INVESTIGATION INTO THE USE OF FELINE CD40 LIGAND AS AN ADJUVANT IN A DNA VACCINE AGAINST FELINE IMMUNODEFICIENCY VIRUS

ABIGAIL LOUISE BROWN, BVMS

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Department of Veterinary Pathology

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Feline immunodeficiency virus (FIV) is a pathogenic virus that causes disease in the domestic cat. FIV is found worldwide and can give rise to an infection that is very similar to human immunodeficiency virus (HIV) and the syndrome that is aquired immunodeficiency syndrome (AIDS). As FIV is a significant pathogen of cats, is found worldwide and induces similar pathology to that induced by HIV, developing a vaccine to FIV would not only benefit the domestic cat population but would provide an insight into the requirements for a HIV vaccine. To further the research into a FIV vaccine, the main aim of this study was to evaluate feline CD40 ligand (CD40L) as an adjuvant in a DNA vaccine against FIV.

Initially, the biological activity of the previously cloned feline CD40L was demonstrated using proliferation assays and a CD40L feeder layer system. Once the biological activity of feline CD40L had been proven, a FIV DNA construct was selected for use in a DNA vaccine. To determine whether CD40L would enhance the immune response to FIV, an immunogenicity trial was conducted in mice. BALB/c mice were injected with CD40L DNA alone, FIV DNA alone or FIV DNA and CD40L DNA together. A control group was inoculated with phosphate buffered saline (PBS) only. The study demonstrated that CD40L enhanced the humoral immune response in mice to FIV but the results of the cell-mediated immune response to FIV were inconclusive.

Studies were also conducted in the cat. Specified pathogen free (SPF) cats were inoculated with FIV DNA alone, CD40L DNA alone, or FIV and CD40L DNA. A control group was inoculated with PBS only. This study demonstrated that CD40L enhanced the cell-mediated response to FIV, but no anti-viral antibodies were detected in any of the groups post-vaccination. To further test the efficacy of a CD40L adjuvanted FIV DNA vaccine, the vaccinated cats were challenged intraperitoneally (i.p) with the virulent FIV isolate FIV-Glasgow 8 (FIV-GL8). No cats developed sterilising immunity to the FIV challenge as all vaccinated cats became virus isolation positive; however overall the viral loads were lower in the FIV and CD40L vaccinated cats than in the other groups.

In the vaccine trial it was noted that there was a degree of enhancement of infection in the groups that were inoculated with FIV DNA or CD40L DNA alone. The final part of this study investigated potential modes of enhancement of FIV infection.

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Author's Declaration

This presentation is entirely the product of my own efforts except where specifically stated in the text and in the acknowledgements section. This thesis has not been previously submitted for the award of a degree to any university. The following publication contains work included in this thesis:

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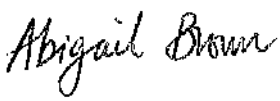
Abigail Louise Brown



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Dedication

In memory of Karen.

Abbreviations

ADE	Antibody-dependent enhancement
AIDS	Acquired immunodeficiency syndrome
AP	Alkaline phosphate
APC	Antigen presenting cell
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BL	Burkitts lymphoma
BSA	Bovine serum albumin
CA	Capsid
CD	Cluster determinant
CD40L	CD40 ligand
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
ConA	Concanavalin A
CpG	Cytidine-phosphate-guanosine
CPM	Counts per minute
CrFK	Crandell feline kidney cells
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbeccos modification of essential medium
DNA	Deoxyribonucleic acid
d.o.c.	Day of challenge
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent antibody cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
GC	Germinal centre
Hb	Haemaglobin
HCT	Haematocrit
HIgM	Hyper-immunoglobulin M
HIV	Human immunodeficiency virus

HSV	Herpes simplex virus
IC	Infected cell
ID	Infective dose
i.d.	Intradermal
IF	Immunofluorescense
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscular
IN	Integrase
i.p	Intraperitoneal
ISCOM	Immune stimulating complexes
kD	kiloDaltons
LAL	Limulus amebocyte lysate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MA	Matrix
mAb	Monoclonal antibody
MACS	Magnetic antibody cell sorting
MAIDS	Murine acquired immunodeficiency syndrome
MCH	Mean cell haemaglobin
MCHC	Mean cell haemaglobin concentration
MCV	Mean cell volume
MHC	Major histocompatibility complex
MIDGE	Minimalistic immunogenic defined gene expression
MLN	Mesenteric lymph node
MM	Mya medium
NBT	Nitro blue tetrazolium
NC	Nucleocapsid
OD	Optical density
PBA	Phosphate buffered saline with azide
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
p.i.	Post infection
PLN	Peripheral lymph node
PR	Protease
PWM	Pokweed mitogen
QVI	Quantitative virus isolation
RBC	Red blood cell count
RPMI	Roswell park memorial institute
RNA	Ribonucleic acid
RSB	Reducing sample buffer
RT	Reverse transcriptase
rVV	Recombinant vaccinia virus
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	Standard error mean
SI	Stimulation index
SIV	Simian immunodeficiency virus
SPF	Specified pathogen free
SU	Surface glycoprotein
TBS	Tris buffered saline
TCID	Tissue culture infective dose
TCR	T-cell receptor
Th	T helper
TM	Transmembrane protein
TNF	Tumour necrosis factor
VNA	Virus neutralising antibody
WBC	White blood cell count
WIV	Whole inactivated virus

Chapter One

INTRODUCTION

1.1. Feline immunodeficiency virus

FIV is a retrovirus of the lentivirus subfamily that infects domestic cats. Closely related viruses infect several species of large felids, cross-reactive antibodies to a FIV-like lentivirus have been found in free-ranging African lions and cheetahs and also in North American pumas (Olmsted et al. 1992). Other lentiviral infections include visna virus in sheep, caprine arthritis-encephalitis virus in goats, equine infectious anaemia virus in horses, bovine immunodeficiency virus in cattle, simian immunodeficiency virus (SIV) in macaques and HIV in man (Coffin, Hughes, and Varmus 1997). FIV was first isolated in 1986 from a pet cat in California (Pedersen et al. 1987) and was classified as a lentivirus due to its many characteristic properties, for example, cell tropism for T lymphocytes and macrophages, magnesium dependent reverse transcriptase enzyme and the induction of a persistent, lifelong infection in the host (reviewed in Miyazawa & Mikami 1993). Analysis of serum samples stored in the 1960s indicated that the virus was prevalent then (Gruffydd-Jones et al. 1988); moreover subsequent phylogenetic studies have suggested that it is even older than that (Rigby et al. 1993; Sodora et al. 1994; Bachmann et al. 1997; Carpenter et al. 1998). Sodora et al. (1994) determined patterns of genetic diversity by studying a 684-nucleotide region encompassing variable regions V3, V4 and V5 of the FIV *env* gene, when the results were compared with a SIV (SIV-AGM) it was found that SIV-AGM subtypes existed within clear geographic boundaries, however FIV subtypes did not suggesting an earlier entry into and/or the greater mobility of the host species. Furthermore, analysis of K_s values (proportion of the potential silent nucleotide changes that have occurred) demonstrated that FIV has been prevalent in cat populations longer than HIV-1 has been in humans. FIV is found worldwide, with the prevalence ranging from as little as 1% in low-risk cats in the United States and Canada to 44% of sick cats in Japan (reviewed in Lee et al. 2002), further evidence that FIV is relatively ancient.

In the UK, Hosie, Jarrett, and Robertson (1989) confirmed that FIV was a significant pathogen of cats, infecting 19% of sick cats compared to 6% of healthy cats. A more recent study concluded that the sero-prevalence of FIV in a group of 517 stray cats sent to a Royal Society for the Prevention of Cruelty to Animals (RSPCA) hospital in Birmingham

was 10.4% in all of the cats, 4.9% in the healthy cats and 16.7% in the sick cats (Muirden 2002). Moreover, in agreement with Hosie, Jarrett and Robertson, Muirden also demonstrated that the seroprevalence of FIV was significantly higher in entire males and neutered males than in females, in cats over two years old compared with younger cats, and in cats suffering disease of non-traumatic origin rather than in healthy cats or cats suffering only from trauma. FIV is shed in the saliva and is transmitted mainly through biting hence the high prevalence in male, free-roaming cats.

In a follow up study of FIV infected cats conducted in July 2000, the outcome of 32 cats that tested seropositive for FIV between February and June that year were categorised into three groups: group 1 were cats that had died or were euthanased due to the severity of their clinical signs; group 2 were cats that had responded to treatment but had shown at least one recurrence of clinical signs since their diagnosis; and group 3 were cats that responded well to treatment and remained free of clinical signs following the initial presentation (Lawson and Hosie 2001). Of the 32 cats in the survey, 53% could be assigned to group 1 and had either died or been euthanased following diagnosis of FIV infection, 25% were in group 2 and were suffering from recurring clinical signs and only 21.9% remained symptom free and could be assigned to group 3. Therefore, it was concluded from these results that FIV is a significant feline pathogen and infection with FIV can result in severe and often fatal illness.

FIV is an important subject to study, not only because of its implications for feline health but also because of the similarities between this feline virus and other lentiviruses causing immunodeficiency, including HIV, the cause of AIDS (Willett, Flynn, and Hosie 1997). Therefore FIV is a useful small animal model for HIV, particularly for research into vaccination. At the present time, 40 million people are living with HIV/AIDS worldwide, of whom 37 million are adults and 2.5 million are children under the age of 15. Five million new infections with HIV occurred in 2003 and a total of 3 million people died from HIV/AIDS related causes in the same year (World Health Organisation and the United Nations Programme on HIV/AIDS, AIDS Epidemic Update, December 2003) (http://www.avert.org/worldstats.htm). It is generally accepted that vaccination is the only way in which the pandemic can be halted. FIV is an animal model which, in terms of clinical pathology and disease progression, is very similar to HIV (Johnson et al. 1994; Miller et al. 2000). Such similarities have promoted the use of FIV as a model for

identifying effective HIV vaccine strategies (Bendinelli et al. 1995). FIV vaccines and treatments can be tested in the natural host and the correlates of protection may be identified. This exchange of ideas and data from FIV research could be vital in reaching the ultimate goal of a vaccine against HIV.

1.1.1. Genomic structure

The FIV genome consists of two identical molecules of single-stranded RNA. Each molecule is flanked by terminal non-coding sequences that include two direct repeats and a unique 5' (U5) or 3' (U3) sequence. Reverse transcription of these non-coding sequences generates identical structures referred to as long terminal repeats (LTRs) found at both ends of the DNA provirus, transcription of the provirus between the upstream U3 and the downstream U5 regions generates RNA with the same terminal organisation as the parental virus (Coffin, Hughes, and Varmus 1997).

The FIV genome contains three major coding regions, *gag*, *pol* and *env*. The *gag* gene encodes the matrix (MA or p15), capsid (CA or p24) and nucleocapsid (NC or p10) proteins, the *pol* gene encodes the protease (PR p14), reverse transcriptase enzyme (RT or p62), integrase (IN or p31) and dUTPase (DU or p15) proteins, and the *env* gene encodes the surface envelope glycoprotein (SU or gp120) and the transmembrane glycoprotein (TM or gp42), see Figures 1.1 and 1.2. There are additional genes, including the *rev* gene, which is involved in transport of incompletely spliced RNA from the nucleus to the cytoplasm, the *vif* gene which controls cell free virus infectivity (reviewed in Miyazawa and Mikami 1993) and the *orf A* gene which is required for virus particle formation and virus infectivity (Gemeniano et al. 2003; Gemeniano, Sawai, and Sparger 2004).

1.1.2. Replication

Once the viral particle has attached to a specific receptor on a susceptible cell, the viral core enters the cell. In order to make the necessary proteins of progeny virions, the viral RNA is reverse transcribed into a linear DNA duplex, which then integrates into the hosts chromosomal DNA and is thereafter transcribed by the hosts own machinery. The integration of the proviral DNA is the basis for the life-long persistence of the virus. Expression of proviral genes leads to the production of progeny virions and may induce death of the cell.

1.1.3. Clinical and pathological findings

$CD4^+$ T-cells are the target of FIV during early infection *in vivo* but, with increased time after infection, $CD8^+$ T-cells and $CD21^+$ B-cells become infected (English et al. 1993; Dean et al. 1996). There are many alterations in T and B-cell function following infection, with a decline in $CD4^+$ T-cells (Ackley et al. 1990; Willett et al. 1991; Hoffmann-Fezer et al. 1992), the expansion of a $CD8^+$ subpopulation (expressing elevated levels of major histocompatibility complex [MHC] class II) (Novotney et al. 1990; Willett et al. 1993), reduced proliferative responses to mitogens, depressed antibody responses and hypergammaglobulinaemia (Ackley et al.1990; Lin et al. 1990; Lawrence, Callanan, and Jarrett 1992).

