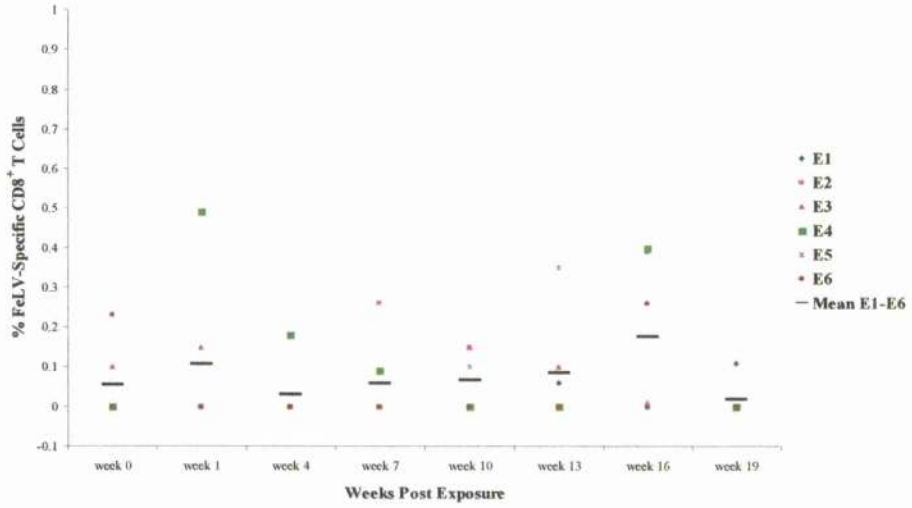
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
Mean E1-E6

D


% FeLV-Specific CD8+ T Cells
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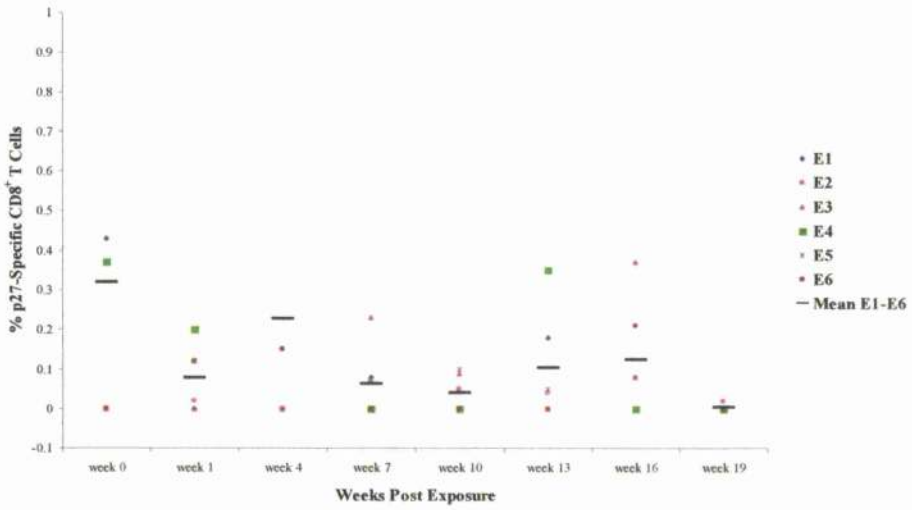
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
Mean E1-E6

E


% p27-Specific CD8+ T Cells
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
Mean E1-E6

F

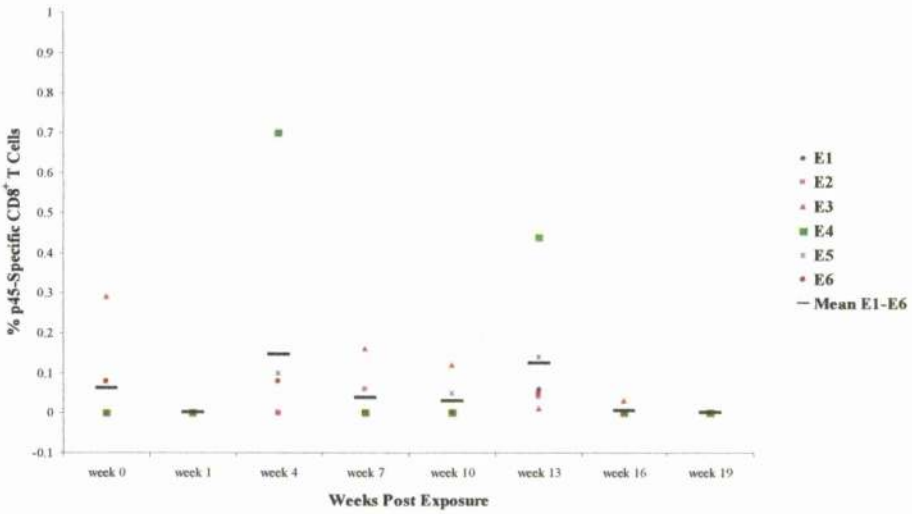

% p45-Specific CD8+ T Cells
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
week 0
week 1
week 4
week 7
week 10
week 13
week 16
week 19
Weeks Post Exposure
E1
E2
E3
E4
E5
E6
Mean E1-E6

Fig. 4.9 Virus-specific $CD4^+$ and $CD8^+$ T cells in the peripheral blood from cats E7-E12, detected using ICS

Graphs depicting the expression of fIFN-γ by $CD4^+$ T cells (A-C) and $CD8^+$ T cells (D-F) in the peripheral blood following a 4 h stimulation with either inactivated FeLV particles (A, D), or recombinant p27 (B, E) or p45 proteins (C, F). The gates that determined fIFN-γ expression were set according to the reactivity of the isotype-matched control-FITC mAb. In total, 10, 000 events were counted. Feline IFN-γ expressing $CD4^+$ and $CD8^+$ T cells within the lymphoblast population were enumerated from cats E7-E12 at regular intervals following experimental exposure to FeLV at week 0. These data were corrected by deducting the percentage of any $CD4^+$ and $CD8^+$ T cells that expressed fIFN-γ, either in the absence of virus-specific stimulation, or prior to FeLV exposure, in order to calculate the percentage of virus-specific $CD4^+$ and $CD8^+$ T cells. The data recorded for unstimulated $CD4^+$ and $CD8^+$ T cells are shown in tables (i) and (ii), respectively. On each sample date, the mean percentage of virus-specific $CD4^+$ and $CD8^+$ T cells was calculated (Mean E7-E12). The data are presented as 'not done' (n.d) when samples were not analysed.

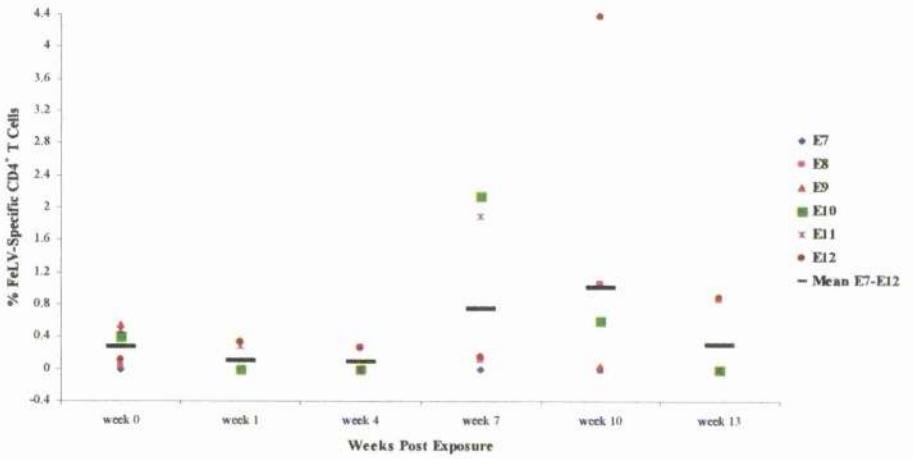
(i)

Cat No.	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13
E7	1.68	0.51	0.66	0.00	0.66	0.93
E8	0.62	0.09	0.00	0.19	0.00	0.40
E9	0.68	0.27	0.71	0.00	0.16	0.00
E10	0.61	0.74	0.65	0.00	0.78	0.52
E11	0.68	0.00	0.30	0.00	4.23	0.56
E12	1.75	0.52	0.19	0.00	0.34	1.67

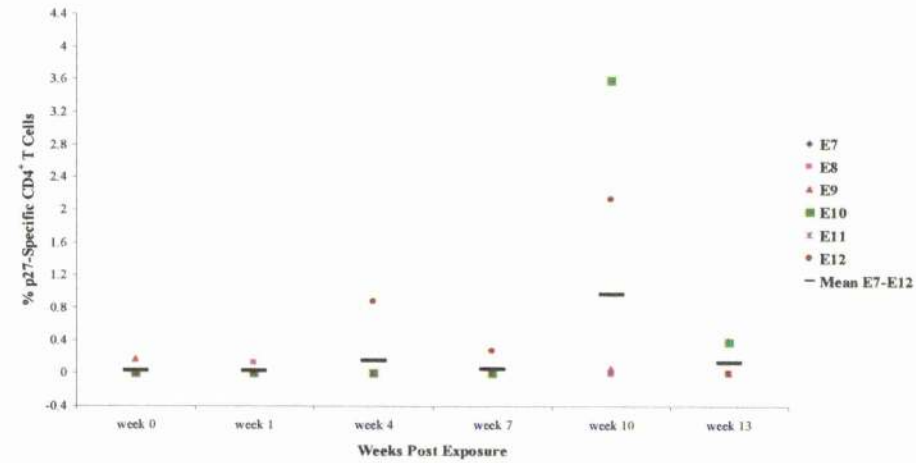
(ii)

Cat No.	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13
E7	1.28	0.30	0.55	0.19	0.53	0.23
E8	2.61	0.19	0.52	0.29	n.d	2.62
E9	0.75	0.16	0.00	0.00	0.36	0.00
E10	0.70	0.28	0.00	0.00	0.56	1.39
E11	1.36	0.13	0.00	0.00	4.66	0.58
E12	0.82	0.33	0.45	n.d	0.56	1.26

A


% FeLV-Specific CD4+ T Cells
4.4
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12
Mean E7-E12

B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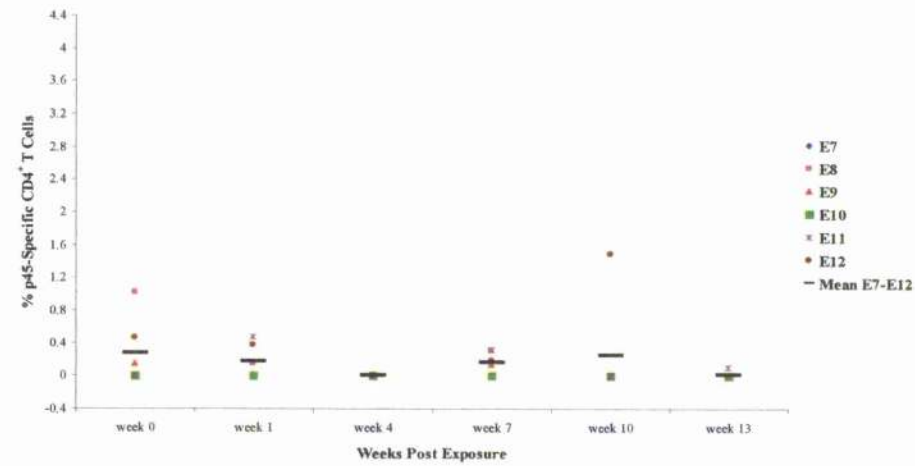
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12
Mean E7-E12

C


% p45-Specific CD4+ T Cells
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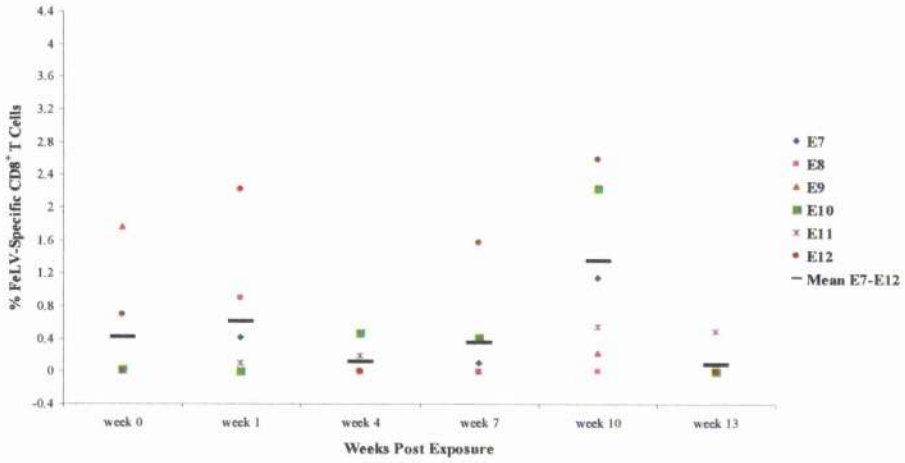
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12
Mean E7-E12

D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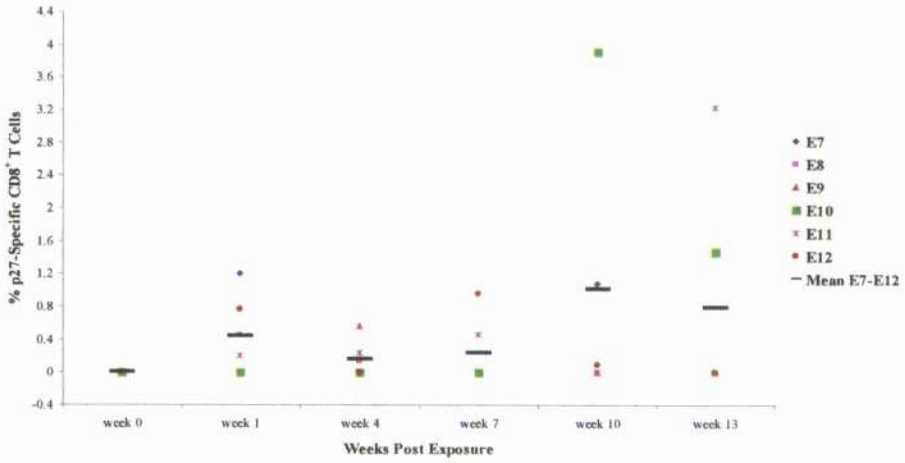
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12
Mean E7-E12

E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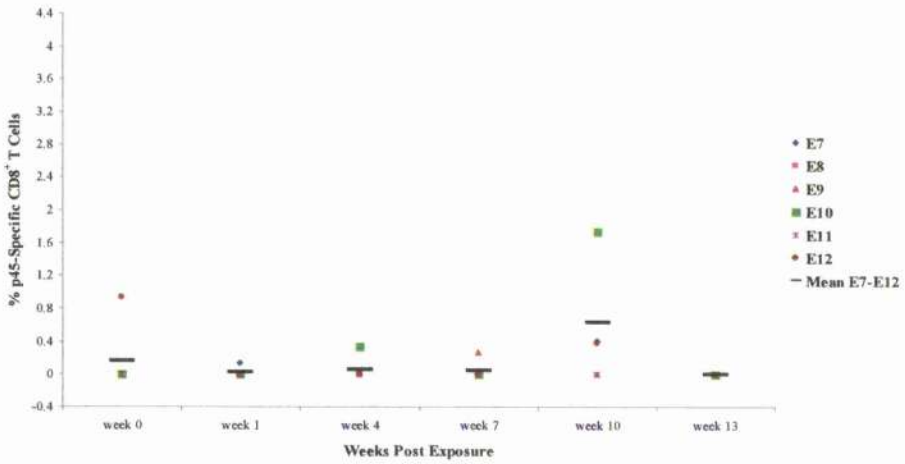
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12
Mean E7-E12

F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4.4
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
week 0
week 1
week 4
week 7
week 10
week 13
Weeks Post Exposure
E7
E8
E9
E10
E11
E12
Mean E7-E12

Fig. 4.10 p27-specific $CD4^+$ and $CD8^+$ T cells in the peripheral blood from cat E12, detected using ICS

Two-colour dot plots A-D displaying the percentage of $CD4^+$ (A-B) and $CD8^+$ T lymphoblasts (C-D) in the peripheral blood from cat E12 that expressed fIFN-γ (quadrants C2 and D2) following a 4 h stimulation with the recombinant p27 protein (B, D) in the presence of BFA. The gates that determined fIFN-γ expression were set according to the reactivity of the isotype-matched control-FITC mAb. In total, 10,000 events were counted. Feline IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells within the lymphoblast population are illustrated from weeks 0-13 following experimental exposure to FeLV. Unstimulated $CD4^+$ (A) and $CD8^+$ T cells (C) were used as controls.