The initial phase of FIV infection of cats can last from a few weeks up to a few months, during which time there is viral growth resulting in clinical signs such as lymphadenopathy and pyrexia (Yamamoto et al. 1988; Dua et al. 1994). Most cats recover from this phase and then enter the asymptomatic phase (the term 'asymptomatic' is not strictly correct for use with animals but has been widely adopted to describe the period during which infected individuals remain in good health, by analogy to the course of HIV infection in man). The asymptomatic phase can last for several years, during which time the virus is controlled by anti-FIV immune responses. However, there is a steady decline in $CD4^+$ T lymphocyte numbers (Ackley et al. 1990; Willett et al. 1991; Hoffmann-Fezer et al. 1992; Etemad-Moghadam et al. 2001), presumably due to cell killing by the virus, and eventually the animal may become immunodeficient and succumb to the terminal phase of the disease, with clinical signs such as stomatitis/gingivitis, cystitis, chronic diarrhoea and chronic upper respiratory tract infections (reviewed in Bendinelli et al. 1995). Such clinical signs are similar to those exhibited by patients with AIDS, following infection with HIV.

The infected animal mounts humoral and cellular immune responses against the virus. Antibodies directed at the major core proteins and the envelope glycoproteins develop within 2-6 weeks of the animal becoming infected and titres remain high throughout infection (Hosie and Jarrett 1990; Avrameas et al. 1992; Avrameas et al. 1993). Virus neutralizing antibodies (VNA) are also evident from around 5-6 weeks post infection (p.i) (Fevereiro et al. 1991; Osborne et al. 1994; Inoshima et al. 1996; Del Mauro et al. 1998). Virus-specific cytotoxic T lymphocyte (CTL) responses are also detected after infection and normally before the appearance of serum antibodies. Thus, Gag-specific cytotoxic

activity is detected as early as 2 weeks p.i, followed by *env*-specific activity (Beatty et al. 1996). The FIV-specific CTLs induced are predominantly MHC-I restricted. Not only are cytolytic cells induced but non-cytolytic anti-viral activity is also evident (Hohdatsu, Okubo, and Koyama 1998; Flynn et al. 1999; Choi, Hokanson, and Collisson 2000). In a recent study, cytolytic and non-cytolytic T-cell responses were compared in a group of cats experimentally infected with the FIV-GL8 isolate (Flynn et al. 2002). Virus-specific effector CTL responses were first detected at 4 weeks p.i, with the emergence of FIV Gag-specific effector CTL responses in the peripheral blood. However, suppression of FIV replication by non-cytolytic T-cells was apparent in all the lymphoid tissues examined as early as one week p.i.

1.1.4. Cell tropism and pathogenesis of FIV

The cell tropism of a virus is a vital determinant of the pathogenesis of the ensuing disease (Lairmore et al. 1987). FIV has been shown to infect several different cell types *in vivo*, including $CD4^+$ and $CD8^+$ T-cells and B-cells (English et al. 1993). To gain entrance into a cell, the viral SU must attach to one or more cellular receptors. Initially, it was considered that FIV might use the same cell receptor as HIV, namely CD4, due to the similarities in tropism and pathogenesis between HIV and FIV. However, although feline $CD4^+$ cells are highly susceptible to FIV infection, it has been demonstrated that FIV also infects feline $CD4^-$ cells, including feline fibroblasts (Yamamoto et al. 1988), macrophages (Brunner and Pedersen 1989) and astrocytes (Dow, Poss, and Hoover 1990). Another putative cellular receptor for FIV was feline CD9, after an anti-CD9 monoclonal antibody was shown to block infection (Willett et al. 1994). However, further studies demonstrated that the inhibition of FIV infection by anti-CD9 antibody did not operate at the level of virus entry but at a subsequent stage in the virus life-cycle (Willett et al. 1997b). The primary cellular receptor for FIV was identified as CD134 (Shimojima et al. 2004), during the completion of this thesis. CD134 is a T-cell activation antigen and a co-stimulatory molecule. Furthermore, it has been demonstrated that although FIV and HIV do not use the same primary cellular receptor, both viruses use the same co-receptor, namely CXCR4 (Willett et al. 1997c; Poeschla and Looney 1998a; Egberink et al. 1999; Richardson et al. 1999). Willett et al. (1997c) demonstrated that cell-culture adapted strains of FIV were able to use the α-chemokine receptor, CXCR4 for cell fusion and later Richardson et al. (1999) demonstrated the use of CXCR4 by primary isolates of FIV. HIV-1 strains that replicate

only in T cell lines, but not in macrophages or monocytes, known as T-tropic viruses, use CXCR4 for cell entry, while HIV-1 M-tropic (specific to macrophages and monocytes) viruses utilise another chemokine receptor, CCR5 (reviewed in Coffin, Hughes, and Varmus 1997).

As would be expected, the *env* gene is the primary determinant of cell tropism for both HIV and FIV. It has been shown that most primary FIV isolates do not normally grow in fibroblast cells of the CRFK line but replicate freely in activated peripheral blood mononuclear cells (PBMC). Conversely, isolates that are cell-adapted will grow in CRFK cells (Siebelink et al. 1995a). A glutamic acid→lysine (E→K) mutation in the V3 hypervariable domain of SU has been shown to be responsible for the change in tropism (Verschoor et al. 1995a). This amino acid substitution causes an increase in charge of the V3 loop. These differences in cell tropism have been associated with differences in virulence between isolates of FIV. In a recent study the cell tropism and virulence of three different molecular clones of FIV, (FIV-pF34, FIV-14, and FIV-pPPR) was investigated (Dean, Himathongkham, and Sparger 1999). FIV-pF34 was shown to replicate in CRFK cells but not PBMC and proved least efficient for establishing persistent infection in cats, whereas FIV-pPPR could infect PBMC but not CRFK and was most efficient for production of a persistent viraemia *in vivo*. In addition, the molecular clone FIV-14, which was shown to replicate in both CRFK and PBMC induced an infection of low virulence *in vivo*.

Virulence of individual FIV isolates may play a major role in assessing the efficacy of vaccines. Protection with a vaccine based on FIV-Petaluma (PET) was demonstrated against challenge with the homologous isolate and later the heterologous FIV-Dixon isolate (Yamamoto et al. 1993), but these isolates of FIV are believed to be of low virulence compared to the primary FIV-GL8 isolate, which requires a period of adaptation for replication in CRFK cells. However, it is likely to be necessary for a commercially available vaccine to confer protection against primary, and presumably more relevant isolates, such as FIV-GL8. Hosie et al. (1995) conducted whole inactivated virus (WIV) and inactivated cell (IC) vaccine trials in the early 1990s using the FIV-GL8 isolate, but could not reproduce the successful results of Yamamoto et al. (1993) using a FIV-PET WIV vaccine. Since then, a variety of immunogens have failed to induce good protection

against the FIV-GL8 isolate (Hosie et al. 1992; Hosie et al. 1995; Bishop et al. 1996; Hosie et al. 2000; Dunham et al. 2002).

1.2. Vaccination against FIV

The genes of FIV encode proteins against which an immune response can be made. Vaccine trials have been conducted using a variety of immunogens, including whole inactivated virus (WIV), inactivated infected cells (IC), recombinant subunits, peptides, recombinant live viruses and DNA representing different structural and functional FIV genes.

Some vaccine trials have been successful in inducing protection against FIV infection. As indicated above, Yamamoto et al. (1991b) demonstrated protection against the FIV-PET isolate of FIV using paraformaldehyde-inactivated FIV-PET-infected FeT or FL4 cells or paraformaldehyde-inactivated virus from FL4 cell culture fluid (Yamamoto et al. 1991a). Hosie et al. (1995) confirmed the latter result. In a subsequent study, Hosie et al. (1998) induced protection against the FIV-PET isolate by vaccinating with a DNA construct with an in-frame deletion in the RT region of *pol* (FIVΔRT). This construct, administered together with a feline gamma-interferon (IFN-γ) genetic adjuvant, was the first DNA vaccine to confer protection against a lentivirus. However, the result of vaccination is often not as simple as protection or non-protection against viral challenge. There may be other beneficial outcomes after vaccinating against FIV, including decreased viral loads post challenge, slowed rate of inversion of the CD4:CD8 ratio, slowed rate of development of the $CD8\beta^{low}$ cell population or even, unfortunately, enhancement of infection after vaccination. For this reason, more research is required to define the enhancing and protecting immune responses against FIV. Moreover, if a sterilising vaccine cannot be developed, studies into the effects of a non-sterilising HIV vaccine have demonstrated that it may still be very beneficial (Davenport et al. 2004). Davenport et al. (2004) used an epidemiological model to determine the effects of a disease-modifying HIV vaccine and predicted that a decrease in viral load of 1 $\log_{10}$ copies/ml would be sufficient to reduce HIV-associated mortality in the first 20 years after the introduction of vaccination by 30%. Accompanying the variations in vaccine trial outcome are the many variations in vaccine trial design itself, for example, type of vaccine administered (WIV or DNA), injection site of vaccine (intradermal [i.d], subcutaneous [s.c] or intramuscular [i.m]) and challenge inoculum (biological isolates or molecular clones of FIV or natural exposure).

1.2.1. Whole inactivated virus and infected cell vaccines

Since the discovery of FIV in 1986, many combinations of vaccine type and challenge inoculum have been assessed. WIV and IC vaccines have induced the greatest level of protection thus far. WIV vaccines have induced protection against homologous (Hosie et al. 1995; Yamamoto et al. 1991b) and heterologous isolates of FIV (Yamamoto et al. 1993). The results with IC vaccines have been more variable. Protection against infection has been achieved using infected FeT or FL4 cells (Yamamoto et al. 1991b) and MBM (feline $CD3^+$, $CD4^-$ and $CD8^-$ PBMC) cells (Matteucci et al. 1996). In a third study, Bishop et al. (1996) demonstrated protection against a challenge administered soon after vaccination with an FL4-PET IC vaccine, but experienced later infection of FIV-PET in 6/8 vaccinated cats at 50 weeks post-challenge. A further group tested the efficacy of an IC vaccine using infected CRFK cells and infected thymocytes as immunogens (Verschoor et al. 1995b). The cells were infected with the Dutch isolate, FIV-UT113. After challenge with the homologous isolate, 8/10 cats vaccinated with IC vaccines became virus isolation positive. Nonetheless, it was shown that the cats vaccinated with infected CRFK cells had lower viral loads (as determined by quantitative virus isolations [QVI]) after challenge when compared with the unvaccinated controls, suggesting that a degree of protection had been achieved.

IC vaccines have also induced enhancement of infection. Karlas et al. (1998) used autologous fixed FIV-infected PBMC as the vaccine. The FIV isolate used to infect the PBMC was the molecular clone 19K1, and the challenge inoculum was the homologous virus. Although antibodies against Gag proteins were detected after vaccination, antibodies against Env proteins were not, VNA were also detected at low levels. However, 4 weeks after challenge, vaccinated and control cats all became viraemic and, more alarmingly, the infection in the vaccinates was accelerated. Thus, vaccinated cats became viraemic a week before the controls and developed higher viral loads overall. Moreover, provirus was detected by PCR at 2 weeks post-challenge in vaccinated cats as opposed to 4 weeks post-challenge in controls. Giannecchini et al. (2002) also demonstrated enhancement after IC vaccination with infected-autologous lymphoblasts. However, the same group showed promising results with an IC vaccine tested conventionally in SPF cats challenged parenterally (Matteucci et al. 1996) and then by contact in field cats (Matteucci et al. 2000), a study which was more representative of natural infection than those studies

natural infection than those studies conducted in SPF cats. Subject cats were housed in a private shelter where FIV was endemic, with a prevalence of FIV infection of 29-58% over an 8-year observation period. Cats were able to roam freely in the areas surrounding the shelter. After cats were designated FIV negative, they were immunised with 6 doses of an IC vaccine consisting of MBM cells infected with the FIV-M2 Italian isolate over a 16 month period and were observed for 28 months after the commencement of the study. Immunised cats developed both antibodies and cellular immunity to FIV, although the immune responses varied greatly between cats. At the end of the study 0/12 immunised cats had evidence of FIV infection by virus isolation and PCR in comparison with 5/14 control cats. These data are encouraging, suggesting that a vaccine against naturally occurring FIV infection can be developed and evoking the question: should more vaccine trials be conducted in this manner? It may be that parenteral challenge delivers an unnaturally high dose of virus to the host or may circumvent the innate barriers to natural challenge.