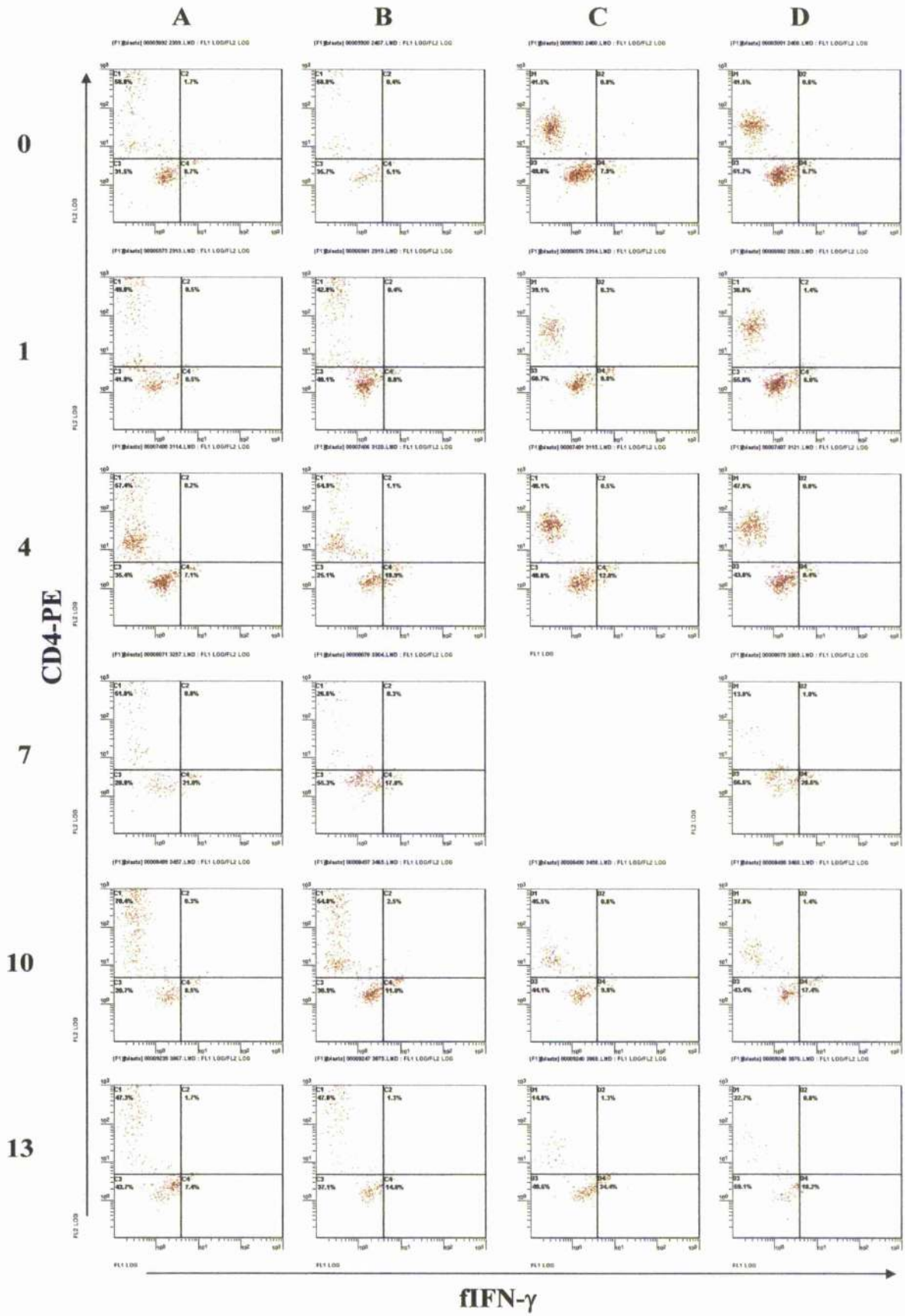
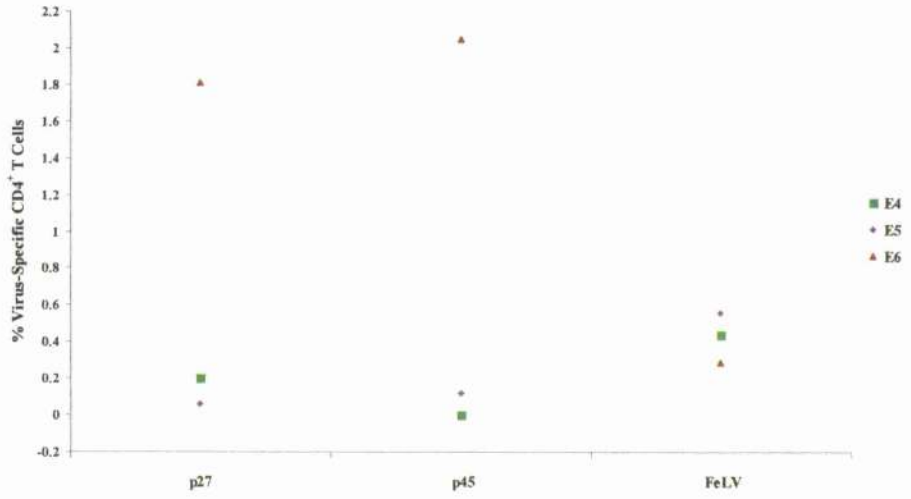

A
B
C
D
0
1
4
7
10
13
CD4-PE
fIFN-γ

Fig. 4.11 Virus-specific $CD4^+$ T cells in the PLN tissues from cats E4-E12, detected using ICS

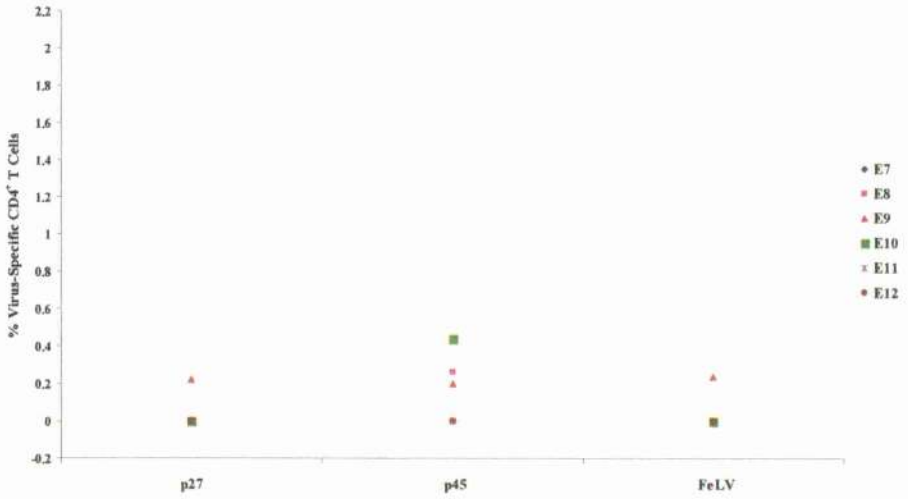
Graphs depicting the expression of fIFN-γ by virus-specific $CD4^+$ T cells in the PLN tissues from cats E4-E6 (A) and cats E7-E12 (B) following a 4 h stimulation with either recombinant p27 or p45 proteins, or inactivated FeLV particles, in the presence of BFA. Feline IFN-γ expressing $CD4^+$ T cells within the lymphoblast population were enumerated in the PLN tissues post mortem, 13 (cats E4-E6) and 19 weeks (cats E7-E12) following oronasal exposure to FeLV. The gates that determined fIFN-γ expression were set according to the reactivity of the isotype-matched control-FITC mAb. In total, 10,000 events were counted. The percentage of virus-specific $CD4^+$ T cells was calculated by deducting the percentage of fIFN-γ expressing cells detected within unstimulated cells; the data from unstimulated cells are displayed in the table below. The data were presented as 'not done' (n.d.) when samples were not analysed.

Cat No.	$CD4^+$
E4	0.25
E5	0.28
E6	0.00
E7	0.00
E8	0.52
E9	0.30
E10	n.d
E11	1.35
E12	0.45

A


% Virus-Specific CD4+ T Cells
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
p27
p45
FeLV
E4
E5
E6

B


% Virus-Specific CD4+ T Cells
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
p27
p45
FeLV
E7
E8
E9
E10
E11
E12

4.4 Discussion

The primary aim of the longitudinal study was to correlate immune responses with viral burden, and in so doing, establish the role of host immunity in disease outcome. As previously mentioned, there are three possible outcomes following exposure of cats to FeLV, namely persistence, recovery and latency. However, the results presented in Chapter 3 clearly demonstrate that eleven out of twelve cats in this study became persistently viraemic and one cat became latently infected. This occurred in spite of the fact that around fifty *per cent* of the cats were expected to recover, given their age at exposure and the exposure dose of virulent FeLV (Hoover *et al.*, 1976; Jarrett *et al.*, 1982a; Flynn *et al.*, 2002b). It is not clear why no cats spontaneously recovered. Indeed, other studies in our laboratory conducted at the same time as this study did show recovery, despite using higher challenge doses (Donovan, L., PhD thesis, University of Glasgow). Thus, in this chapter the FeLV-specific immune responses in peripheral blood were compared with the levels of proviral DNA in the blood throughout infection, and proviral DNA loads in lymph nodes and spleen were compared with the FeLV-specific cellular immune responses present in those lymphoid tissues post mortem.

The recombinant p27 protein expressed in *E. coli* is identical in size to the FeLV CA protein p27, as indicated in a Coomassie Brilliant Blue-stained SDS-PAGE gel (Fig. 4.1.I). Serum from an FeLV recovered cat, which was shown to harbour VNA, and is likely to contain additional anti-FeLV antibodies (Lutz *et al.*, 1980b; Charreyre and Pedersen, 1991; Hawks *et al.*, 1991; Flynn *et al.*, 2002b), recognised and bound to antigenic determinants within the recombinant p27 protein in a Western blot. These results demonstrate that the recombinant p27 protein shared antigenic determinants with FeLV; therefore, this recombinant protein has the potential to elicit antigen-specific responses in any cat exposed to this protein (Fig. 4.1.II). The FeLV SU protein gp70 expressed in *E. coli* is non-glycosylated, and is approximately 45 kDa in size. The recombinant p45 protein contained antigenic determinants recognised by sera from FeLV-recovered cats in a Western blot, indicating that the recombinant p45 protein also has the potential to be antigenic, and is therefore useful in this study (Fig. 4.1.V). Furthermore, a recombinant p45 protein forms the basis of a vaccine commercially available in the U.K. ('Leucogen', Virbac S.A, France),

which is known to elicit protective immunity against FeLV (Marciani *et al.*, 1991; Jarrett and Ganière, 1996).

The recombinant FeLV proteins generated in this thesis were not tested for the presence of endotoxin. An endotoxin is a microbial protein that acts as a superantigen, *i.e.* a potent non-specific polyclonal activator of $CD4^+$ T cells; in addition, endotoxin-primed $CD4^+$ T cells may enhance antigen-specific responses (Torres et al., 2002). Thus, any endotoxin present in the recombinant protein preparations would be expected to exert a mitogenic effect on the $CD4^+$ T cells present in the LPA cultures. However, the data generated using these recombinant proteins in the proliferation assays were consistently low, suggesting the absence of endotoxic activity (Figs. 4.2-4.3 and 4.7).

Data from the LPA can be expressed in two principal forms, as S.I., or as the net count (cpm). The data generated from the proliferation assays in this thesis were presented as S.I. There are advantages associated with both forms of data presentation. Significant inter-laboratory variation in net cpm can occur due to the variations in the efficiency of liquid scintillation counters; therefore, for comparative purposes, the S.I. is a more useful unit. Furthermore, variations in cell preparation and culture conditions are minimised by calculating the S.I., as both the unstimulated and stimulated cells should be similarly affected. However, under certain circumstances, the S.I. may obscure important information. Since the S.I. is a ratio, it is particularly vulnerable to changes in the background data. High levels of background proliferation may cause misleadingly low S.I. results. Such high counts may indicate that cultured cells were activated *in vivo* prior to sampling, or that APC within the mononuclear cell preparation were loaded with viral antigen *in vivo* and therefore were capable of activating $CD4^+$ and $CD8^+$ T cells *ex vivo*. These phenomena are particularly relevant in this thesis, where the majority of cats were persistently viraemic. However, the S.I. are calculated from the raw cpm data; thus, aberrant background counts should be readily detectable and reported in the analyses. In this thesis, background cpm levels were generally very low (Figs. 4.2-4.3 and 4.7). At week 10, cats E10 and E12 generated higher levels of background cpm than previously recorded. However, the antigen-specific responses in these cats were also proportionately higher, which suggests that the cells were subject to between-week

laboratory variations. Because of the low background counts recorded over the course of the study, it was considered that the S.I. was an appropriate means of data presentation in this thesis.

Lymphocyte proliferation assays were conducted to monitor the development of virus-specific cell-mediated immunity, particularly $CD4^+$ T cell responses, in the peripheral blood and lymphoid tissues from cats E1-E12, following oronasal exposure to FeLV. In addition, the general immune competence of these cats was evaluated by assessing the ability of mononuclear cells to proliferate in response to Con A stimulation (Torten *et al.*, 1991; Lawrence *et al.*, 1992). When feline mononuclear cells are cultured with Con A *in vitro*, the proportion of $CD4^+$ and $CD8^+$ T cells within the proliferating population increases, compared to unstimulated cells, whereas $CD21^+$ B cell numbers decline (Figs. 4.4.C and 4.5.C). Thus, Con A-induced proliferation is a good indicator of T cell function in the cat.

Marked variations were observed in the ability of mononuclear cells in the peripheral blood and lymphoid tissues to proliferate in response to Con A (Figs. 4.6 and 4.7.D). Differences were detected in between-week samples, as well as between-cat samples. Several reasons are proposed in explanation; first, periodic discrepancies may be accounted for by the occasional use of frozen mononuclear cells, i.e. in the peripheral blood from cats E1-E6 (weeks 0-1), cat E2 (weeks 0-19) and cat E6 (week 19); and in the PLN tissues from cats E1-E6. However, on occasions where only fresh mononuclear cells were used, proliferative responses to Con A were still distinctly diverse, e.g. the S.I. recorded using PBMC from cats E7-E12 at week 1 ranged from S.I. 16.24 (E12) to S.I. 93.5 (E9); moreover, a four-fold increase in proliferative responses were detected in PBMC from cat E7 over weeks 1 (S.I. 17.8) to 4 (S.I. 79.33).

Second, PBMC from FeLV-infected cats have been shown to be immunologically compromised *in vivo* (Olsen *et al.*, 1977; Mathes *et al.*, 1979; Trainin *et al.*, 1983), with a reduced ability to respond to Con A stimulation in the LBT *in vitro* (Cockerell *et al.*, 1976). These findings suggest that the FeLV-infected cats in the present study may also be subject to FeLV-induced immunosuppression *in vivo,* which could compromise the ability of mononuclear cells to proliferate in response to mitogenic stimulation *ex vivo*. Variations in the level of mitogen-induced proliferation among

cats E1-E12 in this study may reflect viral burdens, and therefore the degree of immunosuppression within each cat. Mitogenic proliferative responses in the peripheral blood and lymphoid tissues were greater in cats that also recorded the lowest proviral DNA loads in each of those tissues (Section 3.3.1.3). However, such proliferative responses did not significantly correlate with proviral DNA loads using the Spearman r-Test.

Third, inactivated FeLV particles, particularly the p15E constituent, have been shown to have an immunosuppressive effect on the proliferative and secretory function of normal feline and human cells *in vitro* (Hebebrand *et al.*, 1977; Mathes *et al.*, 1978; Hebebrand *et al.*, 1979; Copelan *et al.*, 1983; Stiff and Olsen, 1983; Engelman *et al.*, 1984; Orosz *et al.*, 1985; Lafrado *et al.*, 1987). Therefore, any virus present within cell cultures prepared from viraemic cats in this study may have interfered with normal mitogenic responses.

Fourth, it is well known that proliferation assays are subject to great variability as a result of normal biological variation within and between individual humans (Froebel *et al.*, 1999; Picker and Maino, 2000). Mononuclear cells in the peripheral blood and lymphoid tissues from normal cats also demonstrate a wide range of proliferative responses to Con A in the LPA (Flynn, J.N., personal communication). Indeed, variation among individual cats was observed in this study; prior to FeLV exposure, S.I. recorded using fresh PBMC ranged from S.I. 3.19 (cat E12) to S.I. 43.88 (cat E8) (Figs. 4.2 and 4.3). Poor mitogenic responses in some cats might render virus-specific proliferative responses more significant; cats such as E4 and E6 that recorded high levels of mean mitogenic proliferative responses in the peripheral blood tended to demonstrate strong virus-specific responses, whereas cat E12 demonstrated both poor mitogenic and virus-specific proliferative responses (Figs. 4.3 and 4.4.B).

The magnitude of p27-specific and p45-specific proliferative responses in the peripheral blood was similar in groups of cats exposed to different doses of infectious FeLV. However, a temporal shift in the peaks of mean specific proliferative responses to both recombinant p27 and p45 proteins was observed, which was related to the exposure dose. Thus, cats that received a higher exposure dose of FeLV by a factor of one log, recorded mean peaks in proliferative responses

at weeks 4 and 10, while cats that received a lower exposure dose peaked at weeks 7 and 13. Since proviral DNA loads were significantly higher at weeks 1 and 4 in peripheral blood from cats that received the higher exposure dose (Section 3.3.1.3), these cats might be expected to develop virus-specific responses earlier. In another study, cats that developed an early peak in antigenaemia recovered from FeLV, whereas cats that failed to develop an early peak in antigenaemia became persistently viraemic; the authors suggested that the early antigenaemia may have primed the immune response (Flynn *et al.*, 2002b). In this study, early virus-specific proliferative responses were not a good predictor of recovery, in that all five cats that generated significant p27-specific proliferative responses at week 4 became persistently viraemic. Overall, the magnitude of mean p45-specific proliferative response in the peripheral blood was less than that observed for mean p27-specific proliferative responses, irrespective of the exposure dose; however, the difference was not statistically significant (Section 4.3.2.1.1).