1.2.2. Recombinant subunit and peptide vaccines

After initial work with WIV and IC vaccines, attention turned to recombinant subunit and peptide vaccines. These vaccines involved immunising animals with either FIV proteins expressed by recombinant viruses or bacteria, or immuno-affinity purified proteins or synthetic peptides. Several groups tested the efficacy of these vaccines, hoping that targeting the immune system to the immunodominant epitopes of the Env protein for example, would enhance protection. In a previous study, Hohdatsu et al. (1993) demonstrated that serum antibodies against FIV envelope glycoproteins correlated with protective immunity. Thus, when cats were immunised with sera from FIV-PET infected or IC/WIV vaccinated unchallenged cats and later challenged with FIV-PET, they were protected. Again, in a now familiar pattern, vaccination studies with subunit and peptide vaccines had three general outcomes: no noticeable effect on infection, suppression of infection or enhancement of infection.

None of the vaccines that were studied conferred sterilizing immunity in all vaccinates, although some vaccinations did lead to a suppression of infection, manifesting either as a reduction in viral load post-challenge or by a delay in seroconversion (Hosie et al. 1996; Lutz et al. 1996; Tijhaar et al. 1997; Huisman et al. 1998; Richardson et al. 1998; Leutenegger et al. 1998). In addition, many studies did demonstrate the induction of FIV-

specific cell-mediated and humoral immune responses following immunization, particularly with peptide based vaccines (Lombardi et al. 1994; Flynn et al. 1994; Flynn et al. 1995b; Rigby et al. 1996; Finerty et al. 2000). Frustratingly though, none of these vaccines induced protection or even a significant suppression of infection when compared with controls. However, and perhaps more importantly, neither did they facilitate infection.

In contrast, enhancement of infection was observed in cats immunised with a range of vaccines (Hosie et al. 1992; Siebelink et al. 1995b; Flynn et al. 1997; Huisman et al. 1998). In one such study, Hosie et al. (1992), cats were inoculated with purified FIV virions incorporated into immune stimulating complexes (ISCOMS), ISCOMS containing recombinant FIV p24 or an IC vaccine. All of the vaccinates became infected (13/13) in comparison with 7/9 controls. Furthermore, the vaccinated cats were found to be virus isolation positive earlier than the controls. Similar problems were encountered with a recombinant vaccinia virus (rVV) vaccine (Siebelink et al 1995b). FIV envelope glycoproteins were expressed by rVV (in native form or with a deletion of the cleavage site between SU and TM) and were incorporated into ISCOMS. However, two weeks after challenge with the homologous FIV-AM19 isolate, the proviral load proved to be significantly higher in the cats immunized with the rVV than in other cats immunised with β-Gal-FIV Env or SIV-Env ISCOMS or PBS. In another approach, Tellier et al. (1998) tested the efficacy of a canarypox-based FIV vaccine. The efficacy of a HIV-1 canarypox-based vaccine had already been proven in macaques against a heterologous challenge with HIV-2 (Abimiku et al. 1995). The coding regions of the *env, gag* and *protease* genes from the FIV- Villefranche isolate were inserted into a canarypox virus vector (ALVAC) and the ALVAC recombinant (ALVAC-FIV) was then used to immunise SPF cats, with and without an IC vaccine boost. When challenged with a homologous subtype A virus (FIV-PET, which is almost identical to FIV-Villefranche), 2/3 cats inoculated with ALVAC-FIV alone were protected. In addition, 3/3 cats inoculated with ALVAC-FIV and who received an IC vaccine boost were protected. These three cats were then challenged further with the heterologous subtype B virus, FIV-Bangston (FIV-BANG). Partial protection was observed, in that one of the cats remained completely virus negative. Although the other two cats remained virus isolation negative, FIV proviral DNA was detected by PCR. This vaccine trial raises hope that vaccines could be developed that would induce a broader range of protection against more than one subtype of FIV.

1.3. DNA vaccination

A relatively new concept in vaccination strategies is the use of naked DNA as an immunogen. DNA vaccines consist of a foreign gene, for example an FIV structural gene, cloned into a bacterial plasmid. The plasmid also contains an origin of replication to allow growth in bacteria, a selectable marker (such as antibiotic resistance), a eukaryotic promoter to drive expression of the gene of interest in mammalian cells (for example the human cytomegalovirus [CMV] promoter), and a polyadenylation signal to stabilise the transcribed mRNA. DNA vaccines provide a mechanism for achieving intracellular synthesis of antigen by the introduction of a coding sequence directly into cells, either via i.m or i.d inoculation or via a gene gun (whereby DNA coated gold particles are introduced into the epidermis). The DNA is taken up by the host cells and transcribed to mRNA, which is then translated into the protein of interest by the cells own machinery. The viral proteins are then recognised as foreign by the host cells and an immune response is induced against them.

DNA vaccines act like a live viral vaccine, by inducing a MHC class I restricted $CD8^+$ T-cell response, because the immunogen is synthesised inside the cell and processed by the endogenous pathway. Precursor CTLs recognise viral peptides of approximately 9 amino acids presented on infected cell surfaces by MHC class I molecules and are activated to become cytolytic. DNA vaccines can also induce a MHC class II response when immunogens are internalised (reviewed in Robinson and Torres 1997). DNA vaccines do not have the major disadvantage of live viral vaccines, in that the growth of potentially dangerous viruses can be avoided.

Already much research has been conducted using DNA vaccination in the continued search for a FIV vaccine. DNA constructs that have been studied include FIV proviruses which have been rendered defective in various ways, with an in-frame deletion in *pol* (FIVΔRT) (Hosie et al. 1998), a deletion in *vif* (FIVΔvif) (Lockridge et al. 2000), a deletion in the AP-1 binding site (FIVΔAP-1) (Kohmoto et al. 1998) or a deletion in the integrase gene (FIVΔIN) (Dunham et al. 2002). Other constructs that have been developed encode for FIV structural proteins, for example, the envelope glycoprotein and the nucleocapsid protein (Richardson et al. 1997; Cuisinier et al. 1997). Boretti et al. (2000) tested the immunogenicity of a vaccination protocol based on minimalistic, immunogenic defined gene expression (MIDGE) vectors coding for gp140 (SU and the extracellular part of TM)

and feline interleukin-12 (IL-12). The vaccine was administered by gene gun. Like most of the DNA vaccines studied, using this system a low level of protection was induced. On the contrary however, DNA vaccines, like the other vaccines studied, are also capable of causing enhancement of infection (Richardson et al. 1997), although the mechanism is not well understood.

1.3.1. Mode of action of DNA vaccines

The mechanism by which the inoculation of muscle cells (DNA vaccines are primarily introduced i.m) with DNA elicits an immune response is not entirely clear, but there are three possibilities by which antigen encoded by a DNA vaccine could be processed and presented to the immune system, (reviewed in Gurunathan, Klinman, and Seder 2000):-

(a) Direct priming by muscle cells.

(b) Cross-priming in which DNA is taken up by muscle cells or antigen-presenting cells (APCs) and the secreted protein is then processed by other APCs and presented to T-cells.

(c) Direct priming by APC for example, Langerhans cells in the case of i.d injection.

DNA vaccines have been shown to elicit both cellular and humoral immune responses. The predominant isotype detected after DNA vaccination in mice is IgG2a, indicative of a T-helper 1 (Th1) response, although other isotypes produced include IgG1, IgA and IgM. DNA vaccines can also induce Th2 responses in mice when using the gene gun method of immunisation (Feltquate et al. 1997).

There are many factors that influence the type and efficacy of the immune responses provoked by individual DNA vaccines in particular species of animals. It will be important to determine which factors work best to produce a protective immune response against primary field isolates of FIV (such as FIV-GL8). The factors involved include the promoter used to regulate viral gene expression, the mode of administration, any co-stimulatory molecules or adjuvants included in the vaccine (most DNA vaccines developed to date have only been successful in terms of protection when administered in combination with a vector expressing a co-stimulatory molecule) and the presence of cytidine-phosphate-guanosine (CpG) sequences/motifs. CpG motifs are specific nucleotide sequences that contribute to the immunogenicity of DNA vaccines. They occur naturally

in bacterial DNA and have many beneficial immunological effects, such as inducing proliferation of B-cells, induction of APCs to secrete cytokines and stimulation of T-cells. The immune response induced in mice by CpG motifs is characterised by the production of IL-6, IL-12, TNF-α and TNF-γ (Klinman, Yamshchikov, and Ishigatsubo 1997).

In an attempt to define the factors influencing cellular immune responses to FIV DNA vaccination, Flynn et al. (2000) administered FIV-PETΔRT to cats. Feline IFNγ was included as an adjuvant in some groups in an attempt to boost the cell-mediated immune responses. The promoters used in the vaccine trial were either a human CMV promoter or the FIV 5' long terminal repeat (LTR) promoter. The delivery routes of the vaccine were i.m and i.d. The results showed that the highest level of FIV-specific CTL activity occurred in cats inoculated with FIVΔRT + IFNγ, administered i.m, with the CMV promoter. However, the highest level of protection was achieved using a similar construct but with the FIV 5'LTR promoter, again administered i.m.

The fundamental properties of a protective vaccine against FIV have not yet been identified. An attempt was made to define the immune correlates of protection by establishing an immunisation regimen that induced suboptimal protection against FIV-PET using a WIV vaccine (Hosie et al. 1996b). Cats were vaccinated twice with WIV as opposed to three times, the regime shown previously to induce protection. In a preliminary experiment this protocol resulted in virus isolation following challenge from 2/5 vaccinated cats. It was intended that a comparison of the humoral and cellular immune responses in the viraemic and non-viraemic cats would provide an indication of which type of response was required for protection. In the definitive study, only 2/10 vaccinated cats became virus isolation positive. Thirty-five weeks after the initial challenge, the 8 protected cats were then rechallenged with a higher dose of virus (FIV-PET), to assess the duration of immunity conferred by vaccination. After the second challenge only one of the challenged cats became virus isolation positive. The results of the study demonstrated that after the initial challenge, protection correlated positively with the VNA titre induced before challenge. However, the ability to resist the second challenge was more closely related to the initial induction of Env-specific CTL activity following vaccination. These results indicated that protection might be conferred by both humoral and cellular components.

Similar correlates of immunity may not apply to the FIV-DNA vaccine systems. Thus, Lockridge et al. (2000) induced protection against the FIV-PPR isolate using a DNA vaccine that did not induce cellular responses in all of the protected cats. Only one of four immunised protected cats exhibited a measurable CTL response to Env and one to Gag post-inoculation. Conversely, Hosie and co-workers later showed that DNA vaccination could confer protection against a homologous FIV-PET challenge in the absence of a detectable antibody response (Hosie et al. 1998). However, it appears that both cellular and humoral immunity will be required to protect against FIV-GL8 infection and therefore it is important to develop vaccines that may elicit high levels of these types of immune responses.

1.4. CD40L

In order to extend protection against FIV-GL8, the effect of the co-stimulatory molecule CD40L (CD154, gp39, T-BAM or TRAP [tumour necrosis factor related activation protein]), as a genetic adjuvant to a DNA vaccine was investigated. Ligation of the T-cell receptor (TCR) is insufficient to trigger activation of naïve T-cells and both the engagement of the TCR with MHC/antigen and a second signal are required for complete activation. This second, co-stimulatory signal takes the form of a co-stimulatory molecule, which is delivered by the APC to which the naïve T-cell is bound (Parham 2000). APCs can be distinguished from other cells by the presence of co-stimulatory molecules on their surfaces. The CD28/B7 receptor/ligand system is one of the dominant co-stimulatory pathways in the immune system and others include CD40/CD40L.

Kim et al. (1998) demonstrated in mice and chimpanzees that the efficacy of DNA vaccines could be enhanced by co-stimulatory molecules, using CD86 as a co-stimulatory molecule in a DNA vaccine against HIV-1. DNA vaccine constructs expressing HIV-1 *env* and HIV-1 *gag/pol,* as well as constructs expressing human CD80 and CD86 were prepared. CD80 and CD86 belong to the B7 co-stimulatory molecule family and blocking of this signal leads to T-cell anergy. A dramatic increase in CTL induction was observed as well as Th cell proliferation with the co-administration of the gene coding for CD86. This enhancement of CTL response was MHC class I restricted and $CD8^+$ T-cell dependent.

CD40L plays a role in cell-mediated immunity and is also involved in the production of antibodies. Since the components of a protective immune response are not known, it is desirable for FIV vaccines to induce both cell-mediated and humoral immunity. CD40L is a 33kDa type II glycoprotein of the tumour necrosis factor (TNF) family, which is assembled in trimers on the cell surface. CD40L was originally cloned from the murine EL-4 thymoma cell line (Armitage et al. 1992). A cDNA encoding CD40L was isolated from EL-4 cells after enrichment of the cells that bound to CD40-Fc, a biotin-labelled soluble fusion protein consisting of the extracellular domain of CD40 linked to the Fc region of human IgG1. CD40 is the receptor for CD40L and is expressed on B-cells, dendritic cells, endothelial cells, fibroblasts and epithelial cells. The human CD40L gene was isolated by screening stimulated human blood T-cell libraries with the murine CD40L probe (Spriggs et al. 1992; Hollenbaugh et al. 1992). The nucleotide sequences of murine and human CD40L (Genbank accession numbers X65453 and X67878 respectively) predict proteins of 260 and 261 amino acids respectively. CD40L is expressed primarily on activated but not resting $CD4^+$ T-cells and is also expressed on skin mast cells, blood eosinophils and basophils.

1.4.1. CD40L functions in B-cell maturation

CD40L has many functions in the immune system, including the induction of B-cell proliferation, maturation and isotype switching, a role in tolerance at the level of the thymus, and in T-cell priming. The critical role of CD40L in the humoral immune response was confirmed by the discovery that hyper-immunoglobulin M (HIgM) syndrome in humans was caused by defects in the CD40L gene (Allen et al. 1993). Since the CD40L gene is found on the X chromosome, HIgM syndrome is a sex-linked condition and is characterised by increased levels of IgM and decreased amounts of all other isotypes, leading to increased occurrence of bacterial infections in affected individuals. CD40L complementary DNA from three of four patients contained mutations. The proteins encoded by these DNAs were unable to bind the CD40 receptor or to induce proliferation of B-cells or IgE secretion from normal B-cells. Activated T-cells from the four affected patients failed to express wild-type CD40L, although their B-cells were able to respond normally to wild-type CD40L. These findings demonstrated that CD40L is required on activated T-cells to bind CD40 on B-cells to induce proliferation of B-cells, and that CD40L plus the cytokine IL-4 are required for the production of IgE (Spriggs et al. 1992).

Noelle et al. (1992) demonstrated that CD40L induces B-cell maturation, proliferation and differentiation using monoclonal antibodies (mAb) and CD40-Ig (a soluble fusion protein of CD40 and human Ig). The addition of CD40-Ig to cultures of plasma membrane fractions from activated Th cells and B-cells caused a dose-dependent inhibition of B-cell RNA synthesis, whereas a control fusion protein had no effect. Figure 1.3 illustrates the effect of the ligation of CD40 (expressed on B-cells), by CD40L (expressed on T-cells).

CD40L interacts with IL-4 to induce production of IgE (Jabara et al. 1990; Zhang, Clark, and Saxon 1991; Spriggs et al. 1992) and therefore CD40L could possibly be implicated in the cause and treatment of allergies as IgE is produced in allergic reactions (Markert et al. 1999; Mehlhop et al. 2000). CD40 is also expressed on carcinomas (Bussolati et al. 2002; Voorzanger-Rousselot and Blay 2004) and there are exciting new developments with regard to using CD40L as a therapy for cancer patients (Gurunathan et al. 1998; Sotomayor et al. 1999; Hirano et al. 1999; Kikuchi et al. 2000; Vonderheide et al. 2001). Also, CD40L may act directly to suppress tumour growth and indirectly to enhance anti-tumour immune responses (reviewed in Tong and Stone 2003).

1.4.2. CD40L functions in T-cell priming

CD40L promotes the cellular immune response by activating APCs to allow CTL priming. Schoenberger et al. (1998) demonstrated that CD40L expressed on $CD4^+$ T-cells primes CTLs. Ligation of CD40 on APCs such as dendritic cells (DC), macrophages and B-cells by CD40L (expressed on activated $CD4^+$ T-cells), greatly increases their antigen presenting and co-stimulatory capacity. DCs process foreign antigen and present it in a complex with MHC Class II molecules to naïve antigen-specific T-cells. DCs also express B7, which interacts with CD28 on T-cells to cause early T-cell activation and the expression of CD40L by T-cells. The CD40L expressed binds to CD40 on the DCs, resulting in expression of T-cell stimulatory molecules such as IL-12, and upregulation of B7 production. The expression of these molecules culminates in T-cell expansion and maturation into Th-cells or CTLs, as illustrated in Figure 1.4.

1.4.3. Identification of feline CD40L

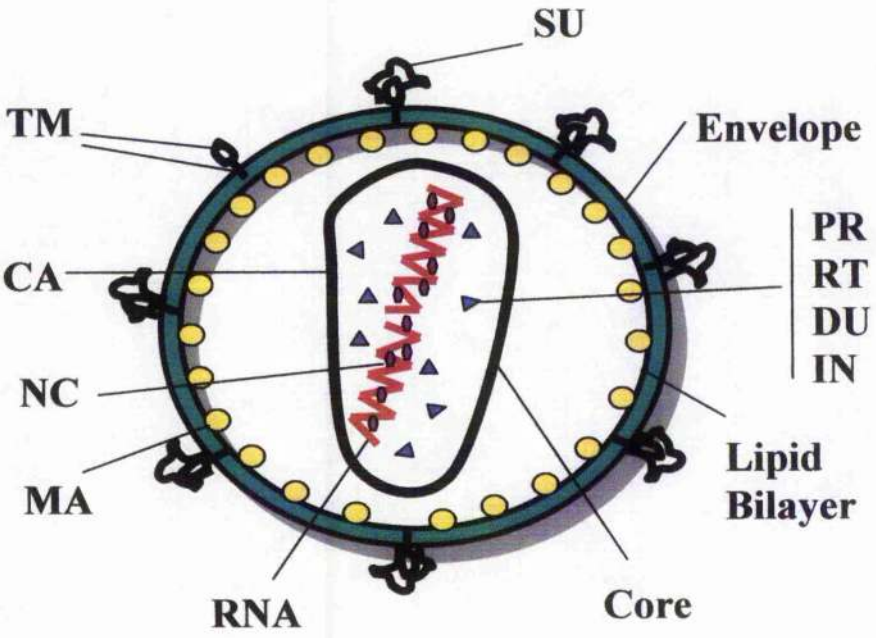
A biologically active homologue of CD40L has been identified in the domestic cat (Brown et al. 2002). Briefly, cDNAs encoding the feline homologue of CD40L were isolated from mRNA from feline thymus and peripheral blood lymphocytes. Primers designed from

regions of consensus sequence identified in the published human and murine CD40L sequences were used to amplify feline CD40L using the polymerase chain reaction (PCR). The cDNAs encoded a single open reading frame 780bp in length giving rise to a polypeptide of 260 amino acids with a predicted molecular mass of 28.6kDa. The nucleic acid sequence of feline CD40L has been deposited in GenBank, accession number AF079105. The biological activity of feline CD40L was demonstrated using 3T3 cells (murine fibroblast cell line) (Todaro and Green 1963) stably transfected with feline CD40L (3T3.F154) as targets in proliferation assays and it would appear that feline CD40L has similar properties and functions as murine and human CD40L. Therefore, it may have the potential to induce humoral and cellular immune responses if used as a co-stimulatory molecule in feline DNA vaccines. As described above, CD40L mediates its antiviral effects via interaction with CD40. In addition it may have direct antiviral activity itself, since Ruby et al. (1995) demonstrated that rVV that expressed CD40L were readily cleared from a variety of immunodeficient mice.

CD40L has been considered as an adjuvant for HIV vaccination. Ihata et al. (1999) conducted a study in mice whereby a plasmid expressing human CD40L and plasmids encoding gp160 of HIV-1IIIB and HIV-1 *rev* were inoculated by the i.m route. Expression of human CD40L enhanced Th1 and Th2 type cytokine production, increased the production of antigen-specific antibody of both IgG and IgM isotypes and induced delayed-type hypersensitivity and CTL activity in a dose-dependent manner.

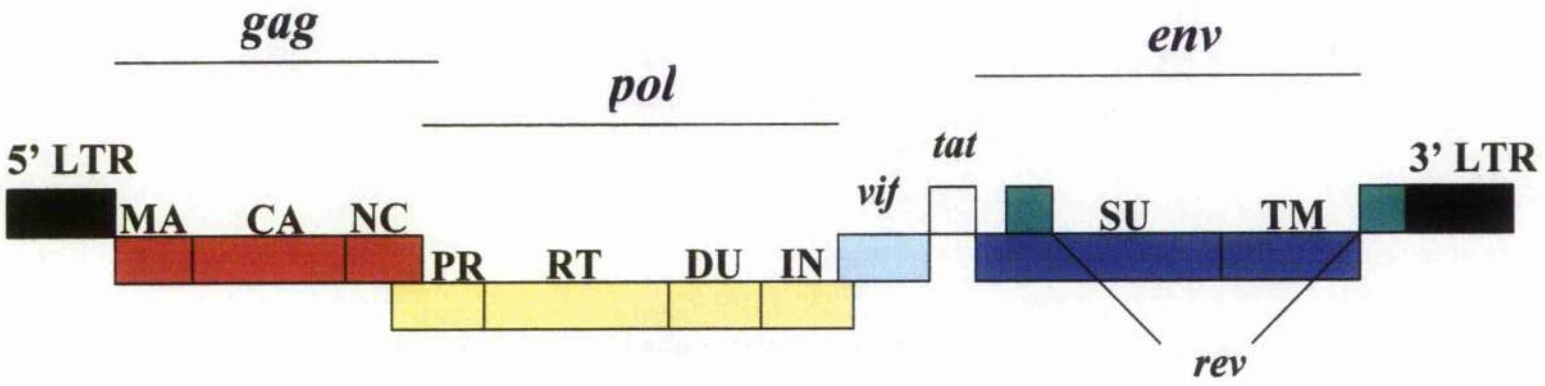
The aim of this project was to evaluate the use of CD40L as an adjuvant for FIV DNA vaccination. As a preliminary, the biological activity of feline CD40L was assessed. The immunomodulatory capacity of feline CD40L in mice was tested and a vaccine trial against FIV was conducted in cats. The advantage of testing the adjuvant effects of CD40L in conjunction with an FIV DNA vaccine is that the FIV cat model represents a challenge system in which the protective efficacy of the induced immune responses may be tested. Thus, research into the use of CD40L as an adjuvant in DNA vaccination is an important step in the continuing search for a vaccine against FIV and HIV.

Figure 1.1. Schematic cross section of a retrovirus particle



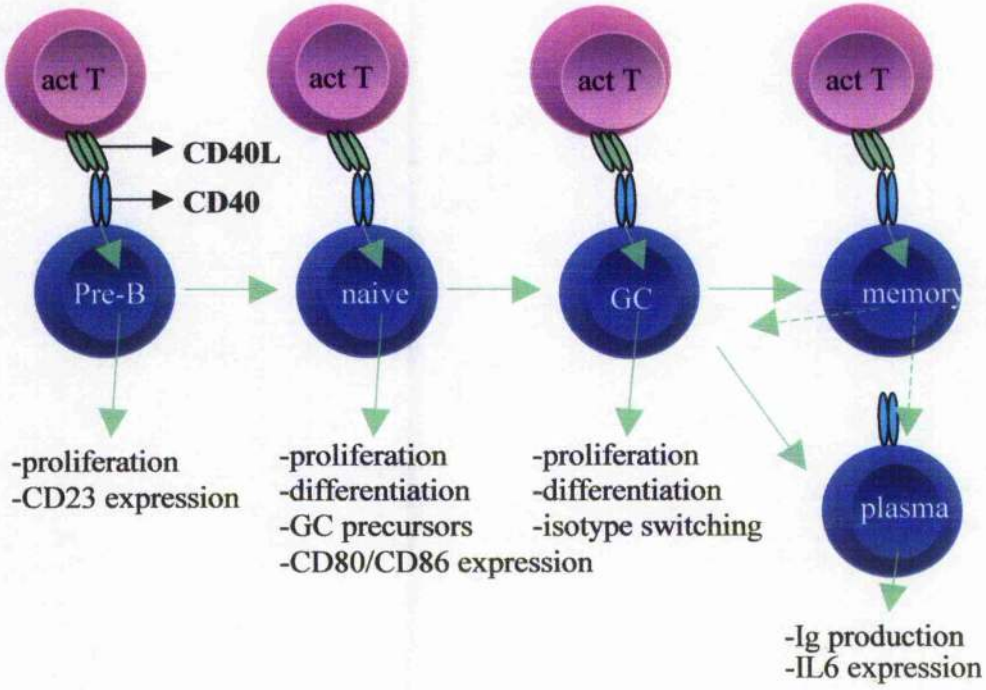
Proteins encoded by the *env* region of the viral genome are SU and TM, the *gag* region of the viral genome encodes MA, CA and NC and the *pol* region encodes RT, PR, IN and DU. For details of abbreviations, see text. Adapted from Bendinelli et al. 1995.