The inability of inactivated FeLV particles to induce a S.I. greater than 2 in any cat over the course of the study was surprising, given that the principal viral constituents, p27 and p45 respectively, were able to induce specific proliferative responses in most cats. A number of explanations can be offered. First, immunosuppressive factors may have been present within the inactivated FeLV particles. p15E, a recognised immunosuppressive element (Mathes *et al.*, 1979; Copelan *et al.*, 1983; Lafrado *et al.*, 1987), was visible on the Coomassie-stained SDS-PAGE gel, indicating that this protein was retained within the inactivated FeLV particles used in these studies (Fig. 4.1.I). To ascertain whether inactivated FeLV particles were in fact immunosuppressive, the degree to which these particles inhibited the mitogen-induced proliferation of normal feline cells in the LPA could be evaluated. Second, virus-specific proliferative responses would be subject to the same immunosuppressive factors as described for mitogenic proliferative responses. Third, key antigenic determinants may have been denatured by heat inactivation. The protein structure of the virus is severely disrupted by the boiling process; and important antigenic determinants may not have been preserved. It may have been more prudent to inactivate FeLV using UV light. Nucleic acids are the most important absorbers of UV light; therefore, such treatment causes photochemical damage to viral RNA, which inhibits viral replication. Since the protein structure of

the virus is preserved, the antigenic determinants should remain intact. Nonetheless, using ICS, specific $CD4^+$ and $CD8^+$ T cell responses to inactivated FeLV particles were detected in the majority of cats, suggesting that antigenic epitopes were retained within the inactivated FeLV particles. Fourth, inactivated FeLV particles were used at the same concentration (25 $\mu g/10^6$ cells) as both recombinant p27 and p45 proteins; thus, the relative amounts of viral core protein and envelope glycoprotein corresponding to recombinant p27 and p45 proteins, respectively, would be much lower. However, titration studies conducted using the inactivated FeLV particles demonstrated that increased viral concentrations did not elicit greater proliferative responses.

It has previously been shown that FeLV recovered cats generate high levels of Gag/Pro-specific CTL in the peripheral blood shortly after oronasal exposure to FeLV, whereas persistently viraemic cats develop delayed and reduced levels of Gag/Pro-specific CTL (Flynn *et al.*, 2002b). On this evidence, we proposed that Gag-specific $CD4^+$ T cells might be important in eliciting protective immune responses following oronasal exposure to FeLV. To investigate this, we evaluated the ability of $CD4^+$ T cells in the peripheral blood from cats E1-E12 to proliferate, and to express fIFN-γ, in response to stimulation with the recombinant p27 protein. Concurrent virus-specific $CD8^+$ T cell responses were analysed using ICS, while the numbers of proliferating $CD8^+$ T cells, and either $CD21^+$ B cells or $CD45R^+$ WBC, were recorded in the LPA.

Using ICS, high frequencies of p27-specific $CD4^+$ T cells were detected in the peripheral blood from latently infected cat E12 four weeks following exposure to FeLV, which were maintained until week 13. Moderately high numbers of p27-specific $CD8^+$ T cell responses were detected in the peripheral blood from this cat at weeks 1 and 7-10. Conversely, in the LPA, p27-specific $CD4^+$ or $CD8^+$ T cells were not detected in the peripheral blood from latently infected cat E12 at any time throughout the study. p27-specific $CD4^+$ T cell responses were detected in ten persistently infected cats following oronasal exposure to FeLV using ICS; three of which, E3, E6 and E8, had developed such responses by week 4. However, these responses either rapidly declined, or disappeared altogether. The remaining persistently infected cats generated transient levels of p27-specific $CD4^+$ T cell

responses after week 10. All persistently infected cats generated p27-specific $CD8^+$ T cells in the peripheral blood; seven of which had generated such responses by week 1. Cat E11 maintained p27-specific $CD8^+$ T cells responses over weeks 1-7; however, such early responses were transient in each of the remaining cats. Stimulation indices greater than, or in excess of, 2 were recorded in sixteen peripheral blood samples from seven persistently infected cats throughout the study in response to p27-specific stimulation. In ten of these samples, from cats E4, E6, E9, E10 and E11, such responses were associated with p27-specific $CD4^+$ T cells. These cells were detected early following exposure in just two cats, E4 and E6.

In a previous study, differences in Env-specific CTL in the peripheral blood between recovered and persistently viraemic cats were not significant (Flynn *et al.*, 2002b). In this study, Env-specific $CD4^+$ T cell responses in the peripheral blood were investigated by assessing the ability of mononuclear cells from cats E1-E12 to proliferate, and to express fIFN-γ, in response to stimulation with recombinant p45 protein.

Moderate frequencies of p45-specific $CD4^+$ T cells were detected in the peripheral blood from latently infected cat E12 shortly after exposure and over weeks 7-10, using ICS. Concurrent $CD8^+$ T cell responses were not detected in the peripheral blood from this cat until week 10. However, in the LPA, neither p45-specific $CD4^+$ nor $CD8^+$ T cells were detected in the peripheral blood from cat E12 at any time throughout the study. Using ICS, p45-specific $CD4^+$ T cells were detected in the peripheral blood from nine persistently infected cats; four of which had developed such responses by week 1. However, no persistently infected cat generated high levels of p45-specific $CD4^+$ T cells. Nine persistently infected cats developed p45-specific $CD8^+$ T cell responses in the peripheral blood. High frequencies of such cells were detected in the peripheral blood from cats E4 (weeks 4 and 13), E10 (weeks 10-13) and E11 (week 13). Stimulation indices greater than, or in excess of, 2 were recorded in ten peripheral blood samples from eight persistently viraemic cats in response to p45-specific stimulation. These were associated with p45-specific $CD4^+$ T cells in six samples, from cats E1, E8, E9, E10, E11. However, in this assay, p45-specific $CD4^+$ T cells were not detected in any cat until week 7 (cats E9 and E11).

Although S.I. greater than, or in excess of 2, were not recorded in the peripheral blood from any cat in the LPA in response to stimulation with inactivated FeLV particles, such responses were identified using ICS. Overall, specific $CD4^+$ T cell responses to inactivated FeLV particles were higher than to the recombinant p45 protein, irrespective of the exposure dose (Section 4.3.2.2.1). Specific $CD4^+$ T cell responses to inactivated FeLV particles were detected throughout the course of the study in the peripheral blood from latently infected cat E12 with high levels of concurrent specific $CD8^+$ T cell responses to inactivated FeLV particles at weeks 1 and 7-10. Four persistently infected cats developed early peaks in specific $CD4^+$ T cell responses to inactivated FeLV particles; cat E3 maintained these responses over weeks 1-10. Cat E10 developed high frequencies of specific $CD4^+$ T cell responses to inactivated FeLV particles at week 7, with concurrent high levels of $CD8^+$ T cell responses.

FeLV-recovered cats developed higher levels of Gag-specific CTL in lymphoid tissues, compared to persistently viraemic cats (Flynn *et al.*, 2000a). In this study, virus-specific responses were examined in the lymphoid tissues from cats E1-E12 post mortem in the LPA and using ICS. p27-specific proliferative responses were detected in the MLN tissues from cat E12. In addition, p27-specific $CD8^+$ T cell responses were detected in the PLN tissues from latently infected cat E12 and from persistently infected cats E4, E5, E6 and E9, using ICS. p45-specific proliferative responses and p45-specific $CD8^+$ T cells were detected in the MLN tissues from latently infected cat E12. Persistently infected cats E7 and E9 recorded S.I. greater then, or equal to 2, in response to p45-specific stimulation. Using ICS, p45-specific $CD4^+$ T cells were detected in the PLN tissues from persistently infected cats E5, E6, E8, E9 and E10, which were particularly high in cat E6.

The virus-specific $CD4^+$ T cell responses presented for recovery and latent infection were based on the data generated by a single cat, E12. As we have seen, a wide range of virus-specific $CD4^+$ T cell responses were recorded for persistently infected cats; thus, it is important that virus-specific $CD4^+$ T cell responses are compared among a significant number of recovered cats. In addition, no results were presented for spontaneously recovered cats.

The data generated in the present study indicated that there was a poor correlation between results obtained using the LPA and ICS in the detection of virus-specific $CD4^+$ T cells. Intracellular cytokine staining is a highly sensitive assay, which facilitates the enumeration of individual virus-specific cells. Mononuclear cells are stimulated for a mere four hours *ex vivo*; therefore, $CD4^+$ T cells detected using this assay are in the early stages of a virus-specific response when fIFN-γ expression and secretion is optimal. Effector T cells rapidly produce high levels of cytokines upon activation, particularly IFN-γ (Fitzpatrick *et al.*, 1998). A four-hour period of restimulation was used in this thesis to generate these data. However, as discussed in Chapter 2, this may have been insufficient time to elicit optimal fIFN-γ expression. Thus, levels of fIFN-γ expresssion recorded by cats in this thesis may not reflect optimal fIFN-γ production by $CD4^+$ and $CD8^+$ T cells.

Results from the LPA are acquired after three days of culture. In the interim, fIFN-γ expression by virus-specific effector cells has ceased, and other cytokines characterised by different secretion profiles predominate, such as IL-2 (Swain, 1999). Therefore, the profile of the virus-specific cells detected using each of these techniques is fundamentally different. Second, results from the LPA reflect both the absolute numbers of responsive cells plated out and the ability of these cells to exponentially expand *in vitro,* which may be altered by factors such as the presence of inhibitory viral elements, viral destruction of responding cells and apoptosis of responding cells. The relevant antigen-specific $CD4^+$ T cells may be present but dysfunctional, reflecting either an inability to proliferate *in vivo,* or a novel dysfunction acquired *in vitro* (Palmer *et al.*, 2002). As we have seen in the present study, idiosyncratic variation among individual cats may affect the results. Third, the processing and presentation of Gag and Env soluble antigens with MHC class I molecules *in vitro* may be distinct from pathways that express the same proteins *in vivo* during natural infection. Fourth, virus-specific $CD4^+$ T cell precursors may be present but difficult to expand *in vitro,* or present at such low frequencies that they are not detected in LPA (Musey *et al.*, 1999). The LPA may underestimate the frequencies of virus-specific cells, particularly when the starting proportion of $CD4^+$ T cells in the mononuclear preparation is low, thereby decreasing the absolute numbers of responding cells in each well (Picker and Maino, 2000). Fifth, LPA require multiple days of culture *in vitro*, and are susceptible to factors that

differentially influence cell viability during these long incubations (Pitcher *et al.*, 1999).

Although both assays are vulnerable to the immunosuppressive factors previously outlined, the enhanced sensitivity of ICS used in conjunction with flow cytometry in the detection of individual antigen-specific cells means that low frequencies of virus-specific cells consequent to inadequate antigen concentration or immunosuppression can be identified and enumerated.

Moderate levels of fIFN-γ secretion were recorded for the unstimulated mononuclear cells in the peripheral blood and lymphoid tissues from cats E1-E12 over the course of the study (Figs. 4.8-4.11). Common causes of background staining were discussed in Chapter 2. In addition, fIFN-γ expression was detected at week 0 in many cats E1-E12. In cats E1-E6, good separation of the cell populations was not achieved at week 0; thus, it was difficult to gate solely on the lymphoblast population. In cats E7-E12, a different batch of D9-FITC mAb was used compared to the rest of the study, and appeared to detect much higher levels of fIFN-γ in both the unstimulated and the stimulated cells than the subsequent batch of mAb, suggesting the presence of high levels of non-specific staining. Therefore, it is highly probable that the data recorded at week 0 from either group of cats were inaccurate.

In the dot plots presented for cat E12, the majority of fIFN-γ expressing cells in the antigen-stimulated populations were not $CD4^+$ T cells (Fig. 4.10). Similar results for mitogen-activated cells were reported and discussed in Chapter 2. Natural killer cells are a population of large granular lymphocytes, which constitute important effectors and regulators of the innate immune response, particularly against intracellular pathogens. Thus, these cells are particularly relevant to FeLV infection. Natural killer cells are readily activated via specialised surface receptors, and can only be inactivated through the detection of MHC class I molecules by cell surface inhibitory receptors (Moretta. *et al.*, 2002a; Moretta. *et al.*, 2002a). In humans, two subsets of NK cells with diverse effector functions have been identified based on expression of the CD56 molecule. $CD56^{bright}$ NK cells are major producers of IFN-γ and other cytokines, whereas $CD56^{dim}$ NK cells can lyse target cells in the absence of prior

activation. $CD56^{bright}$ NK cells constitutively express multiple monokine receptors, and can produce IFN-γ and other cytokines rapidly in response to stimulation by monokines, such as IL-12, IL-18 and IL-15 (Cooper *et al.*, 2001). Natural killer-like cells with cytotoxic function have been described in the spleen and peripheral blood from normal cats (Tompkins and Tompkins, 1985). Thus, these cells, which may be incorporated into the gated lymphoblast population, may be partly responsible for the high levels of fIFN-γ expression detected using these reagents.

Some significant differences in virus-specific proliferative responses were observed between groups of cats that were exposed to different doses of infectious FeLV. Cats exposed to a lower exposure dose of FeLV by a factor of one log, developed significantly greater p27-specific and p45-specific proliferative responses in the PLN and MLN tissues respectively, than cats exposed to a higher exposure dose; these cats also generated significantly greater mitogenic responses in the MLN tissues. In addition, cats that were exposed to the lowest FeLV exposure dose also recorded the lowest proviral DNA loads in the peripheral blood over much of the study (weeks 1, 4, 10 and 13), and in all the lymphoid tissues examined post mortem.

4.5 Summary and Conclusions

All twelve cats developed p27-specific and/or p45-specific $CD4^+$ T cell responses in the peripheral blood following oronasal exposure to FeLV. In persistently infected cats, early virus-specific $CD4^+$ and $CD8^+$ T cell responses were frequently transient or low, whereas higher frequencies of p27-specific and p45-specific responses were often detected later in the study. Only persistently infected cats generated virus-specific proliferative responses in the peripheral blood. Four persistently infected cats generated p27-specific responses in the PLN and MLN tissues, while seven cats generated p45-specific responses in those tissues.

Latently infected cat E12 developed high frequencies of p27-specific $CD4^+$ T cells in the peripheral blood over weeks 4-10; p27-specific $CD8^+$ T cells were detected at weeks 1 and 7-10. Furthermore, p45-specific $CD4^+$ T cell responses were detected in the peripheral blood from this cat at weeks 1 and 7-10, with concurrent p45-specific

$CD8^+$ T cells at week 10. This cat maintained specific $CD4^+$ T cell responses to inactivated FeLV particles over the course of the study (weeks 1-13), with concurrent CD8 responses at weeks 1 and 7-10. Low numbers of p27-specific and p45-specific $CD8^+$ T cells were detected in the PLN tissues from latently infected cat E12 post mortem. In addition, p27-specific and p45-specific proliferative responses were detected in the MLN tissues from this cat.

CHAPTER 5

LONGITUDINAL IMMUNOPATHOGENESIS STUDY

III FeLV EXPRESSION IN THE BONE MARROW AND PLN

5.1 Introduction

The bone marrow and lymphoid tissues are critical sites of FeLV integration, replication and expression. Following oronasal exposure, FeLV initially replicates in tonsillar lymphocytes and macrophages, before being conveyed to the draining lymph nodes of the head and neck (Rojko *et al.*, 1979). Exposed cats may recover at this point, indicating that virus-specific immune responses have successfully eliminated virus within the lymph nodes. Conversely, failure to purge the lymph nodes of virus precipitates a cell-associated viraemia, which disseminates FeLV to the bone marrow, gut and other lymphoid organs (Rojko and Kociba, 1991). In the absence of antiviral immunity, extensive replication in these tissues precedes the dispersal of virus to epithelial and glandular tissues throughout the host; these events can occur as early as 3 weeks following exposure (Jarrett, 2001).

Virus replicates easily in the actively dividing precursor cells within the bone marrow. Using the IFA test on bone marrow smears prepared from FeLV-infected cats, FeLV CA protein p27 was detected in neutrophil and erythrocyte precursor cells (Hoover *et al.*, 1977b); in addition, immunohistochemical studies identified the FeLV CA protein p27, and SU protein gp70, chiefly in association with megakaryocytes (Kovacevic *et al.*, 1997). In lymphoid tissues, FeLV has a predilection for the follicular B cell (Rojko *et al.*, 1979; Rojko *et al.*, 1981). In lymph node sections from FeLV-infected cats, expression of the FeLV CA protein p27 was mainly restricted to the lymph node follicles, in close association with the FDC network, while the FeLV SU protein gp70 was demonstrated in the follicles, paracortex and medulla (Kovacevic *et al.*, 1997; Kipar *et al.*, 2000).

Many degenerative and proliferative diseases arise because of FeLV activity in the bone marrow and lymphoid tissues. Bone marrow haematopoiesis is impaired; in addition to the direct cytopathic effect of replicating virus on infected cells, some FeLV-infected cats experience a maturation arrest at the myelocyte and metamyelocyte stage in granulocytic haematopoiesis (Shelton and Linenberger, 1995). Thus, neutropenia is a common symptom of persistent infection (Latimer, 1995), and may be accompanied by neutrophil dysfunction (Lewis *et al.*, 1986).

Furthermore, FeLV-C greatly impairs the differentiation of early erythroid progenitor cells within the bone marrow, inhibiting erythrocyte production and causing a primary non-regenerative anaemia (Onions *et al.*, 1982; Hoover and Mullins, 1991). Moreover, bone marrow and lymphoid tissues are important sites of FeLV-associated oncogenesis. Myeloid and lymphoid tumours can originate in either tissue; however, 90% of the tumours characterised in the U.S. were lymphoid, or LSA (Hardy, 1981b).

These pathogenesis studies illustrate that the outcome of infection is influenced by early encounters between FeLV and the antiviral immune response in the bone marrow and lymphoid tissues. Furthermore, they demonstrate that virus integration, replication and expression in the bone marrow and lymphoid tissues have important disease consequences. Impaired haematopoiesis may interfere with the capacity of infected cats to overcome active FeLV infection and combat secondary infection; moreover, virus activity in these tissues may trigger the development of a fatal FeLV-associated disease, such as non-regenerative anaemia, or LSA.

Following oronasal exposure to FeLV, eleven cats in this study became persistently infected, and one cat retained a latent infection in the bone marrow and lymphoid tissues (Chapter 3). In this chapter, we investigated whether the expression of FeLV CA protein p27 in the bone marrow and PLN tissues correlated with the disease outcome or the antiviral immune response characterised in Chapters 3 and 4. In addition, we attempted to identify the cells that were associated with viral proteins in the bone marrow and PLN tissues, to determine whether this was relevant to the immunopathogenesis of FeLV-associated disease.