Figure 1.2. The proviral FIV genome



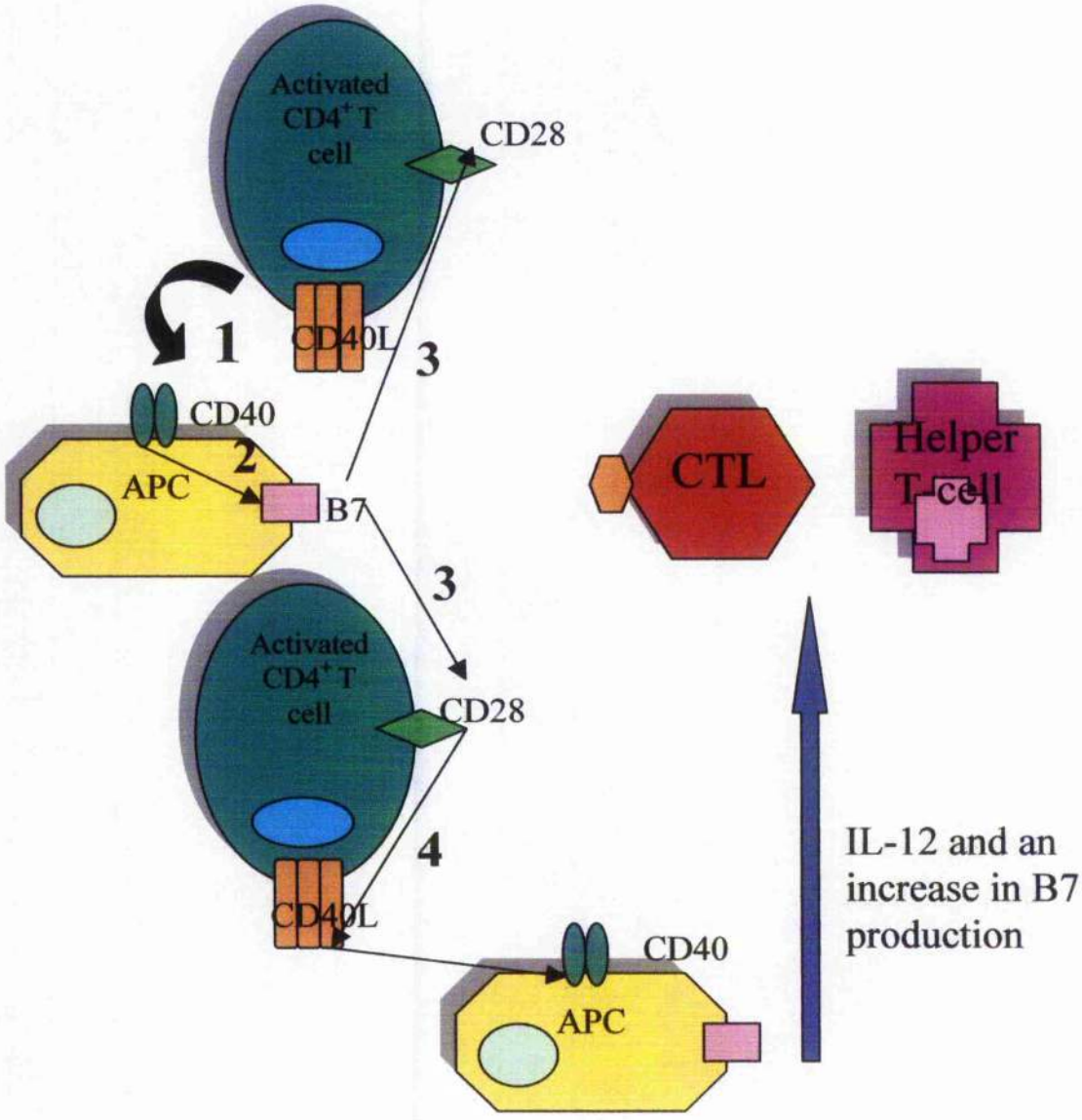
See text for abbreviations. Adapted from Encyclopedia of Virology, Second Edition, Volume One, A.Granoff and R.G.Webster, Academic Press

Figure 1.3. Biological effects of CD40 activation on different stages of B-cell development



The figure illustrates the effect of CD40 ligation on B-cells with CD40L expressed on activated T-cells. The consequences differ depending on the stage of development of the B-cell. Transition from naïve to germinal centre (GC) and from GC to memory is thought to be promoted by CD40 activation. CD40 activation of memory B-cells either results in a new round of germinal centre reaction and/or an accelerated differentiation into immunoglobulin producing plasma cells. Adapted from Kooten and Banchereau 2000.

Figure 1.4. CD40L functions in T-cell priming



CD40L expressed on activated CD4+ T-cells primes cytotoxic T-lymphocytes (CTL), in association with antigen presenting cells (APCs). Ligation of CD40 on APCs by CD40L expressed on CD4+ T-cells, greatly increases their antigen presenting and co-stimulatory capacity. These events culminate in T-cell expansion and maturation into helper T-cells or CTLs.

Chapter Two

COMMON MATERIALS AND METHODS AND STATISTICAL ANALYSIS

2.1. Common materials and methods

2.1.1. Cells and culture media

2.1.1.1 Mya-1 cells

Mya-1 cells (Miyazawa et al. 1989) represent a feline T lymphocyte cell line that was selected for its susceptibility to FIV infection and was maintained in Roswell park memorial institute (RPMI)-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 100 IU/ml penicillin and 100µg/ml streptomycin, 5 x 10^{-5}M 2-mercaptoethanol (complete RPMI) and 100 IU/ml human recombinant IL-2 (a kind gift from M. Hattori, University of Tokyo).

2.1.1.2. 3T3 cells

The murine fibroblast cell line 3T3 (Todaro et al. 1963) was maintained in Dulbecco's modification of minimal essential medium (DMEM) (Life Technologies) supplemented with 10% FCS, 2mM glutamine, 100 IU/ml penicillin and 100µg/ml streptomycin (complete DMEM). The 3T3.F154 cell line derived from the 3T3 cell line was maintained in complete DMEM, with the addition of 400µg/ml G418-sulphate (Geneticin, Life Technologies).

2.1.1.3. 293T cells

The human kidney epithelial cell line 293T (DuBridge et al. 1987) was also maintained in complete DMEM supplemented with 400µg/ml G418-sulphate (Geneticin, Life Technologies).

2.1.2. Viruses

The FIV-GL8 isolate (Hosie 1991), first isolated in 1988 was used in this study, both to develop a DNA vaccine and also as the challenge inoculum. The challenge virus (Hosie et al. 1992; Hosie et al. 1995) was prepared from the biological isolate and was previously

titrated *in vivo* to establish the infective dose 50% (ID_{50}) and 10 ID_{50} was used as the challenge inoculum.

2.1.3. DNA vaccines

2.1.3.1. CD40L DNA vaccine

For the purposes of inoculating into skeletal muscle, the feline CD40L cDNA (previously amplified from cDNA prepared from feline thymus using PCR) was sub-cloned into the VR1012 plasmid vector (Vical Inc., San Diego, CA, U.S.A), to produce VR1012.F154. Plasmid DNA was purified using an endotoxin free plasmid maxi kit (Qiagen, Hilden, Germany) and was subsequently dialysed extensively against PBS (Slide-a-Lyser, Pierce, Perbio Science UK Ltd, High Street, Cheshire, UK) to ensure it was safe for inoculation. If endotoxins are not removed prior to inoculation *in vivo*, the recipients can suffer from pyrexia. Therefore, to confirm that the DNA was endotoxin free, endotoxin testing was conducted using a Limulus Amebocyte Lysate (LAL) gel clot endotoxin testing kit (BioWhittaker Walkersville, MD) and demonstrated that the endotoxin content of the DNA was sufficiently low for use as a DNA vaccine. Endotoxin levels < 5 Endotoxin Units/ml were considered acceptable (Q-One Biotech Ltd., Glasgow, UK).

2.1.3.2. FIV DNA vaccine

Plasmid DNA was purified using double caesium chloride-ethidium bromide gradient centrifugation, butanol extraction and ethanol precipitation. To prepare large scale plasmid preparations, 500ml cultures of E.coli containing the plasmid DNA were centrifuged at 6K for 15 minutes and the supernatant discarded. The bacterial pellet was resuspended in 50ml of solution I (see Appendix B) and incubated on ice for 30 minutes, 80ml of solution II (see Appendix B) was then added and incubated on ice for a further 15 minutes. To this 40ml of solution III (see Appendix B) was added and incubated on ice again for 15 minutes. This suspension was then centrifuged at 7K for 25 minutes at a temperature of 14°C. The resultant supernatant was filtered through filter paper and 170ml of isopropanol was added to this, this solution was centrifuged at 7K for 30 minutes and at a temperature of -2°C. The resultant pellet was resuspended in 20ml Tris- Ethylenediaminetetra-acetic acid (EDTA) (TE, 10mM Tris/1mM EDTA). To this, 21g caesium chloride and 2ml 3mg/ml ethidium bromide was added. This suspension was then ultracentrifuged at 55K overnight at 20°C. The resultant ethidium bromide stained DNA band was removed from

the ultracentrifuge tubes with a needle and syringe. The DNA was ultracentrifuged again at 100K for 4 hours at 18°C to increase purity. To extract the DNA, the ethidium bromide band was again removed with a needle and syringe and to this an equal volume of butanol was added and centrifuged at 3K for 10 minutes. This step was then repeated. To precipitate the DNA, 2 volumes of TE were added, a $1/10^{th}$ of the volume of 3M NaOAc was added and mixed by inverting the tube. To this, 2 volumes of 100% ethanol were added. This suspension was placed on ice for 30 minutes to precipitate the DNA and then centrifuged at 3K for 10 minutes. The DNA pellet was then washed in 70% ethanol and centrifuged again at 3K for 10 minutes. The supernatant was removed, the DNA pellet was air-dried and re-suspended in TE buffer and then dialysed extensively against PBS (Slide-a-Lyser, Pierce). Endotoxin testing was conducted using a LAL gel clot endotoxin kit (BioWhittaker) as for the CD40L plasmid. To confirm the results a DNA sample was also sent to the European Endotoxin Testing Service (BioWhittaker, Europe).

2.1.4. Proliferation assays

3T3 cells expressing feline CD40L (3T3.F154) and control 3T3 cells were used as target cells in proliferation assays. ^{60}Cobalt irradiated (3000 rads) 3T3.F154 and 3T3 cells were added in decreasing numbers (10^5, $3x10^4$, 10^4, $3x10^3$, 10^3, 300 and none) in triplicate to a 96-well flat-bottomed plate (Falcon, Becton Dickinson Labware Europe, France, S.A). PBMC from a SPF cat were obtained by overlaying Ficoll-Hypaque (Pharmacia LKB, Biotechnology Inc., Pistcataway, NJ, USA) with a heparinised whole blood sample diluted 1 in 4 in serum free RPMI (Life Technologies) and centrifuging at 2000rpm for 10 minutes, after which time the PBMC had collected at the interface above the red blood cells and could be removed by pipetting. The PBMC were then washed in serum free RPMI (Life Technologies) and centrifuged again at 1000rpm for 5 minutes. After centrifugation the PBMC were resuspended in complete RPMI (Life Technologies) and 10^5 cells were added to each well of the 96-well plate. The plates were incubated at 37°C in 5% CO_2 for 4 days, after which time 20µCi/well of tritiated thymidine (^{3}H-thymidine) (Amersham Biosciences, Buckinghamshire, UK) was added for the final 18 hours of incubation. A positive control of concanavalin A (conA) (Sigma Aldrich Co Ltd., Poole, Dorset, UK) was included. The cells were harvested from the 96-well plates onto absorbent filter-plates using the Packard Filtermate 196 (Perkin Elmer, Beaconsfield, Buckinghamshire, UK) and the amount of incorporated radioisotope was determined by a

microplate scintillation counter (Topcounter, Packard). Results are expressed as the mean of the triplicate value ± standard error mean (SEM) counts per minute (cpm).