Bone marrow, lymphoid tissues and peripheral blood were sampled post mortem, thirteen (cats E7-E12) and nineteen (cats E1-E6) weeks following oronasal exposure to FeLV. To detect virus expression in the bone marrow and PLN tissues, cytospin preparations of bone marrow, and cryostat sections of PLN tissues, were probed with a sheep anti-p27 pAb in an immunofluorescent, and an immunohistochemical, technique, respectively. The impact of virus expression on haematopoiesis was determined through concurrent cytological and haematological analyses of bone marrow and peripheral blood smears, respectively. Bone marrow cytospin

preparations were stained with May-Grünwald-Giemsa for cytological assessment; peripheral blood smears were similarly stained to calculate the WBC differential count.

An abnormally large population of mature lymphoid cells was identified within the bone marrow cytospin preparations from nine cats. To characterise these cells, paraffin-embedded sections were prepared from formalin-fixed bone marrow tissue, and stained with anti-T and anti-B cell mAbs, in an immunoperoxidase technique.

5.2 Materials and Methods

5.2.1 Bone Marrow and Peripheral Blood

Bone marrow tissue was harvested from the femoral bones of cats E1-E12 post mortem (Section 3.2.4). The tissue was distributed among sterile containers containing 5% BSA in PBS, 10% formalin, or BMTM, for cytological and immunofluorescent analysis, immunohistochemistry, and VI, respectively. A description of the VI techniques conducted on bone marrow tissues harvested in BMTM was given in Chapter 3; the remaining techniques will be described below.

While under deep terminal anaesthesia, a 1 ml sample of peripheral blood was collected from cats E1-E12 in EDTA blood tubes, for haematological analysis.

5.2.1.1 Immunofluorescence

The expression of the FeLV CA protein p27 in the bone marrow from FeLV exposed cats E1-E12, was determined using a sheep anti-p27 pAb in an immunofluorescent technique on bone marrow cytospin preparations.

Bone marrow harvested into bijoux containing 5% BSA in PBS was transported on ice to the Clinical Diagnostics Laboratory. Bone marrow spicules were gently washed from the tissue in a test tube using a 1 ml syringe. The suspended marrow was then pelleted, and resuspended in approximately 50 µl 5% BSA in PBS.

Conventional wedge smears were prepared from the marrow pellet using a cytocentrifuge, and air-dried; a proportion of which were retained for cytological analysis (Section 5.2.1.2). The remainder were air-dried overnight, and then fixed in ice-cold acetone for ten minutes. Slides were wrapped in 'Parafilm' and aluminium foil, and stored at -20°C until use.

Immediately before staining, frozen slides were brought to RT while still wrapped in 'Parafilm'. A small area of cells was encircled with the 'Pap pen' to minimise reagent wastage, then hydrated in wash buffer (PBS-T), for five minutes. The slides were subsequently incubated with 50 µl sheep anti-p27 pAb, diluted 1:500 in wash buffer, in a humidified chamber for one hour at 37°C. Following two, five-minute washes in PBS-T, the slides were incubated with a FITC-conjugated anti-sheep Ig pAb (Diagnostics Scotland, U.K.), diluted 1:1000 in PBS-T, for one hour at 37°C. The slides were washed twice over ten minutes in PBS-T and rinsed in deionised water. The slides were then stained in 0.02% Evans Blue for seven minutes to eliminate autofluorescence. Slides were rinsed in running tap water, mounted under water, and examined for fluorescent staining using a UV microscope (Leica Instruments, Germany).

5.2.1.2 Cytology and Haematology

Cytospin preparations (Section 5.2.1.1) were stained with May-Grünwald-Giemsa for cytological assessment. A standard wedge smear was prepared from each peripheral blood sample, and stained with May-Grünwald-Giemsa to calculate the differential WBC count.

5.2.1.3. Immunohistochemistry

An unusually large population of mature lymphoid cells was observed within May-Grünwald-Giemsa stained cytospin preparations of bone marrow from nine persistently infected cats in this study (Section 5.3.1.2). These cells were further characterised using mAbs that recognise T and B cell antigens, in an immunoperoxidase technique.

Formalin-fixed bone marrow tissue was submitted to the Histopathology Laboratory, for the preparation of paraffin-embedded tissue sections. Formalin-fixation preserves cellular morphology very well; however, it has a tendency to mask antigens due to the formation of methylene bridges between reactive sites in different areas of the same molecule, or on adjacent proteins (Mason and O'Leary, 1991). Superheating in denaturing solutions is effective in rupturing some of these cross-links, re-exposing antigens masked by fixation (Cattoretti *et al.*, 1993). This technique is known as antigen retrieval, and is required for the detection of some cell surface antigens. It was known that the feline CD4 and CD8 antigens recognised by the species-specific anti-CD4 and anti-CD8 mAbs (vpg34 and vpg9, respectively) are masked following formalin-fixation (J.J. Callanan, personal communication). In the absence of established antigen-retrieval methods that could be used to re-expose these particular antigens, alternative antibodies were used.

The anti-CD3 and anti-CD79α mAbs (Dako A/S, Glostrup, Denmark), which detect human T and B cells respectively, were used in these experiments. The CD3 and CD79α antigens recognised by these mAbs are also masked by formalin fixation; however, superheating in a 0.01 M sodium citrate buffer pH 6.0, is known to be effective in re-exposing these antigens (C. Nixon, personal communication). The anti-CD3 mAb recognises the human CD3 molecule, which is associated with the TCR, and is therefore highly specific for T cells (Campana *et al.*, 1987). The anti-CD3 mAb also cross-reacts with the CD3 molecule from other species (Jones *et al.*, 1993), including canine CD3. CD79α represents the mb-1 polypeptide, which forms a disulphide-linked heterodimer with the B29 polypeptide. This heterodimer is non-covalently associated with membrane-bound Ig on B cells, forming the B cell antigen receptor complex (van Noesel *et al.*, 1991). The anti-CD79α mAb was raised to the human mb-1 polypeptide, but has been shown to stain B cells in a number of mammalian species, including cattle, horses, monkeys, rats and mice (Mason *et al.*, 1991), as well as dogs.

Bone marrow tissue sections from cats E1-7, E9 and E12 were deparaffinised by immersing in 'Histoclear' for two minutes, then passed through graded alcohols and placed in water. Bone marrow tissue sections were also prepared from an SPF cat,

T10, which was not part of this FeLV immunopathogenesis study. Cat T10 was a control animal in another group of experimental cats; the bone marrow from this cat was used here to assess the normal distribution of B and T cells in an age-matched SPF cat. Endogenous peroxidase activity was blocked by immersing the slides in 0.5% H_2O_2 in methanol for thirty minutes, before returning to water. The sections were subsequently pressure cooked in 0.01 M sodium citrate buffer, pH 6.0, at 15 lb of pressure for seventy-five seconds, followed by three, five-minute washes in TBS-T. All subsequent incubations were conducted in a humidified chamber at RT, and were followed by three, five-minute washes in TBS-T. The tissue sections were encircled using a 'Pap pen'. Non-specific background staining was minimised by a thirty-minute incubation with 1% NRS or 1% normal swine serum (NSS), prior to a two-hour incubation with either anti-CD3 (diluted 1:500 in TBS-T) or anti-CD79α (diluted 1:1000 in TBS-T) mAbs, respectively. This was followed by a thirty-minute incubation with either biotinylated rabbit anti-mouse Ig pAb (Dako A/S, Denmark), or biotinylated swine anti-rabbit Ig pAb (Dako A/S, Denmark), to detect CD3 and CD79α, respectively; both pAbs were diluted 1:200 in TBS-T. Finally, all sections were incubated with streptavidin-peroxidase (Dako A/S, Denmark), diluted 1:1000 in TBS-T, for one hour, before developing colour with DAB. Slides were counterstained using Gills' Haematoxylin; then dehydrated in graded alcohols, cleared and mounted using DPX. Control slides were incubated with either 1% NRS, or 1% NSS in TBS-T, in place of the primary antibody.

5.2.2 Peripheral Lymph Nodes

Peripheral lymph node tissues were harvested from cats E1-E12 post mortem. These tissues were snap frozen in liquid nitrogen and stored at -70°C for future immunohistochemical studies and qRT-PCR analysis, or placed into RPMI 1640 medium for the preparation of single cell suspensions. The methods used to conduct qRT-PCR analysis and prepare single cell suspensions were described in Chapter 3.

5.2.2.1 Single Immunohistochemistry Staining

The expression of the FeLV CA protein p27 in PLN tissues was determined using a sheep anti-p27 pAb in an immunoperoxidase technique on cryostat PLN tissue

sections. In addition, the distribution of the FDC network in PLN tissues was determined using the Ki-M4 mAb (a kind gift from Prof. Parwaresch, University of Kiel), in an immunophosphatase technique. The FDC population resides within primary and secondary lymph node follicles; their cytoplasmic processes extend between neighbouring B cells, forming a complex meshwork (van Der Valk and Meijer, 1992). The FDC retains antigen-antibody complexes bound to Fc receptors, and antigen-antibody-complement complexes bound to complement receptors (Szakal *et al.*, 1983; Schriever and Nadler, 1992). The Ki-M4 mAb recognises the long isoform of the CD21 molecule (CD21L), which is selectively expressed on the FDC population in humans (Parwaresch *et al.*, 1983; Liu *et al.*, 1997) and in felines (Bach *et al.*, 1994; Graham, E.M., MVM Thesis, University College Dublin).

The Histopathology Laboratory prepared cryostat sections of PLN tissues from four cats, E4, E5, E7 and E12. Frozen PLN tissues were embedded in 'O.C.T.', from which 5-8 μm sections were cut using a cryotome and placed onto 'sticky' slides ('SuperFrost slides', Menzel Glaser, Braunscheig, Germany). Slides were fixed in ice-cold acetone for ten minutes, wrapped in 'Parafilm' and aluminium foil, and stored at -20°C until use. Immediately prior to staining, frozen slides were brought to RT while still wrapped in 'Parafilm'. The tissue sections were encircled using a 'Pap pen' and gently agitated in TBS for five minutes. All incubations were carried out at RT in a humidified chamber, and were followed by two, five-minute washes in TBS to remove unbound reagents. The sections were initially incubated with 0.3% H_2O_2 and 0.1% NaN_3 in TBS for twenty minutes, to block endogenous peroxidase activity. To minimise non-specific binding, sections were incubated for twenty minutes with 10% NRS. Excess serum was shaken from the sections, which were subsequently incubated with the primary antibodies, sheep anti-p27 pAb or Ki-M4 mAb, diluted 1:1000 in 1% NRS in TBS, for two hours. This was followed by a one-hour incubation with the secondary reagents, biotinylated rabbit anti-sheep Ig pAb (Southern Biotechnology, U.S.) and AP-conjugated rabbit anti-mouse Ig pAb (Southern Biotechnology, U.S.), diluted 1:2000 and 1:1000 respectively, in 1% NRS in TBS.

Alkaline phosphatase activity was developed using the chromogen Fast Blue BB[30] (FBBB, Sigma-Aldrich, U.K.). A final one-hour incubation with streptavidin-peroxidase, diluted 1:500 in TBS, preceded the development of peroxidase activity using a commercial kit based on the chromogen AEC (Vector Laboratories, U.S.). Sections were washed with tap water to halt colour development, and mounted using an aqueous permanent mounting medium ('UltraMount', Dako A/S, Denmark). Negative control slides were prepared to examine the specificity of the primary mAb; in these slides, the primary mAb was omitted and replaced with 10% NRS.

5.2.2.2 Simultaneous Double Immunoenzyme Staining

The association of the FeLV CA protein p27 with the FDC population in the PLN tissues was investigated using a double immunoenzyme technique. Two unlabelled primary antibodies of different animal host origin were used in an indirect/indirect simultaneous double immunoenzyme staining, based on the original concept described by Mason and Sammons (1978). The protocol used follows many of the procedures described in Section 5.2.2.1. Following the blocking of endogenous peroxidase and non-specific binding, tissue sections were incubated for two hours with a primary antibody cocktail comprising sheep anti-p27 pAb and Ki-M4 mAb, diluted 1:1000 and 1:500 respectively, in 1% NRS in TBS. Sections were further incubated for one hour with a cocktail of secondary pAbs, biotinylated rabbit anti-sheep Ig and AP-conjugated rabbit anti-mouse Ig, diluted 1:2000, and 1:1000 respectively, in 1% NRS in TBS. Streptavidin-peroxidase, diluted 1:500 in 1% NRS in TBS, was added for a further hour prior to the development of colour. Alkaline phosphatase activity was then developed using the chromogen FBBB. The sections were washed with TBS twice over ten minutes before peroxidase activity was developed using the chromogen AEC. Sections were washed with tap water to halt colour development, and mounted using 'UltraMount'. Negative control slides were prepared by replacing the primary antibody cocktail with 10% NRS. The individual reactivity of each primary monoclonal antibody was assessed using single immunohistochemical stainings in the same experiment.

[30] FBBB: 1 mg of FBBB was added to 5 ml of 0.1M tris buffer containing 0.5 mM levamisole and 1% naphthol-AS-MX-phosphate in N, N dimethylformamide; the solution was filtered and applied.

5.3 Results

5.3.1 Bone Marrow and Peripheral Blood

5.3.1.1 Immunofluorescence

Immunofluorescent techniques were employed to identify the FeLV CA protein p27 within bone marrow cytospin preparations. Slides were examined using a UV microscope and photographed; some images are shown in Fig. 5.1. Negative control slides did not show any positive staining. Red blood cells did not emit UV light when examined using the UV microscope but did appear red. The imaging system used displayed all objects in the field in a green light; therefore, green RBC are evident in some of the images shown. This is a technical limitation of the equipment used to photograph these images.

The FeLV CA protein p27 was expressed in bone marrow cytospin preparations from all persistently infected cats E1-E11 (Fig. 5.1). The level of FeLV CA protein p27 expression varied markedly, with distinctly greater numbers of positive cells detected in cats E1-E6 and cat E9. It was difficult to definitively identify each cell that expressed the FeLV CA protein p27 in the bone marrow; cells in the early stages of haematopoiesis cannot easily be distinguished. However, the majority of positive cells were large mononuclear cells, principally of the granulocytic lineage, such as promyelocytes, myelocytes and metamyelocytes. Polymorphonuclear (PMN) cells that expressed FeLV CA protein p27 were also evident in the bone marrow from several cats; these represent more differentiated cells of the same lineage (Fig. 5.1). Positive plasma cells were also frequently detected. A single large vacuolated cell that expressed FeLV CA protein p27 was also pictured, which may represent either a macrophage or a megakaryocyte (Fig. 5.1).

No fluorescence was detected in bone marrow preparations from latently infected cat E12, indicating that the FeLV CA protein p27 was not expressed in the bone marrow from this cat thirteen weeks post exposure (Fig. 5.1).

5.3.1.2 Cytology and Haematology

Cytospin preparations of bone marrow tissues were stained with May-Grünwald-Giemsa post mortem. Images of the bone marrow preparations are displayed in Fig. 5.2. The myeloid:erythroid (M:E) ratios, the approximate percentage of lymphoid cells enumerated within the cytospin preparations, and summaries of the cytology reports are displayed in Table 5.1. The differential WBC count was calculated manually from blood smears prepared from post mortem peripheral blood samples; the results are shown in Table 5.2.

Preliminary naked eye examination revealed marked differences in the cellularity of bone marrow from persistently infected cats, compared to the latently infected cat, E12 (Fig. 5.3). This initial finding suggested that the bone marrow from persistently infected cats was subject to decreased haematopoiesis and/or increased cell death. Indeed, granulocytic hypoplasia was observed in five persistently infected cats, E1 and E8-E11, associated with a depleted pool of mature segmented neutrophils (Table 5.1). The haematological picture showed that four of these cats, E1 and E9-E11, presented with a concurrent neutropenia; this was moderately severe in cat E9 and was accompanied by a leukopenia (Table 5.2). Cat E11 also suffered a concurrent mild neutropenia and leukopenia. However, all cell lines were present at all stages of differentiation in six persistently infected cats, E2-E6 and E7, indicating normoplasia.

Under normal circumstances, the bone marrow lymphoid population chiefly comprises circulating lymphocytes and maturing B cells. Occasional lymphoblasts, prolymphocytes and plasmacytoid cells are also considered normal. In the literature, the reported proportion of lymphoid cells in normal bone marrow varies widely; ranging from 0.2-15% (Keller and Freudiger, 1983), or averaging either 11.2% (Latimer, 1995) or 16.13% ± 2.92 (Clinkenbeard and Meinkoth, 2000). The values from all cats, except E1, comply with the most stringent normal range, albeit at the upper extremes of normal. However, an increased proportion of small, mature lymphoid cells was observed in the bone marrow from nine persistently infected cats in this study (cats E1-E8 and E10) (Table 5.1). These cells, 6 μm-9μm in diameter,

were identified by the presence of round to slightly indented nuclei, surrounded by a thin rim of light blue cytoplasm (Tyler *et al.*, 1999; Steffans, 2000).

The M:E ratio, which is the ratio of cells in the myelopoietic line to those in the erythropoietic line, varied widely between cats, ranging from 0.51:1 in cat E9, to 2.9:1 in cat E12 (Table 5.1). The normal reference range cited in the literature also varies widely, from 0.65-4.29:1 (Keller and Freudiger, 1983) or from 0.9-2.5 (Latimer, 1995), or averaging 1.63 ± 0.35:1 (Clinkenbeard and Meinkoth, 2000). Using the broadest published criteria, three cats, E1, E4 and E9, recorded M:E ratios below the normal range. A low M:E ratio is associated with either decreased leukocyte precursor cells, or increased erythrocyte precursors. In the affected cats, a low M:E ratio could be attributed to a reduced numbers of leukocyte precursor cells; a granulocytic hypoplasia was identified in both cats E1 and E9. Furthermore, there was no evidence of increased erythrocyte precursor cells in the bone marrow from any cat; in fact, a mild erythroid hypoplasia was detected in the bone marrow from cat E9.