2.1.5. Flow cytometry

For analysis by flow cytometry lymphocytes were stained with the appropriate antibody. Thus, 10^6 cells were washed in PBA (phosphate buffered saline/ 1% bovine serum albumin [BSA]/ 0.1% azide). After centrifugation at 1000rpm for 5 minutes, the resulting cell pellet was resuspended in 50µl of PBA and the appropriate fluorescent conjugated antibody was added. Staining was conducted by incubating with the antibody for 30 minutes at 4°C. After incubation, the cells were washed twice in PBA and resuspended in 1ml PBA. Lymphocytes were "live-gated" on the basis of size (forward scatter) and granularity (side scatter) and 5000 events were collected for each sample.

2.1.6. Serology

2.1.6.1. TM and p24 ELISA

A peptide-based enzyme-linked immunosorbent assay (ELISA) was employed to determine titres of antibodies recognising an immunodominant epitope in the TM glycoprotein (CNQNQFFCK) of FIV (Avrameas et al.1993; Sibille et al. 1995). TM ELISAs were conducted at Companion Animal Diagnostics, Department of Veterinary Pathology, University of Glasgow, UK. Briefly, a 96-well ELISA plate (Greiner High Binding, Greiner Bio-One, Stroudwater Business Park, Glos, UK) was coated overnight with 500ng/well TM peptide in a volume of 100µl Tris buffered saline (TBS), supplemented with 0.05% Tween 20 (TBS/Tween) at 4°C and with the plate continuously shaking. After washing five times in TBS/Tween, the rest of the assay was conducted at room temperature. Wells were blocked with 100µl TBS/Tween, supplemented with 10% normal goat serum (blocking buffer) for 45 minutes and the plates were washed again. Dilutions of the serum samples to be tested were made in blocking buffer and 100µl of sample was added per well and incubated for 30 minutes. After incubation, plates were washed with TBS/Tween and then incubated with biotinylated Protein A (4µg/ml) (ICN Pharmaceuticals Ltd., Basingstoke, Hants.) diluted in blocking buffer for 30 minutes. The plates were again washed and incubated for a further 30 minutes with streptavidin-alkaline phosphatase, before being washed again. Alkaline phosphatase substrate tablets were

dissolved in ethanolamine buffer and 50µl substrate was added per well. The reaction was stopped after 15 minutes by adding 50µl/well of 0.5M NaOH. The optical density (OD) of the wells was read on a Multiskan Ascent microplate reader (Thermo Labsystems, Middlesex, UK) at 405nm. The assay cut-off was determined by adding 0.15 to the mean OD of the negative controls at a dilution of 1:5. The titre of a sample was taken to be the reciprocal of the highest dilution that equalled or was greater than this figure.

Antibodies recognising the FIV p24 core protein were also measured by ELISA using a similar method to the TM ELISA (Reid et al. 1991) and were also conducted at Companion Animal Diagnostics, University of Glasgow, UK.

2.1.6.2. Immunofluorescence

Antibodies recognising FIV-infected CRFK cells were detected by an indirect immunofluorescence (IF) technique using fluorescein isothiocyanate (FITC)-conjugated anti-IgG secondary antibody. The IF technique was performed using previously prepared plates consisting of a 1:1 mixture of uninfected CRFK cells and CRFK cells chronically infected with FIV which had been fixed and stored in methanol at -20°C. 10-fold dilutions of the sample serum from 1:10 to 1:10000 were made using PBS containing 5% FCS and 25µl of each sample was added to the previously prepared plates. The plates were then incubated at 37°C in moisture chambers for 90 minutes. After incubation, the plates were washed with PBS for 2 minutes then rinsed in water and left to dry. 25µl of FITC-conjugated anti-IgG is added to each well and the plates were then incubated for a further 60 minutes at 37°C. After incubation, the plates were again washed and dried and then examined under a UV microscope using a 10x objective. The endpoint was taken as the last dilution showing fluorescence. IF was conducted at Companion Animal Diagnostics, University of Glasgow, UK.

2.1.6.3. Western blotting

For Western blotting a cell lysate was prepared from Mya-1 cells infected with FIV-GL8. Thus, 10^7 Mya-1 cells that had been infected with supernatant previously removed from infected Mya-1 cells, filtered and frozen, were centrifuged at 1000rpm for 5 minutes, to obtain a cell pellet. The resultant cell pellet was resuspended in 50µl of lysis buffer (1% NP40, 150mM $NaCl_2$, 10mM Tris/HCl and 2mM HCl) and incubated for 10 minutes at room temperature. Following incubation, the cell suspension was centrifuged at 1000rpm

for 5 minutes and the resulting supernatant was removed and the pellet discarded. To 20µl of the supernatant (cell lysate) 50µl of reducing sample buffer (RSB) (0.5M Tris pH 6.8, glycerol, 2-mercaptoethanol, 10% SDS and 0.2% bromophenol blue) was added and the sample was boiled for 3 minutes and then resolved on a 4-20% gradient Tris-HCl SDS polyacrylamide single well prep gel (SDS-PAGE) (Biorad Laboratories, Hercules, CA). The proteins were transferred onto a nitrocellulose membrane (Biorad), using the Biorad wet tank transfer system and the resultant blots were blocked overnight with 0.5% non-fat milk powder in PBS (blocking buffer). Blots were cut into 2mm strips and incubated for 2 hours at room temperature with mouse serum or feline plasma diluted 1:10 with PBS supplemented with 1% non-fat milk powder and 0.5% Tween-20. Controls of known FIV-positive and FIV-negative cat serum were included. After incubation the strips of membrane were washed with 0.5% Tween-20 in PBS (washing buffer) and the feline plasma samples and the FIV-positive and FIV-negative samples were incubated for 1 hour with biotinylated protein A (ICN Pharmaceuticals Ltd.) at a concentration of 4µg/ml. The secondary antibody used for membranes incubated with mouse serum was a biotinylated anti-mouse IgG antibody (Vector Laboratories, Inc., Burlingame, CA) (1µg/ml). After incubating with the secondary antibodies the membranes were washed again and incubated for a further hour with streptavidin-alkaline phosphatase (Bio-Rad) diluted 1 in 500 with PBS supplemented with 1% non-fat milk powder and 0.5% Tween-20. Membranes were then washed and incubated in alkaline phophatase buffer (AP buffer) (100mM diethanolamine/5mM MgCl/100mM NaCl, pH 9.5) for 10 minutes before staining was developed using AP buffer containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50mg/ml) (Sigma) and nitro blue tetrazolium (NBT) (50mg/ml) (Sigma).

2.1.7. Detection of a cell-mediated immune response

A proliferation assay was employed to detect T-cell proliferation in response to either FIV p24 core protein or WIV. Either FIV p24 core protein or WIV was added in decreasing concentrations (25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml and none) in triplicate wells to a 96-well U-bottomed tissue culture plate (Falcon). A volume of 100µl of each concentration of p24 or WIV was added per well and incubated overnight at 37°C and 5% CO_2. A positive control of con A (10µg/ml) (Sigma) and a negative control of complete RPMI only were also included. After incubating overnight, either $2x10^5$ mouse spleen cells or feline PBMC were added to each well. The plates were then incubated at 37°C and

5% CO_2 for a further 3 days after which time 20μCi/well of ^{3}H-thymidine in complete RPMI was added for the final 5 hours of incubation. The cells were then harvested on to filter plates (Packard) using the Packard Filtermate 196 and the incorporated radioactivity was read on a microplate scintillation counter (Topcounter, Packard). The stimulation index (SI) was determined from the formula: SI = experimental count/spontaneous count. Spontaneous count wells included 10% FCS, which serves as an irrelevant protein control. A SI of ≥ 2 was regarded as significant.

2.2. Statistical analysis

Where possible results were analysed using the software packages SigmaPlot 2001 for Windows version 7.0 (Copyright© 1986-2001 SPSS Inc.), and SigmaStat for Windows 2.03 (Copyright© 1992-1997 SPSS Inc.). The Student's t-test was used to compare two groups. *P* values ≤0.05 were considered significant (*) and ≤0.01 highly significant (**).

Chapter Three

DEMONSTRATION OF THE BIOLOGICAL ACTIVITY OF FELINE CD40L

3.1. Introduction

After the murine (Armitage et al. 1992) and human (Spriggs et al. 1992; Hollenbaugh et al. 1992) CD40L genes were cloned, CD40L and its receptor CD40 were shown to play an important part in immune regulation (Kooten and Banchereau 1996). In addition, the efficacy of CD40L-adjuvanted vaccines was demonstrated, including those against HIV-1 (Ihata et al. 1999), *Leishmania major* (Gurunathan et al.1998), bovine herpesvirus 1 (Manoj et al. 2003) and herpes simplex virus (HSV) type 2 (Sin et al. 2001). In order to determine the efficacy of a DNA vaccine against FIV adjuvanted with feline CD40L DNA, the feline homologue of CD40L had first to be identified and cloned and then its biological activity assessed. Prior to this study, the feline CD40L gene was cloned and murine fibroblast 3T3 cells were stably transduced with a retroviral vector expressing feline CD40L, enabling the cells to express CD40L on their cell surface (3T3.F154). These cells were used as the basis for *in vitro* studies of the biological activity of feline CD40L.

To determine the biological activity of the cloned feline CD40L, proliferation assays were conducted whereby the results relied on the interaction between B and T-cells through CD40 and CD40L. Thus, feline PBMC or PBMC cell fractions were co-cultured with 3T3.F154 cells in 96-well plates and the extent of cell proliferation was determined by measuring the incorporation of radiolabelled thymidine. The immunophenotype of proliferating cells was determined using flow cytometric analysis.

The capacity of CD40L to maintain B-cells in longer-term culture was investigated. SPF cat PBMC were cultured to establish whether stimulation with CD40L could maintain a normal feline B-cell line in culture, as CD40L expressed on human and murine T-cells has been shown to interact with CD40 on B-cells to cause proliferation and differentiation of resting B-cells (Noelle et al. 1992; Spriggs et al. 1992). Previous studies had shown that it was possible to maintain murine B-cells in culture by stimulating with anti-CD40 antibody and IL-4 (Rush and Hodgkin 2001). However, there is no record of a similar study with

feline B-cells. Flow cytometry was performed to determine the immunophenotype of the cell populations that resulted from stimulation of SPF cat PBMC with CD40L.

An opportunity to include the study of neoplastic cat PBMC arose because a domestic cat suspected to be suffering from chronic lymphocytic leukaemia (CLL) was admitted to the small animal clinic at Glasgow University Veterinary Hospital. Blood was collected from a peripheral vein for diagnostic studies. From the leukaemic cells a B-cell line was established and maintained using 3T3.F154 cells as a feeder layer. This cell line was investigated further using flow cytometry and Southern blotting techniques. CLL is a disease of the bone marrow that can affect either the T or B- lymphocytes. In this case the cat had a B-lymphocytosis (Tebb et al. 2004). Crawford and Catovsky (1993) had previously demonstrated that it was possible to maintain a human leukaemic B-cell line in culture with IL-4 and antibodies to CD40. In these studies, a feline leukaemic B-cell line was maintained with CD40L stimulation alone.

3.2. Materials and methods

3.2.1. Production of a cell line stably expressing feline CD40 Ligand

Prior to this study, feline CD40L was amplified by PCR from cDNA prepared from feline thymus (Brown et al. 2002). Briefly, PCR products were cloned into the pCRII cloning vector and several clones containing the CD40L insert were identified. Plasmid DNA from these clones was isolated. This DNA was then sub-cloned into the pDON-AI retroviral vector (Takara Bio Inc., Shiga, Japan) and transfected into the Phoenix-Eco ecotropic murine leukaemia virus-based packaging cell line (Nolan Laboratory, University of Stanford). Supernatant fluids from the transfected cells were collected 48 hours post-transfection and used to infect the 3T3 cell line. The infected cells were cultured for a further 2 days before being sub-cultured and re-seeded in complete DMEM supplemented with 800μg/ml G418-sulphate. G418-resistant cells containing the CD40L gene, known as 3T3.F154, were amplified and maintained in medium containing 400μg/ml G418-sulphate and were used for feline CD40L studies in vitro.

3.2.2. Proliferation assays (whole PBMC and PBMC cell fractions)

The proliferation assay protocol is described in detail in section 2.1.4. The phenotype of the proliferating cells was determined in a parallel proliferation assay, in which a further 10^6 feline PBMC were cultured in each well of a 12-well plate (Falcon) with decreasing numbers (10^6, 10^5 and none) of 3T3.F154 or 3T3 cells. After 5 days in culture, PBMC were removed from the wells for analysis by flow cytometry and were stained with phycoerythrin (PE)-conjugated anti-CD5 antibody (F43, Southern Biotechnology Associates Inc., Birmingham, AL 35226, USA), which is a pan-T cell marker, and FITC-conjugated anti-B220/CD45R antibody (RA36B2, BD PharMingen, A Becton Dickinson Co., San Diego, California) which is an anti-mouse B-cell antibody that has been shown previously to cross-react with feline B-cells (Brown et al. 2002). The data were analysed using WinMDI v2.8 (Dr. J. Trotter, Scripps Research Institute, La Jolla, CA, USA).

Similar proliferation assays were conducted using cell fractions of PBMC. The fractions were obtained by depleting B and T lymphocytes from a mixed population of PBMC using magnetic antibody cell sorting (MACS). To deplete B-cells, 10µl of anti-B220-antibody coated magnetic beads (MACS beads) (MACS reagents, Miltenyi Biotec, 51429 Bergisch Gladbach, Germany) were added for every 10^7 PBMC and the cells were incubated for 30 minutes at 4°C in MACS buffer (PBS, pH 7.2 with 0.5% BSA and 5mM EDTA). After incubation, cells were washed and resuspended in 500µl MACS buffer. Cells were then added to a MACS separation column (Miltenyi Biotec), attached to a magnet and the B cell-depleted PBMC were allowed to pass through the column. The column was then removed from the magnet and the B cell-enriched fraction was flushed through with MACS buffer. To obtain the T cell-enriched and depleted fractions, the PBMC were first incubated with mouse anti-feline CD3 antibody (NZMI, kindly supplied by Dr. Y. Nishimura, University of Tokyo) for 30 minutes at 4°C. After incubation, cells were washed in 1ml MACS buffer, resuspended in 20µl of goat anti-mouse IgG-coated MACS beads (Miltenyi Biotec) and incubated for a further 15 minutes at 4°C, at which time the PBMC were washed again and resuspended in 500µl MACS buffer. The T-cell fractions were then obtained in a similar manner to the B-cell fractions using the MACS separation column. The proliferation of the different cell fractions in response to incubation with 3T3.F154 cells was then assessed using proliferation assays as described in section 2.1.4.

3.2.3. In vitro stimulation of SPF and CLL PBMC with feline CD40L

PBMC from a SPF cat were isolated from a sample of heparinised venous peripheral blood by centrifugation over Ficoll-Hypaque. In order to increase the number of PBMC *in vitro* for further analysis and to determine if a B-cell line could be established from these cells, a 3T3.F154 cell feeder layer system was adopted (Buhmann et al. 1999). PBMC, 5 x 10^6 in number, were cultured either directly (CON PBMC), co-cultured with 2 x 10^5 ^{60}cobalt irradiated (3000 rads) 3T3 cells (CON.3T3) or co-cultured with 2 x 10^5 ^{60}cobalt irradiated 3T3.F154 cells (CON.F154) in complete RPMI or in complete RPMI that had been conditioned by the Mya-1 cell line (Mya medium [MM]). It has been demonstrated that MM contains granulocyte/macrophage colony-stimulating factor, macrophage CSF, stem cell factor, IL-10 and TNF-α (Linenberger and Deng 1999) and these factors might promote the growth of normal and neoplastic B-cells. PBMC isolated from a cat diagnosed with CLL (Tebb et al. 2004) were set up in parallel, these cultures were designated CLL PBMC, CLL.3T3, and CLL.F154 respectively. Every 4 days the cells were transferred to clean flasks with a fresh feeder layer and fresh medium.

After 58 days in culture the remaining viable cell populations were separated into B-cell enriched and B-cell depleted populations using anti-B220 MACS beads (Miltenyi Biotec) as described above. $B220^+$ and $B220^-$ cultures were obtained and maintained in MM for a further 13 days at which time cells were frozen in aliquots in liquid nitrogen, using a controlled rate freezer, Planer Kryo 10 Series II (PLANER plc, Sunbury, Middlesex, UK).

After 28 days in culture, IL-4 (R & D Systems Europe Ltd., Abingdon Science Park, Abingdon, UK) was added to the CLL.F154 cell populations at a final concentration of 25µg/ml to determine whether stimulating the B220 enriched cell population with both CD40L and IL-4 would increase the number of B-cells.

3.2.4. Flow cytometric analysis of CD40L stimulated SPF cat PBMC and CLL PBMC

To determine the phenotype of the individual cell populations, the SPF and CLL cat PBMC that had been set up in culture alone or with 3T3 or 3T3.F154 cells were processed for flow cytometry every 7-21 days, using the methods described in section 2.1.5. Analysis was

conducted on a Beckman Coulter EPICS using the EXPO analysis software package (EXPO 32 ADC software, Beckman Coulter, Kingsmead Business Park, High Wycombe, Buckinghamshire, UK). Lymphoblasts and lymphocytes were "live-gated" on the basis of size and granularity and 5000 events were collected for each sample.

After 7 and 30 days in culture, PBMC were labelled with FITC-conjugated anti-mouse B220 (BD Pharmingen) and PE-conjugated anti-feline CD5 (Southern Biotechnologies). In parallel at 7 days, PBMC were incubated with an antibody that recognises feline CD21 (CA2.1D6, Serotec Ltd., Kidlington, UK), a marker used widely for the estimation of feline B-cell numbers. After incubation for 30 minutes, the cells were washed in PBA and incubated for a further 30 minutes with FITC-conjugated anti-mouse IgG (F0257, Sigma Chemical Co., St. Louis, USA). On day 16 in culture, PBMC were stained with FITC-conjugated anti-feline CD3 (NZMI, Dr. Y. Nishimura, University of Tokyo), PE-conjugated anti-feline CD5 (Southern Biotechnologies) and cy-chrome-conjugated anti-mouse B220 (RA36B2, BD PharMingen). On days 36 and 44 in culture, PBMC were stained with FITC-conjugated anti-feline CD4 (VPG34, Serotec Ltd.), PE-conjugated anti-feline CD8 (VPG9, Serotec Ltd.) and cy-chrome-conjugated anti-mouse B220 (BD Pharmingen).

After the PBMC were separated by MACS beads into B-cell-enriched and B-cell- depleted populations, to determine the purity of each fraction, flow cytometry was performed on day 64 in culture. The cell populations were stained with FITC-conjugated anti-feline CD4 (Serotec Ltd.), PE-conjugated anti-feline CD8 (Serotec Ltd.) and cy-chrome-conjugated anti-mouse B220 (BD Pharmingen) to identify T-cells expressing CD4 or CD8 and B-cells.

3.2.5. Southern blotting

To determine the clonality of the suspected CLL cells, Ig gene rearrangements were investigated by Southern blot hybridisation (Southern 1975). One million cells from the B220 enriched cell populations that were obtained from the cultures, CON.3T3, CON.F154, CLL.3T3 and CLL.F154 were centrifuged at 1000rpm for 5 minutes and DNA was isolated from the resulting cell pellet using a Blood DNA Kit (Qiagen). The DNA was digested using the HindIII enzyme (Invitrogen Life Technologies, Paisley, UK) at 37°C overnight. The digested DNA was resolved on a 0.8% agarose gel alongside a λHind III marker (Invitrogen) and blotted overnight onto a Hybond membrane (Amersham

Biosciences), using the Southern blotting upward capillary method. Blots were fixed using UV irradiation (Spectrolinker, Spectronics Corporation, Westbury, New York) and probed with a feline Cμ probe (kindly supplied by Anne Terry, Molecular Oncology Laboratory, University of Glasgow, UK) (Terry et al. 1995). The probe was labelled with phosphorus 32 (Amersham Biosciences) using the RadPrime DNA Labeling System (Invitrogen) and purified with a Pharmacia NICK Column (Pharmacia). Blots were visualised by autoradiography using Kodak X-OMAT-S film (Kodak, Rochester, NY, USA).

3.3. Results

3.3.1. Generation of a CD40L expressing cell line

A cell line expressing feline CD40L was required for use in interactions with cells bearing CD40. This was achieved using a retrovirus vector, carrying both the feline CD40L gene as well as a gentamycin resistance gene. In order to produce retroviral particles containing the CD40L gene that were capable of infecting target cells (3T3), the retroviral vector pDON-AI containing the feline CD40L gene was transfected into a retroviral packaging cell line (see Figure 3.1). The 3T3 cell-line became stably transduced, as the CD40L DNA was incorporated into the target cell chromosomal DNA. To ensure that only 3T3 cells that expressed CD40L (3T3.F154) were growing, 3T3.F154 cells were positively selected using G418, an aminoglycoside antibiotic (Southern and Berg 1982). To assess the biological activity of feline CD40L, the 3T3.F154 cells and control 3T3 cells were used as target cells in proliferation assays.

3.3.2. CD40L induced T and B lymphocytes to proliferate

CD40L expressed on activated Th-cells is involved in the proliferation and isotype switching of B-cells (Spriggs et al. 1992). Therefore, proliferation assays were conducted in order to assess the biological activity of the feline CD40L DNA that had been cloned.

Irradiated 3T3.F154 and 3T3 cells were added in decreasing numbers ranging from 10^5 to none to a 96-well plate and incubated with SPF cat PBMC for 4 days. As shown in Figure 3.2, feline PBMC were induced to proliferate to a maximum value of 50958 ± SEM 5126 cpm. This degree of proliferation was highly significant for all wells containing 3T3.F154 cells except those containing 300 or no cells ($P \leq 0.01$), Student's t-test.

To determine whether the cells proliferating in response to CD40L were predominantly B-cells, as expected, a proliferation assay was set up in parallel in a 12 well culture plate. After 5 days of culture, the proliferating PBMC were analysed by flow cytometry. PBMC were stained with antibodies recognising feline CD5 or B220. The results are shown in Figure 3.3. Interestingly, flow cytometric analysis demonstrated that the majority of cells expressed CD5 and not B220, suggesting that the predominant population after stimulation with CD40L comprised of T-cells expressing CD5 rather than $B220^+$ B-cells. Therefore, the B-cells were possibly acting as APCs inducing T-cell proliferation that may have exceeded B-cell proliferation.

In order to determine whether T-cells would still proliferate in the absence of B-cells, fresh PBMC were obtained from a SPF cat and were separated into B-cell enriched or depleted ($B220^+$ or $B220^-$ respectively) and T-cell enriched or depleted ($CD3^+$ or $CD3^-$ respectively) cell fractions using MACS beads. The separated cell fractions were then incubated with 3T3.F154. As shown in Figure 3.4, the $CD3^+$ and $B220^-$ cell fractions did not proliferate markedly. By contrast, B-cells were induced to proliferate to a maximum of 36662 ± 7049 SEM cpm when T-cells were depleted from the PBMC population ($CD3^-$). In addition, proliferation of B-cells was more evident when T-cells were depleted ($CD3^-$), rather than when the population was B-cell enriched ($B220^+$). These results indicated that when T and B-cells were incubated together with CD40L, the B-cells may have acted as APCs and primed the T-cells resulting in T-cell proliferation. However, there was no proliferation of T-cells when the B-cells were no longer present to act as APCs. Furthermore, when B-cells were incubated alone with CD40L there was proliferation of the B-cells as CD40L induces proliferation of the B-cells directly. Also, it was concluded that when T and B-cells were incubated together with CD40L, there was some proliferation of the B-cell population, although the B-cell proliferation may have been masked by the relatively greater numbers of proliferating T-cells.

3.3.3. Long-term cultures were established

In this study, B-cells were shown to proliferate in response to incubation with CD40L for 5 days in the proliferation assays conducted above. Therefore, further studies were conducted to determine whether this proliferation could be maintained longer term. SPF cat PBMC were cultured together with 3T3.F154 cells. As mentioned previously, PBMC from a cat suspected of suffering from CLL were also studied in this manner. B-CLL cells

display low spontaneous growth in vitro and respond poorly to B-cell mitogens, such as lipopolysaccharide (LPS) or pokeweed mitogen (PWM) (Sadamori et al. 1983). Therefore, to expand the CLL cell population, enabling further analysis to be conducted, CLL PBMC were co-cultured (in parallel with SPF PBMC) with 3T3.F154. SPF and CLL cat PBMC were co-cultured with a feeder layer of irradiated 3T3 or 3T3.F154 cells in complete RPMI and MM. MM was used because it is rich in cytokines that were expected to aid the growth of B-cells (Linenberger and Deng 1999). After 58 days in culture, the only cell populations that remained viable were the SPF cat PBMC and CLL PBMC co-cultured with 3T3 or 3T3.F154 cells in MM. These cultures were then separated into B220-enriched or B220-depleted populations using MACS beads coated with anti-B220 antibody, and after a further 10 days in culture, the cells were frozen and stored in liquid nitrogen. The cells had been successfully maintained in culture for a total of 68 days.