Normal haematology reports were cited for cats E2, E3, E7, E8 and E12. A mild leukocytosis was demonstrated in the peripheral blood from cat E6, accompanied by a slight increase in the proportion of band neutrophils. These findings were reflected in a high M:E ratio. The numbers of mature neutrophils and lymphocytes in this cat were also high, but within the normal reference range (Table 5.2). Two cats, E1 and E4, presented with lymphocytosis.

5.3.1.3 Immunohistochemistry

An increased number of small, mature lymphoid cells was detected in the bone marrow tissues from nine cats in this study (Table 5.1). These lymphoid cells were characterised using anti-human CD3 and CD79α mAbs on formalin-fixed bone marrow sections, in an immunoperoxidase technique. The anti-CD3 and anti-CD79α mAbs are known to cross-react with the CD3 and CD79α molecules, respectively, expressed by many other species. No attempt was made to ascertain whether these mAbs definitively recognised the CD3 and CD79α molecules expressed by feline

cells. However, each mAb identified a distinct homogenous cell population defined by disparate distribution and staining patterns (Fig. 5.4); thus, it was considered that the cell populations recognised by these mAbs corresponded to feline T and B cells, respectively. Bone marrow tissue sections were prepared from cats E1-E6, E7, E9, E12 and SPF cat T10 post mortem. Stained sections of bone marrow tissue from each cat were photographed; representative images are shown in Figs. 5.4 and 5.5. Megakaryocytes within the sections were susceptible to non-specific staining, particularly when the anti-CD79α mAb was used. No staining was detected on the negative control slides.

Few $CD3^+$ T cells were detected in the bone marrow sections from SPF cat, T10 (Fig. 5.4). However, bone marrow tissue sections from all FeLV-exposed cats demonstrated strong reactivity to the anti-CD3 mAb. In particular, large numbers of $CD3^+$ T cells were apparent in the bone marrow sections from cats E1 and E5. $CD3^+$ T cells were often distributed at the periphery of the bone marrow sections. In addition, positive cells were occasionally clustered together; this was particularly evident in cat E2. Bone marrow sections from latently infected cat, E12, contained a greater number of $CD3^+$ T cells, compared to SPF cat T10 (Fig. 5.4).

$CD79\alpha^+$ B cells were distributed widely in bone marrow sections from SPF cat T10 (Fig. 5.4). In FeLV-exposed cats, reactivity to CD79α was less intense than to CD3, and fewer positive cells were detected; indeed, no positive cells were detected in the bone marrow from cat E3. Nonetheless, large numbers of positive cells were evident in bone marrow sections from cat E5. A distinct cluster of $CD79\alpha^+$ B cells was evident within the bone marrow from cat E2 (Fig. 5.5.E); however, in cat E9, this effect was more dramatic. Large numbers of $CD79\alpha^+$ B cells had congregated, forming two large pools of positive cells (Fig. 5.5.C). Both lesions were associated with $CD3^+$ T cells. In cat E2, a small number of $CD3^+$ T cells were distributed throughout the pool of $CD79\alpha^+$ B cells, with a greater number congregated distally (Fig. 5.5.F). In cat E9, a large number of $CD3^+$ T cells had infiltrated the pool of $CD79\alpha^+$ B cells, forming an organised pattern. The lesion was also surrounded by considerable numbers of $CD3^+$ T cells (Fig. 5.5.D).

5.3.2 Peripheral Lymph Nodes

5.3.2.1 Single Immunohistochemistry Staining

The distribution of the FeLV CA protein p27 in the PLN tissues was investigated using a selection of cats in the present study. Cryostat sections were prepared from the PLN tissues from persistently infected cats, E4, E5 and E7, and latently infected cat E12 post mortem. No staining was detected on the negative control slides. Positively stained PLN sections were photographed; representative images are shown in Fig. 5.6.

The FeLV CA protein p27 was not detected in the PLN sections from latently infected cat E12, indicating that FeLV infection was not active in the PLN tissues from this cat thirteen weeks post exposure. However, this viral protein was detected in PLN sections from all the persistently infected cats examined (cats E4, E5, E7). In these cats, the positive cells were largely confined to the lymph node follicles (Fig. 5.6.B). Furthermore, the distribution of positive cells corresponded approximately to the distribution of the FDC network, demonstrated using the Ki-M4 mAb (Fig. 5.6.A). Therefore, it was considered likely that the FDC population might harbour the FeLV CA protein p27, as suggested in previous studies (Kovacevic *et al.*, 1997).

5.3.2.2 Simultaneous Double Immunoenzyme Staining

To investigate whether the FeLV CA protein p27 was retained by the FDC population, the Ki-M4 mAb was used with the sheep anti-p27 pAb in a simultaneous double immunoenzyme technique on PLN cryostat sections from persistently infected cat E4. Peripheral lymph node sections from latently infected cat E12 were also used in a double immunoenzyme technique to compare the findings. The results are shown in Fig. 5.6. Single staining of either the FDC population, or the FeLV CA protein p27, in the PLN tissue sections from cat E4, resulted in a homogenous blue or red staining, respectively, developed with either FBBB or AEC (Fig. 5.6.A and B). However, when the FeLV CA protein p27 and the CD21L antigen were probed simultaneously, a purple staining arose, indicating antigen co-localisation (Fig. 5.6.C and D). Cryostat PLN sections from cat E12 were probed simultaneously with the

same antibody cocktail; however, as cat E12 did not express the FeLV CA protein p27, no co-localisation was evident (Fig. 5.6.E and F).

Fig. 5.1 FeLV CA protein p27 in bone marrow cytospin preparations, detected using immunofluorescence

Cytospin preparations of bone marrow from cats E1-E12 were probed with a sheep anti-p27 pAb followed by a FITC-conjugated secondary antibody. Slides were counter-stained using Evans Blue, mounted and examined using a UV microscope. Representative images from persistently infected cats E1 (A, x20); E4 (B, x40) and E6 (C, x40), and latently infected cat E12 (D, x 40), are presented. Large mononuclear cells, which may represent promyelocytes (E, x40); granulated PMN (F, x40); plasma cell or myelocyte (G, x40) and a large vacuolated cell (H, x40), all expressed the FeLV CA protein p27.

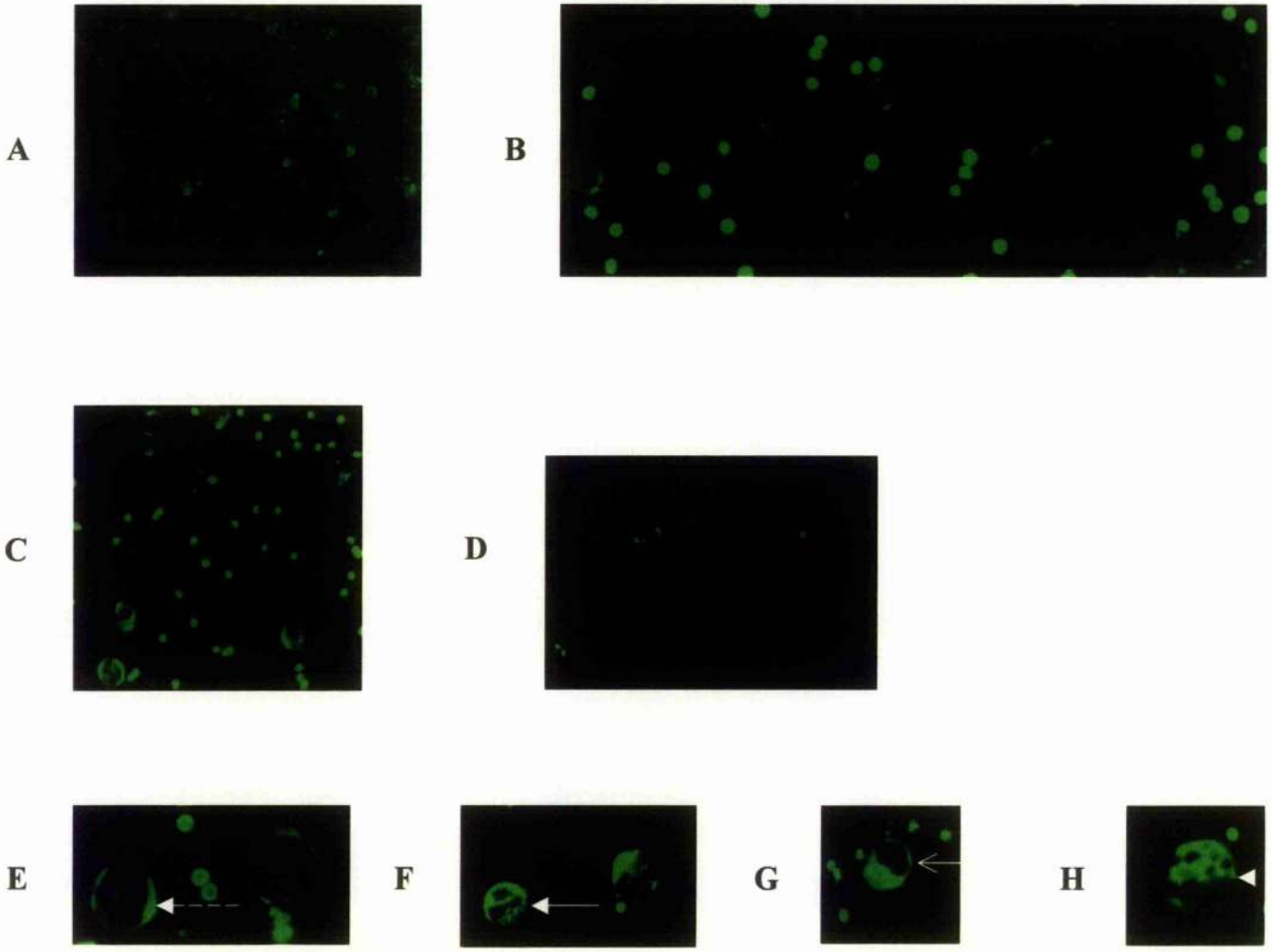
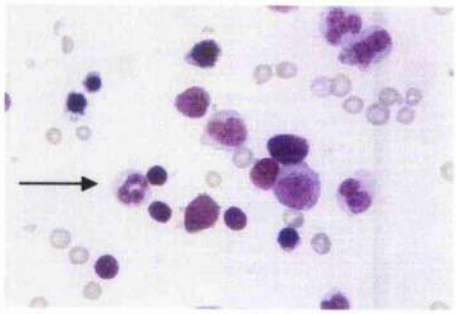

A
B
C
D
E
F
G
H

Fig. 5.2 May-Grünwald-Giemsa stained bone marrow cytospin preparations

Images of bone marrow cytospin preparations from cats E1-E12, stained with May-Grünwald-Giemsa, which illustrate some of the cells and cell series identified within the bone marrow.

(A) Neutrophil series, segmented mature neutrophil (arrow); mature lymphocytes; (x100)
(B) Basophil (arrow, x100)
(C) Erythroid series; mitotic figure (arrow, x100)
(D) Megakaryocyte (open arrow); osteoclast (closed arrow, x40)
(E) Eosinophil series; mature eosinophil (arrow, x100)
(F) Plasma cell (x100)
(G) Megakaryocyte; normoplasia, (x40)
(H) Megakaryocye erupting, dispersing platelets (x100)

A



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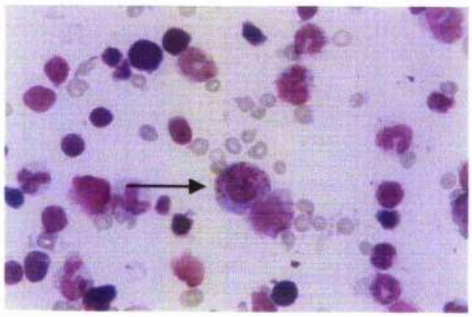

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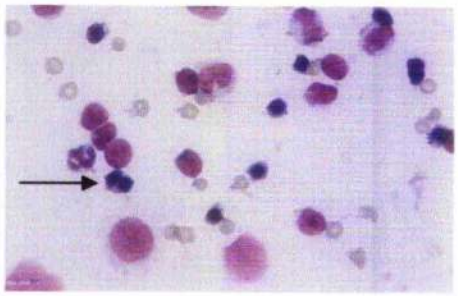

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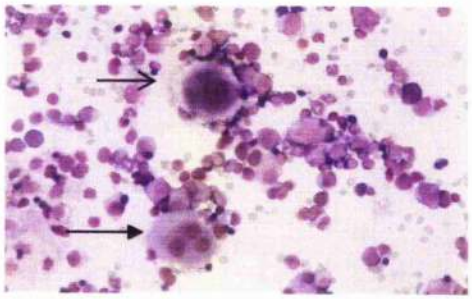

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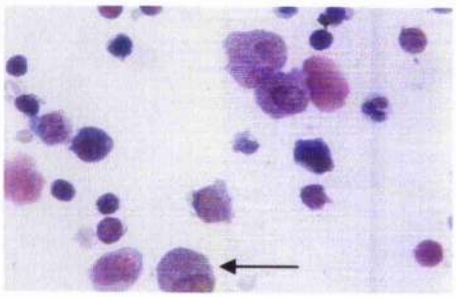

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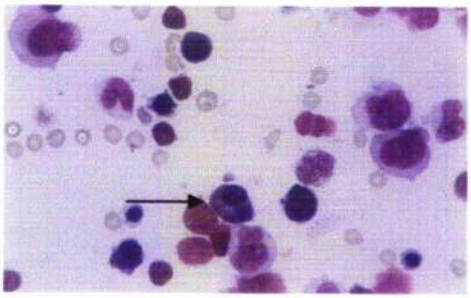
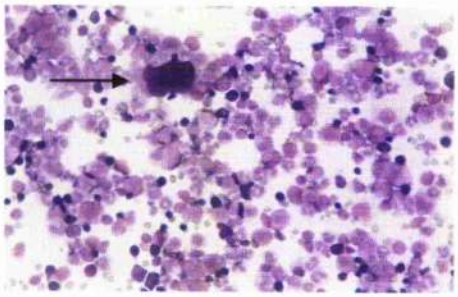


G



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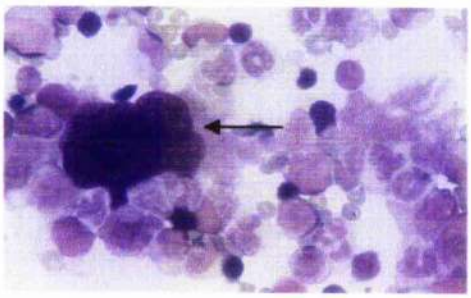
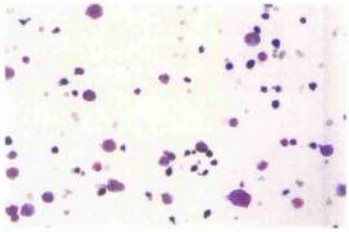
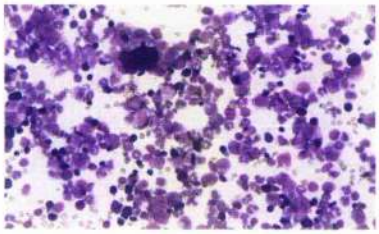


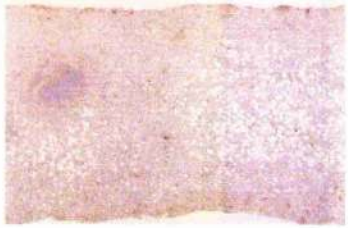
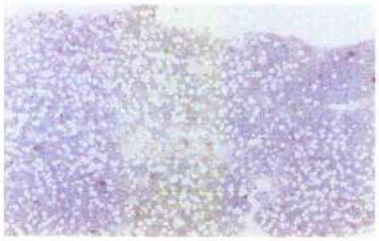
Fig. 5.3 Comparison of bone marrow results from cats E9 and E12

Bone marrow was harvested from persistently infected cat E9 and latently infected cat E12 post mortem. Cytospin preparations were stained with May-Grünwald-Giemsa and examined cytologically (x40). The distribution of T and B cells in the bone marrow was detected using anti-CD3 and anti-CD79α mAbs respectively, in an immunoperoxidase technique on formalin-fixed bone marrow tissue sections (CD3: E9, x2 and E12, x4; CD79α: E9, x4 and E12, x10). Finally, expression of the FeLV CA protein p27 was detected using immunofluorescence on bone marrow cytospin preparations (x40).

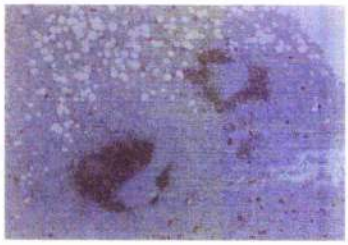
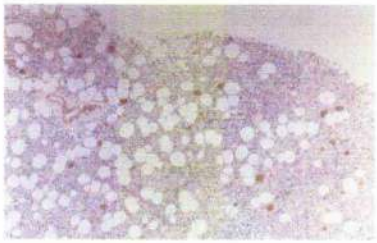
May-Grünwald-Giemsa

CD3

CD79α

FeLV CA p27 protein


Persistently Infected **Latently Infected**

Fig. 5.4 $CD3^+$ and $CD79\alpha^+$ cells in bone marrow, detected using immunohistochemistry

Comparison of formalin-fixed sections of bone marrow from SPF cat T10 (x4), latently infected cat E12 (x10) and persistently infected cat E5 (x10). The sections were probed with anti-CD3 and anti-CD79α mAbs using an immunoperoxidase technique. Colour was developed using DAB, and the sections were counter-stained using Gills' haematoxylin.

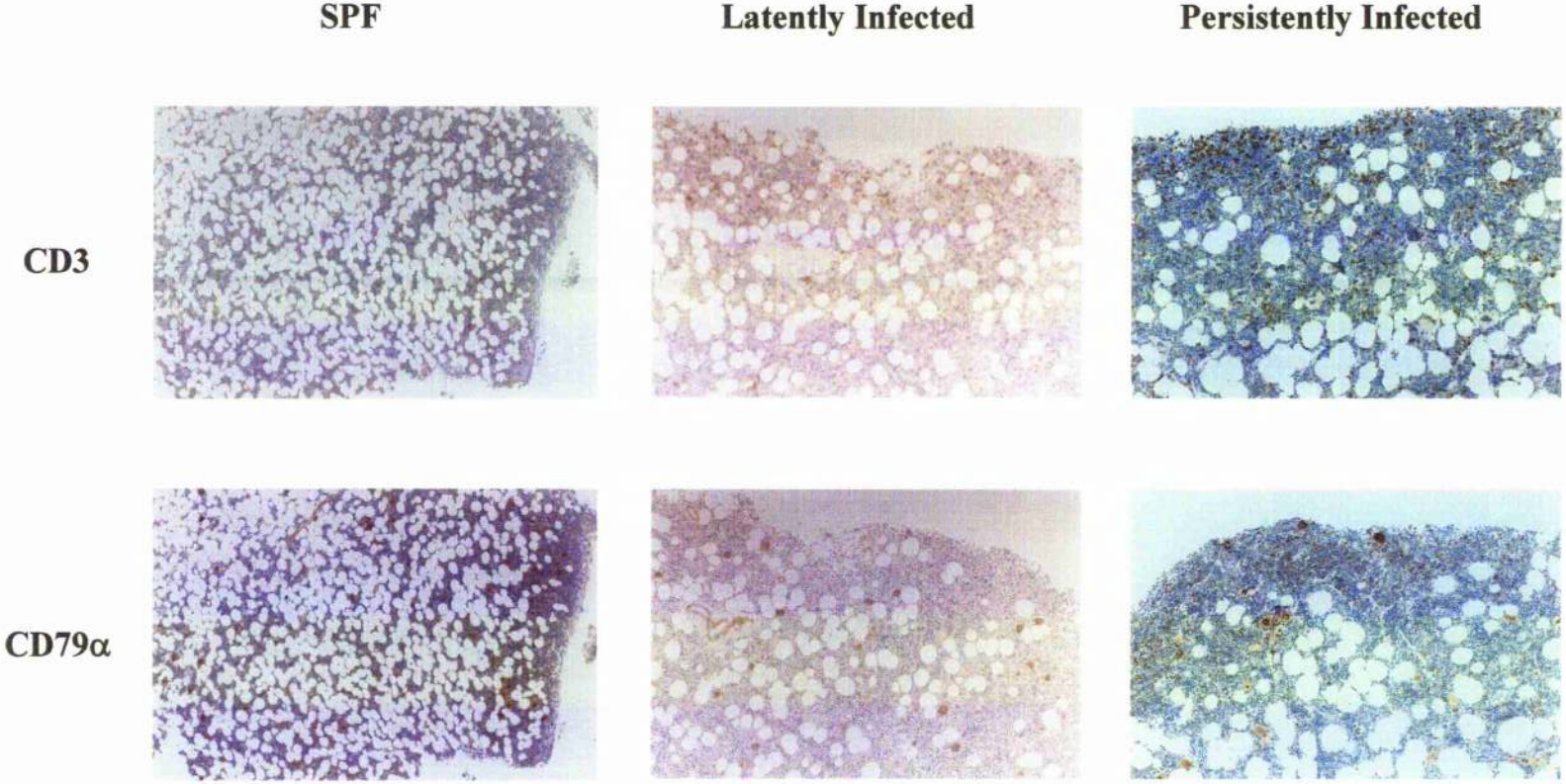
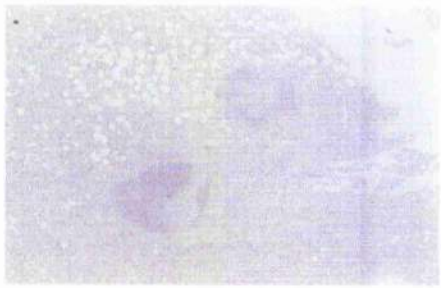

SPF
Latently Infected
Persistently Infected
CD3
CD79α

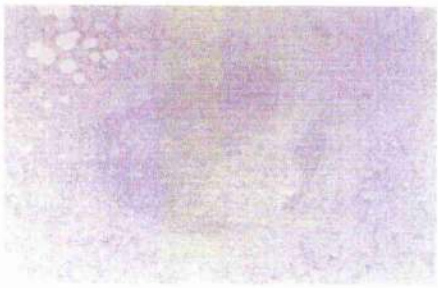
Fig. 5.5 $CD3^+$ and $CD79\alpha^+$ cells in bone marrow from cats E2 and E9

Formalin-fixed sections of bone marrow from persistently infected cats E9 (A-D) and E2 (E and F). The sections were probed with anti-CD79α (C and E, x10) or anti-CD3 (D and F, x10) mAbs using an immunoperoxidase technique. Colour was developed using DAB, and the sections were counter-stained using Gills' haematoxylin. Sections probed with normal serum, acted as negative controls (A, x4 and B, x10).

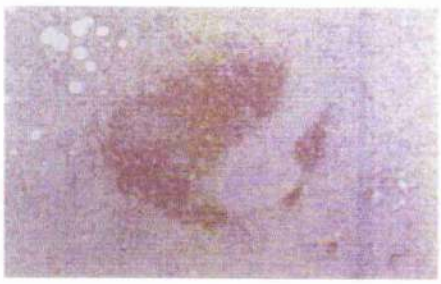
A



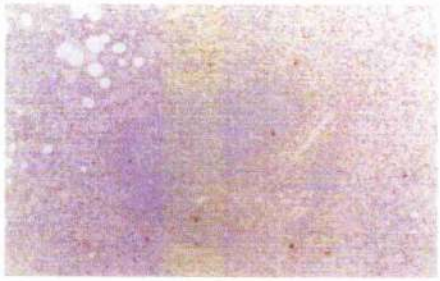
B



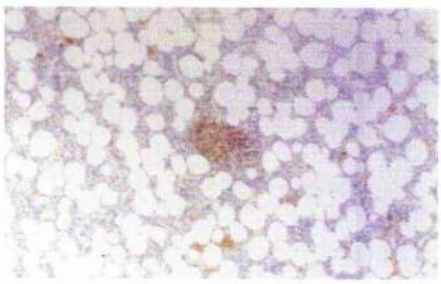
C



D



E



F

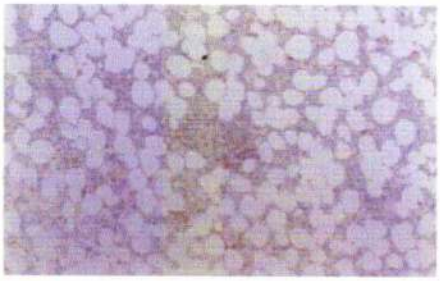
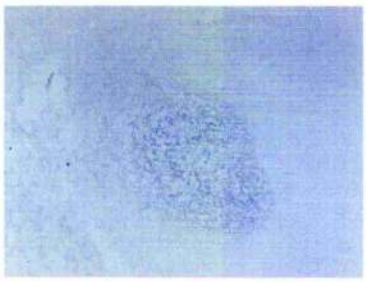


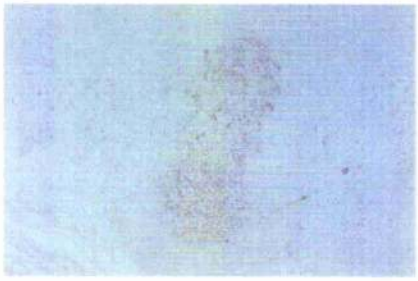
Fig. 5.6 FeLV CA protein p27 and FDC network in cryostat PLN sections, detected using immunohistochemistry

Detection of the FDC network in cryostat section of PLN from persistently infected cat E4, using the Ki-M4 mAb in an immunophosphatase technique; colour was developed using FBBB (A, x10). Detection of FeLV CA protein p27 in cryostat section of PLN from cat E4, using a sheep anti-p27 pAb in an immunoperoxidase technique; colour was developed using AEC (B, x10). Two-colour staining was performed in PLN tissues from the same cat using an antibody cocktail comprising Ki-M4 mAb and sheep anti-p27 pAb; co-expression of FeLV CA protein p27 and the FDC population is illustrated with the colour purple (C, D, x10). Staining of cryostat sections of PLN from cat E12 using the same antibody cocktail resulted in FDC staining only, indicating the absence of FeLV CA protein p27 expression (E, F, x10).

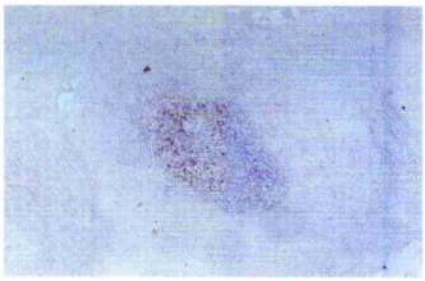
A



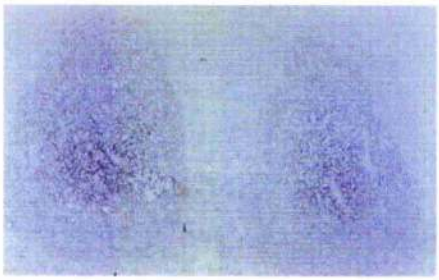
B



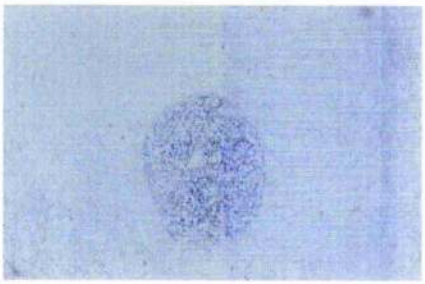
C



D



E



F

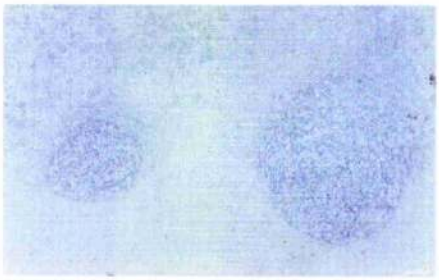


Table 5.1 Myelogram reports for cats E1-E12

This table summarises the myeloid:erythroid (M:E) ratios; the percentage of lymphoid cells, and the cytology report, based on the bone marrow cytospin preparations prepared from cats E1-E12 post mortem.

Cat Number	M:E Ratio	% Lymphoid Cells	Summary of Cytology Report
E1	0.60:1	25	Mild granulocytic hypoplasia, erythroid and megakaryocyte normoplasia with lymphocyte infiltration
E2	0.67:1	11	Normoplasia, mildly increased numbers of lymphocytes present
E3	1.38:1	10	Normoplasia, mildly increased numbers of lymphocytes present
E4	0.59:1	13	Normoplasia, mildly increased numbers of lymphocytes present
E5	1.13:1	15	Normoplasia, mildly increased numbers of lymphocytes present
E6	1.86:1	10	Normoplasia, mildly increased numbers of lymphocytes present
E7	1.10:1	10-12	Normoplasia, mildly increased numbers of lymphocytes present
E8	0.91:1	15	Mild granulocytic hypoplasia, erythroid and megakaryocyte normoplasia with mildly increased numbers of lymphocytes present
E9	0.51:1	5	Granulocytic hypoplasia, mild erythroid hypoplasia
E10	1.13:1	15	Granuloctyic hypoplasia, erythroid and megakaryocyte normoplasia, mildly increased numbers of lymphocytes present
E11	1.43:1	8	Mild granulocytic hypoplasia, erythroid and megakaryocyte normoplasia
E12	2.90:1	6	Normoplasia

Table 5.2 Haematology results for cats E1-E12

Differential WBC counts for cats E1-E12, calculated from cytospin preparations of peripheral blood samples taken from each cat post mortem.

Haematology Results Post Mortem													
Cat Number	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**E10**	**E11**	**E12**	**Ref. Range**
WBC ($x10^9/l$)	10	9.27	7.55	11.6	5.49	17.1	9.01	6.29	2.82	9.15	3.68	8.06	**5.5-15.5**
Band neutrophils ($x10^9/l$)	n.d	0.093	0.075	0	0	0.342	0	0	0	n.d	n.d	n.d	**0-0.3**
Neutrophils ($x10^9/l$)	1.8	6.211	3.322	3.48	3.129	9.405	4.145	3.334	0.338	2.47	1.546	2.821	**2.5-12.5**
Lymphocytes ($x10^9/l$)	7.7	2.503	3.322	7.306	1.867	5.643	4.505	2.642	2.228	6.222	2.098	4.433	**1.5-7.0**
Monocytes ($x10^9/l$)	0.1	0.278	0.378	0.348	0.11	0.684	0.09	0.189	0.169	0.183	0.037	0.161	**0.0-0.85**
Eosinophils ($x10^9/l$)	0.3	1.185	0.378	0.464	0.384	0.684	0.27	0.126	0.085	0.275	0	0.645	**0.0-1.5**
Basophils ($x10^9/l$)	0.1	0	0.075	0	0	0.342	0	0	0	0	0	0	**rare**

5.4 Discussion

Bone marrow and lymphoid tissues have an important role in the immunopathogenesis of FeLV infection. Virus may be rapidly eliminated in the PLN, prior to a cell-associated viraemia; however, uncontrolled viral replication in the bone marrow leads to the dissemination of virus throughout the infected host. In addition, virus integration, replication and expression in these tissues may impair normal cell function in these tissues and precipitate FeLV-associated disease.

Using immunofluorescence on bone marrow cytospin preparations, the FeLV CA protein p27 was detected in the bone marrow cells from each persistently infected cat. This technique, similar to the original IFA technique described by Hardy et al. (1973), detects cell-associated viral protein rather than free viral protein. Thus, this method provides an alternative means of diagnosing viraemia or active infection (Hardy *et al.*, 1973; Jarrett *et al.*, 1982b; Pedersen, 1988). The bone marrow from cats E1-E6, and cat E9, contained the greatest numbers of p27-positive cells. Cats that received the highest exposure dose of FeLV by a factor of one log (E1-E6) generated significantly greater proviral DNA loads in the peripheral blood post mortem, compared to cats that were exposed to a lower dose of infectious virus (Mann-Whitney, P=0.0043). Thus, we can speculate that these cats also expressed higher levels of infectious virus in the bone marrow. The FeLV CA protein p27 was predominately associated with granulocytic precursor cells. These findings are consistent with other reports where virus was principally associated with neutrophil precursor cells (Hoover *et al.*, 1977b); the authors also reported virus expression in erythrocyte precursor cells. However, our findings did not concur with a second study, where the FeLV CA protein p27 and SU protein gp70 were identified chiefly in association with megakaryocytes (Kovacevic *et al.*, 1997).

Bone marrow studies have previously identified a maturation arrest in granulocytic precursor cells at the metamyelocyte stage in some FeLV-infected animals, suggesting a developmental blockade (Shelton and Linenberger, 1995). In this thesis, the majority of p27-positive cells in the bone marrow were granulocytic precursor cells; virus replication in neutrophil precursor cells would directly deplete

the pool of mature neutrophils. These phenomena may account for the granulocytic hypoplasia evident in the bone marrow from five persistently infected cats in this study, cats E1 and E8-E11, reflected in low M:E ratios in cats E1 and E9. The concomitant haematology results revealed that four of these cats, E1, E9-E11, had low levels of circulating mature neutrophils. Neutropenia was particularly marked in cat E9, and was accompanied by a leukopenia; cat E11 also suffered a concurrent leukopenia and neutropenia. Since neutrophils are the predominant circulating leukocyte in cats, neutropenia usually results in a leukopenia (Latimer, 1995). In most FeLV-infected cats, neutropenia is mild, and is associated with relatively normal granulopoiesis. However, in some cats, a moderate neutropenia is accompanied by a granulocytic hypoplasia. Finally, less frequently, FeLV infection may result in a severe persistent neutropenia associated with a severe granulocytic hypoplasia (Latimer, 1995). As neutrophils provide one of the first lines of defence against invading pathogens, particularly bacteria, neutropenia predisposes FeLV-infected cats to secondary opportunistic infections (Latimer, 1995).

It was not possible to detect FeLV CA protein p27 expression in the bone marrow cells from latently infected cat E12 (Fig. 5.1). Failure to detect virus expression in the bone marrow does not necessarily indicate either recovery or latency following exposure to FeLV. In one study, the FeLV CA protein p27 or SU protein gp70 were not consistently detected in the bone marrow from cats suffering from an FeLV-associated disease (Kovacevic *et al.*, 1997). Furthermore, the FeLV CA protein p27 was not detected in the bone marrow from a cat suffering from an FeLV-associated disease, which had a localised infection in the spleen (Hayes *et al.*, 1989). The cytological analysis of the bone marrow from latently infected cat E12 was normal, indicating normoplasia (Table 5.1). Furthermore, no abnormalities were detected following the haematological analysis of the peripheral blood (Table 5.2). These finding are consistent with the fact that bone marrow cells from this cat were not actively producing infectious virus.

An infiltrating population of small mature lymphoid cells was detected in the bone marrow from nine persistently infected cats, while normal numbers of lymphoid cells were detected in the bone marrow from two persistently infected cats, E9 and E11. Both cats suffered a concurrent leukopenia, in addition to a granulocytic hypoplasia

and neutropenia; cat E9 also presented with erythroid hypoplasia. These findings may reflect a general impaired haematopoiesis in these cats; circulating lymphocyte numbers were also low but within the normal range. The infiltrating cells were particularly numerous in the bone marrow from cats E1 and E5, which contained high numbers of p27-positive cells. In addition, these cats had the highest mean levels of proviral DNA in the peripheral blood over the course of the study (4.66 ± 3.65 and 4.82 ± 7.78 copies per cell, respectively). The infiltrating lymphoid cells were characterised using anti-CD3 and anti-CD79α mAbs in an immunoperoxidase technique. It was observed that the SPF cat, T10, harboured very low numbers of $CD3^+$ T cells in the bone marrow; however, $CD79\alpha^+$ B cells were distributed widely. Since the normal range of lymphoid cells within the bone marrow varies widely, according to the literature (Section 5.3.1.1), it might be important to compare the data recorded in the present study with data from a greater number of SPF cats.

Greater numbers of $CD3^+$ T cells were detected within the bone marrow from all cats, compared to SPF cat T10. However, only in cat E5 was it possible to detect markedly increased numbers of $CD79\alpha^+$ B cells in the bone marrow, compared to cat T10 (Fig. 5.4). Thus, for each cat, the majority of infiltrating lymphoid cells were $CD3^+$ T cells. T cells are not normally found in large numbers within the bone marrow; in an early study, only 8% of the small mononuclear cells in the bone marrow classified morphologically as lymphocytes (5-10% of total cells), were erythrocyte-rosette forming cells (E-RFC), regarded as probable T cells (Rojko *et al.*, 1982a). Therefore, the large numbers of $CD3^+$ T cells detected in the bone marrow from cats in this present study exceeded the normal bone marrow T cell population. The bone marrow from all persistently infected cats was actively infected; the FeLV CA protein p27 was demonstrated in the bone marrow tissues from all such cats using immunofluorescence. Furthermore, we have detected virus-specific T cell responses in the peripheral blood from all persistently infected cats at some time over the study (Chapter 4). Therefore, it is likely that the infiltrating T cells chiefly comprised virus-specific effector T cells activated in the lymphoid tissues in response to virus-specific stimulation. To verify this, and further characterise these cells, sophisticated immunohistochemical staining experiments could be conducted. The antigen-specificity and cell phenotype of the infiltrating cells could be

established using tetramer staining *in situ* in combination with cell surface staining. These data would also indicate whether the infiltrating cells retained the capacity to secrete fIFN-γ in response to antigenic stimulation. Finally, FeLV CA protein p27 expression by these cells would indicate whether these cells were actively replicating virus; this could be achieved using double immunoenzyme techniques.

The conclusion that the infiltrating $CD3^+$ T cells represent virus-specific effector T cells is consistent with the low number of $CD3^+$ T cells observed in latently infected cat E12, and SPF cat T10, neither of which were actively infected. Although latently infected cat E12 contained greater numbers of $CD3^+$ T cells within the bone marrow compared to cat T10 (Fig. 5.4), these numbers were still considered normal (Table 5.1). Furthermore, cat T10 was SPF, while cat E12 had been exposed to FeLV under experimental conditions; circulating virus-specific T cells were detected in the blood and lymphoid tissues from cat E12 following oronasal exposure to FeLV (Chapter 4). Therefore, we can speculate that, in the absence of an productive FeLV infection, these infiltrating T cells in cat E12 may comprise effector memory T cells, derived from early effector T cells activated in the lymphoid tissues in response to virus-specific stimulation. These effector memory T cells home to specific viral antigen in the bone marrow and other non-lymphoid tissues (Lefrançois and Masopust, 2002).

The distinctive pooling of B cells in cats E2 and E9 (Fig. 5.5) may represent the formation of germinal centre follicles in the bone marrow, which can occur in response to prolonged antigen stimulation (Valli and Jacobs, 2000). It is known that virus can reach the bone marrow within two days following exposure; therefore, virus may have been expressed in the bone marrow from these cats for up to thirteen and nineteen weeks, respectively. High numbers of p27-positive cells were detected in the bone marrow from both cats post mortem. Continual expression of viral proteins represents a persistent focus for the antiviral immune response.

Neither p27 antigenaemia nor viraemia were detected in cat E12 at any time throughout the study. However, proviral DNA was detected in the PLN tissues and in the buffy coat cells from cat E12 post mortem; in addition, infectious virus was isolated from the bone marrow of this cat after a short *in vitro* culture. Thus, this cat maintained at least three foci of latency, the peripheral blood cells, the bone marrow

and the lymphoid tissues. However, virus was not expressed in the peripheral blood (Chapter 3) or bone marrow (Chapter 5) from this cat post mortem. Furthermore, expression of the FeLV CA protein p27 was not detected in the PLN tissues (Fig. 5.6.E and F). These findings suggest that cat E12 may have a true latent infection, where provirus is quiescent, and not replicating. However, the sensitivity of the assays used to detect virus expression in these tissues may be inadequate. Virus-specific T cells recognise a much greater array of FeLV epitopes compared to the sheep anti-p27 pAb; therefore, the detection of such cells may reflect the current viral status more accurately. A greater number of $CD3^+$ T cells were detected in the bone marrow from cat E12, compared to SPF cat T10. Although we have speculated that these cells may be virus-specific effector memory T cells, further investigations would be necessary to verify their identity.

High levels of proviral DNA were quantified in the PLN tissues from persistently infected cats post mortem (Chapter 3). Furthermore, the FeLV CA protein p27 was identified in the PLN follicles from all persistently infected cats tested. This is in agreement with previous reports where the FeLV CA protein p27 was identified in the MLN follicles in sixteen out of eighteen cats diagnosed with an FeLV-associated disease (Kovacevic *et al.*, 1997). The virus-specific immune responses in the PLN tissues were investigated post mortem using LPA, and ICS analysed using flow cytometry; the results were discussed in Chapter 4. The expression of the FeLV CA protein p27 in the PLN tissues was not associated with the presence of virus-specific immune responses. Virus-specific $CD8^+$ T cell responses were detected in the PLN tissues from latently infected cat E12, which did not express the FeLV CA protein in these tissues. Furthermore, virus-specific $CD4^+$ T cell responses were only detected in cats E4 and E5, although the PLN tissues from cats E4, E5 and E7 all expressed this viral protein. It was suggested in Kovacevic's study (1997) that the FeLV CA protein p27 was associated with the FDC network in the germinal centre of lymph node follicles; however, this was not proven. In the present study, double staining immunoenzyme procedures were used to verify that the FeLV CA protein p27 was associated with the FDC population. It is not known whether the FDC was actively infected, or whether the FeLV CA protein p27 was bound in the form of an immune complex. Antigen retained by the FDC is typically in the form of an immune complex, bound to FcR (Szakal *et al.*, 1983). The FDC can retain the immune

complexes for months or years, and are thought to have a central role in the maintenance of the B cell memory pool (Gray and Skarvall, 1988; Tew *et al.*, 1997). Although speculation exists that the soluble glycosylated p27 protein may induce anergic $CD4^+$ T cell responses, which impede the formation of anti-Gag antibodies, immune complex disease is a common consequence of persistent FeLV infection (Pedersen, 1988). Furthermore, anti-Gag antibodies are known to be produced by persistently infected cats (Lutz *et al.*, 1980b; Charreyre and Pedersen, 1991; Hawks *et al.*, 1991). In studies of FIV infection, it was shown that FIV RNA was co-localised with the FDC population in lymph nodes, using a combination of *in situ* hybridisation and immunohistochemistry; the FDC virus load appeared concurrently with antibodies and persisted for at least three years (Bach *et al.*, 1994).

The implications of the FDC population harbouring the FeLV CA protein p27 are not clear. In the present study, this phenomenon occurred only in persistently infected cats; the FeLV CA protein p27 was not detected in the PLN tissues from latently infected cat E12. Many cats recover following exposure to FeLV with minimal evidence of virus replication; therefore, these cats cannot express and retain significant quantities of the FeLV CA protein p27. Despite the absence of an FDC-p27 network, recovered cats appear to be immune to subsequent exposure to FeLV; however, this may be an age-related phenomenon (Hoover *et al.*, 1976).

5.5 Summary and Conclusions

All persistently infected cats expressed the FeLV CA protein p27 in bone marrow, which was chiefly associated with large mononuclear granulocytic precursor cells. Five persistently infected cats suffered a granulocytic hypoplasia, which was associated with a concurrent neutropenia in four cats. In addition, an infiltrating small, mature lymphoid population was identified in the bone marrow cytospin preparations from nine persistently infected cats. The mature small lymphoid population chiefly comprised T cells, in addition to B cells, defined by CD3 and CD79α molecules, respectively. These mature T cells were particularly numerous in cats E1 and E5. Cats E2 and E9 demonstrated unusual focal congregations of B cells, which may represent follicles formed in response to chronic viral stimulation.

All persistently infected cats examined expressed the FeLV CA protein p27 in PLN tissues; double immunoenzyme staining demonstrated that the FeLV protein p27 was associated with the FDC population in lymph node follicles. Cat E12 retained latent infections in the bone marrow and lymphoid tissues; however, this cat did not express the FeLV CA protein p27 in either tissue. Consequently, cytology and haematology reports were normal.

CHAPTER 6

GENERAL DISCUSSION

Feline leukaemia virus is a naturally occurring γ-retrovirus of domestic cats, predominately associated with proliferative and degenerative diseases of the haematopoietic system. Fortunately, the majority of exposed cats recover and develop lasting protective immunity; however, a number fail to control virus replication, and become persistently viraemic, dying within two-to-three years from an FeLV-associated disease. A large proportion of recovered cats retain FeLV as a latent proviral DNA infection for months or years before eliminating the virus or, more rarely, progressing to a state of persistent viraemia. Virus isolate, dose, host susceptibility, age at the time of exposure and route of exposure all interact to influence the outcome following exposure to FeLV (Hoover *et al.*, 1976). The immune mechanisms that influence host susceptibility have yet to be fully delineated.

The importance of humoral immunity in the control of retroviral disease is controversial, and may vary among individual viruses. Virus neutralising antibodies are a vital correlate of protection against FV; however, VNA alone cannot mediate protection (Hasenkrug and Chesebro, 1997; Dittmer *et al.*, 1999). Recent studies involving chimeric SHIV demonstrated passive protection conferred by anti-HIV Env neutralising antibodies alone *in vivo* (Shibata *et al.*, 1999). However, although high anti-Env antibody titres can be detected in the sera of most HIV-infected individuals, the key events of CD4 binding and fusion are not inhibited, indicating that humoral immune responses are not protective (Matthews *et al.*, 1986). Early FeLV studies showed that the elicitation of VNA correlated with recovery following exposure (Russell and Jarrett, 1978a; Lutz *et al.*, 1980b), and passively protected kittens against FeLV challenge (Jarrett *et al.*, 1977). Nonetheless, in more recent studies, virus was shown to be cleared from the blood prior to, or concurrent with, the appearance of VNA (Flynn *et al.*, 2000a; Flynn *et al.*, 2002b). Thus, in each of these retroviral infections, VNA alone are not sufficient to mediate recovery and/or elicit protective immune responses, implicating a role for cell-mediated immunity.

Indeed, we know that virus-specific CTL are important in the elimination of virus, and in the elicitation of protective immune responses, in each of these retroviral infections. The development of FV-specific CTL correlated with recovery from splenomegaly in FV-infected mice (Robertson *et al.*, 1992); depletion of $CD8^+$ T

cells *in vivo* dramatically reduced the incidence of recovery in these mice. Cytotoxic T cells are important in the control of viraemia in the initial acute phase, and during the asymptomatic period, following infection with HIV and SIV (Koup *et al.*, 1994; Borrow *et al.*, 1997; Ogg *et al.*, 1998; Wilson *et al.*, 1998; Jin *et al.*, 1999). Early Gag/Pro-specific CTL responses in the blood and lymphoid tissues are associated with recovery from FeLV infection (Flynn *et al.*, 2002b); moreover, such responses are implicated in protective immune responses following inoculation of cats with an experimental FeLV DNA vaccine containing *gag, pol* and *env* genes (Flynn *et al.*, 2000a; Hanlon *et al.*, 2001). The adoptive transfer of a single infusion of mixed virus-specific $CD4^+$ and $CD8^+$ T cells significantly reduced the proviral DNA burdens in persistently infected cats, indicating a direct role for virus-specific T cells in the control of FeLV viraemia (Flynn *et al.*, 2002b).

$CD4^+$ T cells have a key role in regulating the humoral and cell-mediated arms of the immune response, through the activation of DC, and the elaboration of cytokines, particularly IFN-γ. Failure of the $CD4^+$ T cell response in HIV and FV infections has been shown to result in inadequate priming, maturation and maintenance of the CTL response; inadequate CTL function culminates in viral persistence. Therefore, the capacity of FeLV-specific $CD4^+$ T cells to activate, prime and maintain virus-specific CTL, and virus-specific B cells, may be a critical component in the immune mechanisms contributing to the control of FeLV viraemia.

The purpose of this thesis was to investigate the role of virus-specific $CD4^+$ T cells in the control of FeLV infection. It was intended to compare such responses among spontaneously recovered, latently infected and persistently infected cats, following oronasal exposure to FeLV. To this end, six sixteen-week old SPF cats were exposed oronasally to 5×10^5 ffu, FeLV-A/Glasgow-1 in the anticipation that each of these three well-defined outcomes would ensue. However, all six cats became persistently infected. A second group of six sixteen-week old SPF cats was exposed to a reduced dose of FeLV under identical conditions as the first group of cats. Five cats in this second group became persistently infected and one cat, E12, retained a latent proviral DNA infection in the bone marrow and lymphoid tissues.

Novel anti-flFN-γ antibodies were generated and characterised to facilitate the detection of virus-specific $CD4^+$ T cells. We have demonstrated the sensitivity of these novel reagents in the detection of both recombinant flFN-γ using ELISA and Western blots, and the production of intracellular flFN-γ in physiologically stimulated cells using flow cytometry; the results were presented and discussed in Chapter 2. These reagents have clear applications in the investigation of other viral and non-viral infections, as well as anti-tumour immune responses.

To define the outcome of infection, and relate virus-specific immune responses with proviral DNA loads, a series of virological parameters were evaluated throughout the study. Sensitive and sophisticated assays, such as the p27 ELISA, VI, and qRT-PCR, were used to detect antigenaemia, viraemia and proviral DNA, respectively, in the peripheral blood throughout the study, and in the lymphoid tissues post mortem; the results were presented and discussed in Chapter 3. In the present study, the qRT-PCR assay was identified as the most sensitive method used in the detection of FeLV. Proviral DNA was detected in the peripheral blood prior to the detection of plasma viraemia or antigenaemia, in ten cats. In addition, this technique identified a persistent proviral DNA infection in the peripheral blood and lymphoid tissues from cat E12, in the absence of detectable antigenaemia or viraemia.

The development of virus-specific cell-mediated immune responses, particularly virus-specific $CD4^+$ T cells, was monitored in the peripheral blood one week following exposure and at regular intervals thereafter. Such responses were also measured in the lymphoid tissues post mortem. Cell-mediated immune responses were assessed using two techniques, the LPA, and ICS analysed using flow cytometry; the results were presented and discussed in Chapter 4. Lymphocyte proliferation assays are technically straightforward to conduct; however, as discussed earlier, these assays do not provide precise information on the frequencies of virus-specific $CD4^+$ T cells, as they reflect both the absolute numbers of responsive cells, and the ability of those cells to expand *in vitro*. Conversely, ICS analysed using flow cytometry is a highly sensitive assay; intracellular IFN-γ expression can be detected at the single cell level following a short four-hour stimulation *ex vivo*, permitting the enumeration and phenotypic analysis of individual virus-specific cells.

The bone marrow and lymphoid tissues are important sites of viral replication and expression; consequently, these are important sources of FeLV-associated disease and act as foci for the antiviral immune response. The expression of FeLV CA protein p27 in the bone marrow and lymphoid tissues was determined using immunofluorescence and immunohistochemistry, respectively; the results were illustrated and discussed in Chapter 5. Where possible, the cells that were associated with the viral proteins were identified, and the consequences for the immunopathogenesis of FeLV disease discussed.

The specificity of the immune response was evaluated by measuring the ability of mononuclear cells in the peripheral blood and lymphoid tissues to proliferate, and to express fIFN-γ, in response to stimulation with FeLV recombinant p27 and p45 proteins, and inactivated FeLV particles. The recombinant p27 and p45 proteins were expressed in *E. coli* bacterial vectors, and purified using affinity chromatography; FeLV particles were purified from F422 cell culture supernatants, and heat-inactivated. The purity, and immunological relevance of these reagents in FeLV infection, was determined using SDS-PAGE and Western blots. In the retroviridae, the principal Gag polypeptide is cleaved to mature forms, MA, CA and NC proteins, by viral PR during and/or immediately after budding (Luciw and Leung, 1992). The CA protein comprises the internal shell, which surrounds the nucleocapsid. The FeLV CA protein p27 has an important role in the immunopathogenesis of FeLV infection; we know that Gag-specific CTL are important in mediating recovery following exposure to FeLV (Flynn *et al.*, 2002b), and in eliciting protection following inoculation with an experimental FeLV DNA vaccine. Furthermore, it has been suggested that the large quantities of glycosylated FeLV CA protein p27 released from infected cells may induce anergic $CD4^+$ T cell responses in infected cats (Jarrett, 1999). The *env* gene product is a polypeptide that is post-translationally modified by cleavage and glycosylation in the ER, resulting in SU and TM domains that form a heterodimer (Vogt, 1997b). The SU protein is dotted along the surface of the lipid envelope and has a crucial role in determining host range and patterns of pathogenesis (Luciw and Leung, 1992). Envelope proteins are important targets for antiviral immune responses, particularly antibodies; major neutralisation epitopes have been identified on the FeLV SU protein gp70, and the FeLV TM protein p15E (Russell and Jarrett, 1978b; Elder *et al.*, 1987).

Neither viraemia nor antigenaemia were detected in the peripheral blood from cat E12 at any sample date throughout the study (Table 3.1). Furthermore, the FeLV CA protein p27 was not detected in the bone marrow, or in the PLN tissues, post mortem. Nonetheless, the appearance of VNA in the peripheral blood at week 13 confirmed that this cat had been exposed to FeLV. It is understood that transient viraemia may last only a few days following oronasal exposure to FeLV (Jarrett *et al.*, 1982a). In the present study, peripheral blood samples were tested for the presence of infectious virus and the FeLV CA protein p27 one week following oronasal exposure to FeLV, and at three-week intervals thereafter; thus, it is possible that viraemia and/or antigenaemia may have occurred in cat E12 between sample dates, and passed undetected. In previous studies using an identical exposure route, transient viraemia was most commonly detected at, or prior to, week 4 post exposure to FeLV (Jarrett *et al.*, 1982a; Flynn *et al.*, 2002b). However, it is unlikely that either antigenaemia or viraemia occurred in cat E12 by week 4, as proviral DNA was not detected in the peripheral blood until week 7. In addition, the proviral DNA loads in the peripheral blood from this cat were very low, compared to most viraemic cats (Fig. 3.2). However, the presence of proviral DNA in the bone marrow post mortem indicates that a viraemia must have occurred in this cat. Since VI and p27 ELISA assays are conducted using plasma samples, a cell-associated viraemia would not have been detected using these methods.

The detection of infectious virus in the bone marrow post mortem, following VI on cultured bone marrow cells *in vitro*, and the detection of proviral DNA in the lymphoid tissues and peripheral blood post mortem, using qRT-PCR, indicated that cat E12 was latently infected at the termination of the study. Other studies have reported the reactivation of FeLV from myelomonocytic precursor cells in the bone marrow, and from certain T cell populations in the lymph nodes of FeLV-exposed aviraemic cats, but not from blood (Rojko *et al.*, 1982b). A high proportion of recovered cats harbour a latent FeLV infection (Rojko *et al.*, 1982b; Madewell and Jarrett, 1983; Pacitti and Jarrett, 1985). It is highly probable that in the absence of stress factors, such as concurrent disease or pregnancy, which are known to precipitate a recrudescence in viraemia *in vivo*, that cat E12 would have totally eradicated FeLV over time. The majority of latently infected cats eliminate infection within thirty months of exposure to FeLV (Pacitti and Jarrett, 1985); therefore,

infected cells within the bone marrow or lymphoid tissues must differentiate to extinction, as suggested by Madewell *et al.* (1983). The persistence of proviral DNA in a latent infection may be advantageous, in that it may aid in the maintenance of immunological memory (Klenerman *et al.*, 1997). However, latently infected cats have been shown to suffer from a degree of immune dysfunction (Lafrado and Olsen, 1986), and are reported to have a higher incidence of FeLV-associated disease (Francis *et al.*, 1979; Rojko *et al.*, 1982b). Cytological and haematological analyses of bone marrow and peripheral blood respectively, from latently infected cat E12 in this study, were normal thirteen weeks post exposure.

In this study, eleven cats became persistently infected following oronasal exposure to FeLV. From weeks 1-7 until the termination of the study, viraemia, antigenaemia and proviral DNA were detected in the peripheral blood from all eleven cats. Furthermore, an active bone marrow infection was detected in each of these cats post mortem, using VI on cell-free bone marrow suspensions, and immunofluorescence on bone marrow cytospin preparations. Persistently infected cats die from an FeLV-associated disease within two-to-three years following exposure. In this study, evidence that normal bone marrow function was compromised after just thirteen or nineteen weeks following exposure to FeLV, was presented and discussed in Chapter 5. Abnormal cytology and/or haematology reports were presented for all such cats post mortem. Infiltrating $CD3^+$ T cells were detected in the bone marrow from nine cats; five presented with granulocytic hypoplasia, four of which developed a concurrent neutropenia. Concomitant abnormalities in the peripheral blood were evident in seven persistently infected cats. Thus, cells that contribute to the immune defence were compromised in these cats as early as thirteen weeks post exposure, enhancing host susceptibility to opportunistic secondary infections.

Virus isolate, dose, host susceptibility, age at the time of exposure and route of exposure all interact to influence the outcome following exposure to FeLV (Hoover *et al.*, 1976). Specific pathogen free cats E1-E6 were exposed to FeLV at an age, dose and route normally expected to result in approximately fifty *per cent* recovery, fifty *per cent* persistent viraemia (Hoover *et al.*, 1976; Jarrett *et al.*, 1982a; Flynn *et al.*, 2002b). Nonetheless, all six exposed cats became persistently viraemic. Cats E7-E12 were exposed to a lower dose of FeLV-A under the same conditions as cats

E1-E6, and one cat, E12, retained a latent proviral DNA infection in the bone marrow and lymphoid tissues. These data suggest a greater inherent susceptibility of all cats to the molecular clone of FeLV-A/Glasgow-1, which was somewhat improved by reducing the exposure dose by a factor of one log. Indeed, cats that were exposed to a lower exposure dose of infectious FeLV harboured lower levels of proviral DNA in the peripheral blood throughout much of the study, and in the lymphoid tissues post mortem. These cats also generated significantly greater p27-specific and p45-specific proliferative responses in the PLN and MLN tissues respectively, in addition to significantly greater mitogenic responses in the MLN tissues. Nonetheless, these studies have demonstrated the difficulty in predicting the responses of individual cats following oronasal exposure to FeLV.

Gag/Pro-specific CTL have been identified as important mediators of recovery and protection following exposure to FeLV. Under experimental conditions, cats that recovered following exposure to FeLV developed an early high peak in Gag/Pro-specific CTL responses, which coincided with a peak in proviral DNA loads; similarly, cats that were protected following inoculation with an experimental FeLV DNA vaccine, generated high levels of Gag/Pro-specific CTL responses shortly after challenge. The magnitude and timing of Env-specific CTL responses were similar in recovered, protected and in viraemic cats. Thus, in FeLV infection, the Gag/Pro proteins are important in the induction of early virus-specific CTL responses associated with recovery and protection.

Similarly, the Gag protein appears to be immunodominant in the induction of CTL responses in HIV-1 infection; only Gag-specific $CD4^+$ T cell responses correlated with Gag-specific CTL responses, *i.e.* for a given level of help, Gag-specific CTL were more frequently detected than CTL specific for other HIV-1 proteins (Kalams *et al.*, 1999). In addition, a highly significant inverse correlation between Gag-specific $CD8^+$ T cells and plasma viral RNA loads was identified in HIV-1 infected patients at different stages of disease (Ogg *et al.*, 1998). Gag-specific $CD4^+$ T cells are also associated with low plasma viral RNA loads in HIV-1 infection; in a cohort of HIV-1 infected individuals with a wide range of viral RNA loads, an inverse correlation was identified between Gag-specific $CD4^+$ T cell proliferative responses and plasma viral RNA loads (Rosenberg *et al.*, 1997). Further studies demonstrated

that the magnitude of the Gag-specific $CD4^+$ T cell proliferative response directly correlated with levels of Gag-specific CTL precursors (Kalams *et al.*, 1999); high frequencies of Gag-specific $CD4^+$ T cells and Gag-specific CTL precursor cells were associated with the control of viraemia. However, when HIV-1 Gag-specific $CD4^+$ T cells were enumerated using ICS to detect IFN-γ expression, Gag-specific $CD4^+$ T cell frequencies did not correlate with either viral RNA loads (Pitcher *et al.*, 1999; Betts *et al.*, 2001; Boaz *et al.*, 2002), the magnitude of the $CD8^+$ T cell response (Betts *et al.*, 2001), or to a good clinical outcome (Boaz *et al.*, 2002).

These findings illustrate discordance between the frequencies of virus-specific $CD4^+$ T cells detected in the LPA, and such responses detected using ICS, where IFN-γ expression is measured. Multiple reasons have been cited which attempt to account for these discrepancies, many of which have been outlined in Chapter 4. In addition, the LPA measures the capacity of $CD4^+$ T cells to proliferate, which greatly reflects IL-2 production. In the peripheral blood, IL-2 secreting $CD4^+$ T cells comprise naïve cells, effector memory cells and virus-specific cells that have returned to a resting state (Boaz *et al.*, 2002). It has previously been reported that only effector memory T cells in the peripheral blood produce IFN-γ upon restimulation with cognate antigen; therefore, ICS assays based on IFN-γ expression alone measure only the effector memory T cell population (Sallusto *et al.*, 1999); naïve $CD4^+$ T cells take around three-four days to produce cytokines such as IFN-γ (Swain, 1999).

When Gag-specific $CD4^+$ T cell responses were defined by the expression of IL-2 and IFN-γ together, rather than IFN-γ alone, Gag-specific $CD4^+$ T cells were associated with a good clinical outcome in HIV infection (Boaz *et al.*, 2002). Moreover, frequencies of the IFN-γ^+ IL-2^+ $CD4^+$ T cell population correlated inversely with virus RNA load, and directly with Gag-specific IFN-γ^+ $CD8^+$ T cell frequencies (Boaz *et al.*, 2002). These results are comparable with the proliferation data; therefore, the populations under examination in each of these assays may be equivalent. So, in HIV-1 infection, while the numbers of virus-specific IFN-γ^+ $CD4^+$ T cells remain constant as disease progresses, the IFN-γ^+ IL-2^+ $CD4^+$ T cell population becomes disrupted; therefore, the IFN-γ^+ IL-2^+ $CD4^+$ T cell population is more relevant to HIV-1 research. It may also be important to establish whether

fIFN-γ secretion alone is sufficient to establish the presence of functional FeLV-specific $CD4^+$ T cells.

In the present study, the frequencies of virus-specific responses detected in the LPA did not significantly correlate with the frequencies of such responses enumerated using ICS and flow cytometry. However, results obtained using the ICS, rather than the LPA, appeared to more accurately reflect the final outcome following exposure. Greater mean p27-specific (S.I. 1.56) and p45-specific (S.I. 1.46) proliferative responses were detected in the peripheral blood from persistently infected cats, than from latently infected cat E12 (S.I. 1.11 and 1.04, respectively), when compared with cats that received a comparable exposure dose of FeLV-A. Using ICS, high frequencies of early p27-specific $CD4^+$ T cell responses were detected in the peripheral blood from latently infected cat E12, which persisted over weeks 4-10. Conversely, persistently infected cats developed transient low levels of p27-specific $CD4^+$ T cell responses; higher levels were detected in some cats later in the study, but did not persist. Nonetheless, neither virus-specific proliferative responses nor virus-specific $CD4^+$ and $CD8^+$ T cell responses significantly correlated with proviral DNA loads using the Spearman r-Test, irrespective of the exposure dose.

The qRT-PCR technique used in this study detects and quantifies cellular DNA; however, the data generated also includes any unintegrated viral DNA (UVD) within the infected cell, in addition to archival DNA (Hofmann-Lehmann *et al.*, 2001). High concentrations of UVD have been detected in the bone marrow, gut and lymphoid tissues from cats infected with FeLV-FAIDS (Hoover *et al.*, 1987); the appearance of UVD coincides with the onset of a clinical immunodeficiency syndrome. Although large concentrations of UVD have not been definitively associated with other FeLV strains, UVD may account for the high proviral DNA levels detected in some persistently infected cats (Hofmann-Lehmann *et al.*, 2001). The RT-PCR method used in this study cannot distinguish between integrated and unintegrated DNA (Hofmann-Lehmann *et al.*, 2001); thus, the presence of UVD would artificially raise proviral DNA loads quantified using this method.

Once integrated, proviral DNA persists in the cellular DNA of infected cells; HIV-1 proviral DNA loads decline much more slowly in response to antiretroviral therapy,

compared to plasma RNA loads (Désiré *et al.*, 2001; Anton *et al.*, 2003). The qRT-PCR technique used in this study detects intracellular viral DNA, which may represent either an active productive infection or a latent infection. In addition, qRT-PCR will also detect archival DNA, indicative of an earlier productive infection. Unfortunately, qRT-PCR cannot distinguish between these forms of viral DNA; thus, the data generated using this method will include viral DNA derived from each of these sources. Furthermore, proviral DNA detected using qRT-PCR may be replication incompetent. In HIV-1 infection, the presence of PBMC-associated viral DNA sequences significantly correlated with replicating virus, detected using a co-culture assay; thus, at least some DNA detected included replication competent virus. However, the number of DNA-containing cells was two-to-three orders of magnitude greater than the number of infectious virus-containing cells; therefore, 99.9 *per cent* of detectable HIV DNA had no replication potential (Anton *et al.*, 2003). Together, these data suggest that since the proviral DNA loads, measured using qRT-PCR, do not accurately reflect the current viral status, these data may not correlate with the virus-specific immune responses. Viral RNA loads, which rapidly respond to host-virus interactions, may correlate more significantly with virus-specific immune responses. Samples have been submitted to the University of Zurich for analysis; we are currently awaiting the results.

It is recognised that both Gag/Pro-specific CTL, and VNA, are important in mediating recovery and protection following oronasal exposure to FeLV; therefore, it was considered that virus-specific $CD4^+$ T cells, which regulate both arms of the specific immune response, might also be an important element in the control of FeLV infection. In this study, latently infected cat E12, generated VNA in the peripheral blood by week 13 following exposure to FeLV; furthermore, low levels of p45-specific $CD4^+$ T cells were detected in the peripheral blood from this cat at weeks 1 and 10. In addition, high levels of p27-specific $CD4^+$ T cells were detected, which persisted from weeks 4-10. Conversely, VNA were not detected in the peripheral blood from any persistently infected cat at any time throughout the study. In addition, transient low levels of p45-specific, and p27-specific, $CD4^+$ T cells were detected by week 4 in the peripheral blood from six, and three, persistently infected cats, respectively. Higher levels of virus-specific $CD4^+$ T cells were briefly detected in the remaining persistently infected cats after week 7.

All cats in this study developed virus-specific $CD4^+$ T cell responses following oronasal exposure to FeLV. However, in persistently infected cats, these responses were either rapidly deleted, blunted, or absent, in the critical early stages following exposure. Feline leukaemia virus can reach the bone marrow within two days following oronasal exposure. Failure to control virus replication in this site leads to widespread dissemination of the virus, and persistent viraemia within three-to-eight weeks. Thus, there is a window of just three weeks in which virus-specific immune responses can develop, eliminate virus and mediate recovery (Jarrett, 2001).

A quantitative and/or qualitative failure of virus-specific $CD4^+$ T cell responses in the early weeks following exposure to FeLV was associated with the development of persistent viraemia. However, the exact nature of this failure is unclear; in addition, the underlying contributing mechanisms are not understood. Nonetheless, we can speculate on the causes of the failure of the virus-specific $CD4^+$ T cell response. First, the activation of virus-specific $CD4^+$ T cells in the absence of adequate costimulation triggers anergic responses; sufficient numbers of competent DC are required to fully activate the virus-specific $CD4^+$ T cell response. The role of DC in the immunopathogenesis of FeLV infection has not been delineated. Following oronasal exposure to FeLV, DC will be important in conveying virus to the draining lymph node (Macatonia *et al.*, 1987), and in the activation of naïve virus-specific $CD4^+$ T cells (Inaba *et al.*, 1990; Steinman *et al.*, 1991; Swain *et al.*, 1999). It is not known whether DC numbers, or function, are compromised in FeLV infection. Widespread replication of FeLV in bone marrow myeloid precursor cells may curb DC numbers; furthermore, infection of DC in the peripheral tissues may impede function, or directly reduce cell numbers.

Second, the large quantity of soluble glycosylated FeLV CA protein p27, released from FeLV-infected cells, may also have a role in the induction of anergic $CD4^+$ T cell responses (Neil *et al.*, 1980b; Jarrett, 1999). In the present study, persistently infected cats either failed to develop early virus-specific $CD4^+$ T cell responses; or developed truncated or delayed responses. In addition, VNA were not detected in any of these cats throughout the study. Although these data illustrate impaired virus-specific $CD4^+$ T cell responses, it was not clear whether the glycosylated FeLV CA protein p27 contributed to these phenomena *in vivo*.

Third, many early studies demonstrated that cell-mediated and humoral immune responses were impaired in FeLV-infected cats. Feline leukaemia virus has been shown to have an immunosuppressive effect on normal cells *in vitro*; this effect was reported to be associated with the FeLV TM protein p15E. Furthermore, mitogenic responses of cells from FeLV-infected cats were shown to be impaired. The ability of mononuclear cells from the lymphoid tissues and the peripheral blood from FeLV exposed cats in the present study to proliferate in response to mitogenic stimulation varied markedly; however, mitogenic proliferative responses in the peripheral blood and lymphoid tissues did not correlate with proviral DNA loads. Thus, it was considered that differences among cats might be idiosyncratic, rather than associated with FeLV-induced immunosuppression. The inactivated FeLV particles used in the present study retained the immunosuppressive TM protein p15E. Mononuclear cells from all cats in the present study proliferated poorly to inactivated FeLV particles *in vitro*. However, using ICS, specific responses were detected in response to stimulation with the inactivated FeLV particles. Therefore, in this present study, these particles did not have an immunosuppressive effect *in vitro*.

Impairment of the quantitative and/or qualitative virus-specific $CD4^+$ T cell responses will compromise the elicitation, priming and maintenance of the virus-specific $CD8^+$ T cell and virus-specific B cell responses. Failure of the virus-specific immune response will permit unlimited virus replication in the critical early weeks following exposure, precipitating a state of persistent viraemia. Neither the quantitative nor the qualitative virus-specific $CD4^+$ T cell responses, required to mediate recovery and protection following oronasal exposure to FeLV, have previously been defined. However, we know that virus-specific $CD8^+$ T cells and B cell effector responses directed towards either the FeLV Gag/Pro or Env proteins are associated with recovery and protection. Now, these studies illustrate that failure to generate and maintain adequate numbers of virus-specific IFN-γ^+ $CD4^+$ T cells in the early weeks following exposure is associated with the development of persistent viraemia.

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