After 28 days in culture, IL-4 was added to a culture of CLL.F154 cells to determine whether this would assist the growth of B-cells. Flow cytometric analysis confirmed an increase in B-cell number in cultures stimulated with IL-4 compared to those without IL-4 stimulation, as shown in Figure 3.5.

SPF and CLL cat PBMC were compared in a proliferation assay to determine whether the neoplastic B-cells would respond to CD40L stimulation to a greater or lesser extent than normal PBMC. The results indicated that the CLL PBMC had an increased response to CD40L when compared with control PBMC. As shown in Figure 3.6, CLL PBMC were induced to proliferate to a maximum value of 54000 ± 6700 SEM cpm in the presence of CD40L. By comparison the SPF cat PBMC proliferated to a maximum value of 29000 ± 3000 SEM cpm, a difference of almost two-fold. As demonstrated in the previous proliferation assays conducted with fractionated PBMC cell subpopulations, it was primarily the B-cells that were induced to proliferate in response to stimulation by CD40L and as the flow cytometrical analysis of the CLL PBMC demonstrated that the lymphocytosis comprised principally of B-lymphocytes (see section 3.3.4), this could account for the increased proliferation in response to CD40L.

3.3.4. Flow cytometry of long-term cultures of cells activated by CD40L

Flow cytometry was conducted to define the cell populations that resulted from co-culturing SPF cat PBMC or CLL PBMC, either alone or with 3T3 or 3T3.F154 cells, in complete RPMI or in MM.

3.3.4.1. FACS analysis after 7 days in culture

Staining of cells at 7 days in culture with FITC-conjugated anti-B220 and PE-conjugated anti-CD5 antibodies demonstrated that there was a large population of blast cells expressing B220 in SPF cat PBMC cultures and cultures of CLL PBMC stimulated with feline CD40L. These blast cells are recognised as being larger and less granular than other lymphocytes, when size (forward scatter) and granularity (side scatter) are compared by flow cytometry (see Figure 3.7). As described in chapter one, the association of CD40L with the CD40 receptor on B-cells can result in the proliferation and differentiation of B-cells (see Figure 1.3). Figures 3.8 and 3.9 demonstrate that more blast cells were present in cultures grown in MM than in those in RPMI. In the lymphocyte populations of SPF.F154 and CLL.F154, there were more B-cells in the CLL PBMC than in the SPF cat PBMC, 39.6% vs 4.5% (see Figure 3.7), consistent with the diagnosis of B-CLL. Very few lymphoblasts were stimulated by control 3T3 cells in RPMI and the lymphocyte populations comprised predominantly of T-cells in SPF PBMC and B-cells in CLL PBMC (Figure 3.10). However, the same cell populations cultured in MM contained a higher proportion of lymphoblasts (28% in CLL 3T3 cultured in MM compared to 5% when cultured in RPMI and 21% in SPF 3T3 cultured in MM compared to 2% when cultured in RPMI) (Figure 3.10), again demonstrating that factors in the MM stimulated the proliferation of lymphoblasts. CLL and SPF cat PBMC cultures that were cultured in RPMI alone contained very few lymphoblasts and these were stimulated to proliferate following the addition of MM, particularly in the CLL PBMC culture (Figure 3.10).

At 7 days in culture the cell populations were also stained with FITC-conjugated anti-CD21 antibody and the results confirmed that the blast population of cells in the cultures SPF.F154 and CLL.F154 contained predominantly B-cells and that the lymphocyte populations were predominantly T-cells in the SPF.F154 culture and B-cells in the CLL.F154 culture (see Table 3.1). Again there were fewer lymphoblasts in 3T3 stimulated

cells and cells cultured alone. Also, in agreement with the staining with FITC-conjugated anti-B220 and PE-conjugated anti-CD5 antibodies, culturing in MM increased the number of blasting cells in both CLL.3T3 and SPF.3T3 and PBMC cultured alone.

3.3.4.2. FACS analysis after 16 days in culture

After 16 days in culture, cells were stained with FITC-conjugated anti-CD3, PE-conjugated anti-CD5 and cychrome-conjugated anti-B220 antibodies. At this time point, the PBMC cultures in RPMI alone and those co-cultured with 3T3 in RPMI were no longer viable. Of the remaining cell cultures that were still viable, the percentage of lymphocytes in the cell populations had decreased significantly, leaving only a population of blasting cells. SPF.F154 and CLL.F154 were still composed predominantly of blasting B-cells. However, the percentage of T and B-cells differed depending on which medium they were cultured in, interestingly, the SPF.F154 cell population was predominantly composed of $B220^+$ lymphoblasts when cutured in RPMI and $CD5^+$ lymphoblasts when cutured in MM, as shown in Figure 3.11. Furthermore, MM also favoured the growth of $CD5^+$ lymphoblasts in the CLL.F154 cell population. This might be accounted for by the MM containing a source of IL-2 (Linenberger and Deng 1999). In the SPF cat PBMC cell population cultured in MM alone, although the T-cell population had some co-expression of CD3 and CD5, not all T-cells expressed CD3 and CD5 as expected. In the SPF.3T3 cell population cultured in MM, there was more co-expression of CD3 and CD5 (80% co-expression in SPF.3T3 compared to 49% co-expression in SPF cat PBMC cultured alone) (see Figure 3.12).

3.3.4.3. FACS analysis after 28 days in culture

After 28 days, virtually no lymphocytes remained in any culture. Lymphoblasts were stained with FITC-conjugated anti-B220 and PE-conjugated anti-CD5 antibodies. CLL.F154 and SPF.F154 cells cultured in RPMI, and CLL and SPF cat PBMC cultured alone in MM were no longer viable. Although CLL.F154 cultured in MM still contained mostly blasting B-cells, SPF.F154, SPF.3T3 and CLL.3T3 cultured in MM were now predominantly T-cells. Therefore, it was concluded that a CD40L feeder system was unable to sustain a normal B-cell population indefinitely.

3.3.4.4. FACS analysis after MACS beads separation

CLL.F154 B220$^+$ lymphoblasts were maintained until the end of the study at 68 days. Their phenotype after separation into B220 enriched and B220 depleted populations using MACS beads was confirmed by staining with FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and cychrome-conjugated anti-B220 antibodies. B220 enrichment led to a less than two fold increase in the percentage of cells expressing B220, as shown in Figure 3.13.

3.3.5. Flow cytometric analysis reveals B220/CD5, B220/CD3 and CD4/CD8 dual positive cell subsets

3.3.5.1. Co-expression of B220 and CD5

After the SPF cat and CLL PBMC had been in culture with 3T3 or 3T3.F154 for 7 days, flow cytometric analysis revealed that the blasting B220$^+$ cell populations were also expressing CD5 (see Figures 3.9 and 3.14). It could be argued that this dual expression was due to activation of the B-cells by CD40L, however, figure 3.15 demonstrates that in CLL PBMC cultured alone, the lymphoblasts also co-expressed B220 and CD5. In addition, as the SPF.F154 B-cell lymphocytes were not co-expressing CD5, it is possible that the co-expression of B220 and CD5 on CLL B-cell lymphocytes was due to the particular immunophenotype of these neoplastic cells (see Figure 3.14). CLL and SPF cat PBMC that were cultured alone in RPMI had very few lymphoblasts that were increased by culturing in MM, more markedly in the CLL PBMC culture where there was a large increase in blasting cells and also an increase in co-expression of B220 and CD5 in lymphocytes and lymphoblasts compared to the corresponding culture containing SPF PBMC (see Figures 3.15 and 3.16). This increase in co-expression in CLL B-cells could be due to their immunophenotype, the fact that there was a higher percentage of B-cells in the CLL PBMC compared with the SPF cat PBMC (so that co-expression was easier to detect), or the higher state of activation of these neoplastic cells compared with normal B-cells. After 28 days in culture the co-expression of B220 and CD5 was restricted to CLL.F154 cultured in MM, since B-cell growth was no longer sustained in the other cell cultures.

3.3.5.2. Co-expression of B220 and CD3

A cell subset co-expressing B220 and CD3 was revealed after 16 days of culture when lymphoblasts were stained with FITC-conjugated anti-CD3, PE-conjugated anti-CD5 and cychrome-conjugated anti-B220 antibodies. This subset was evident in cultures that contained $B220^+$ cells and was more obvious in CLL PBMC cultures, due to their higher numbers of $B220^+$ cells (see Figure 3.17). Therefore, it is tempting to speculate that this B220/CD3 dual expressing cell population was the result of co-culturing B and T-cells.

3.3.5.3. Co-expression of CD4 and CD8

At days 36-64 in culture the remaining populations of cells (SPF.F154, CLL.F154, SPF.3T3 and CLL.3T3 cultured in MM) were defined more specifically using antibodies recognising feline CD4, CD8 and B220. The SPF.F154 cells were defined as a blasting T-cell population, which consisted of mainly $CD8^+$ cells with an unusual sub-population of CD4/CD8 dual positive cells, see Figure 3.18. The CLL.F154 cells were still mainly a blasting $B220^+$ cell population, with the remainder of cells being either $CD4^+$ or $CD8^+$. Similarly, SPF.3T3 and CLL.3T3 cells were $CD8^+$ with the same CD4/CD8 dual positive subset in both cell populations as the SPF.F154 cells. This CD4/CD8 co-expression is not normally observed in feline lymphocytes and could be due to the activation state of the T-cells. However, as the population is also seen in the 3T3 stimulated cells, it is more likely that this co-expression is due to prolonged culture of the T-cells. Unfortunately, this hypothesis cannot be confirmed, as CD4 and CD8 staining was not conducted prior to 36 days in culture. It is interesting that this CD4/CD8 co-expression was not observed in the CLL.F154 population. This could be due to the small numbers of T-cells present in this culture.

3.3.6. No immunoglobulin gene rearrangement was detected in neoplastic B cells

To determine if the CLL PBMC were monoclonal, a Southern blot was performed using DNA prepared from the B220 enriched fraction of CLL PBMC that had been stimulated with CD40L. As shown in Figure 3.19, the analysis revealed the presence of a single 7.5Kb fragment, in both the control and the CLL sample. Therefore, it could be concluded that there was either no re-arrangement of the IgH gene in the expanded B-cell population or if there was, the DNA fragment was the same size as the germ line fragment, which

might occlude the re-arranged gene band. The expansion of a monoclonal population with rearranged immunoglobulin genes would have resulted in the appearance of a second band with a different molecular weight to the germ line fragment. Failure to detect any IgH gene re-arrangement, therefore, would suggest that the chronic lymphocytic leukaemia was not clonal.

3.4. Discussion

This study confirms that the feline CD40L gene that was cloned is biologically active and therefore might be valuable as an adjuvant for a FIV DNA vaccine. Flow cytometric analysis of PBMC proliferating in response to CD40L demonstrated that the majority of cells expressed CD5, consistent with T-cell expansion. However, these cells did not respond as well to CD40L in the absence of $B220^+$ cells, possibly because T-cells require B-cells to act as APCs in order for them to proliferate. The B-cells were most likely activated by a foreign antigen in the culture medium such as a component of foetal calf serum.

SPF cat PBMC were maintained for 68 days by culturing in the presence of CD40L-expressing 3T3 cells. At first B-cells were induced to blast by CD40L. However, the cell population ceased to contain predominantly blasting B-cells after 28 days in culture when T-cells made up the predominant cell population. Consistent with earlier findings (Rush and Hodgkin 2001), this study confirmed that B-cells do not require constant stimulation from CD40L to enable them to blast, but they do require continued bursts of stimulation. Thus, when PBMC that had previously been cultured with 3T3 expressing CD40L were cultured alone for a period of time, their immunophenotype was unaltered. Rush and co-workers used an anti-CD40 antibody and IL-4 to stimulate murine B-cells. IL-4 was added to our CLL PBMC cultures, but not to our SPF cat PBMC cultures. Therefore, further studies will be required to determine whether IL-4 could have sustained the SPF B-cell population indefinitely.

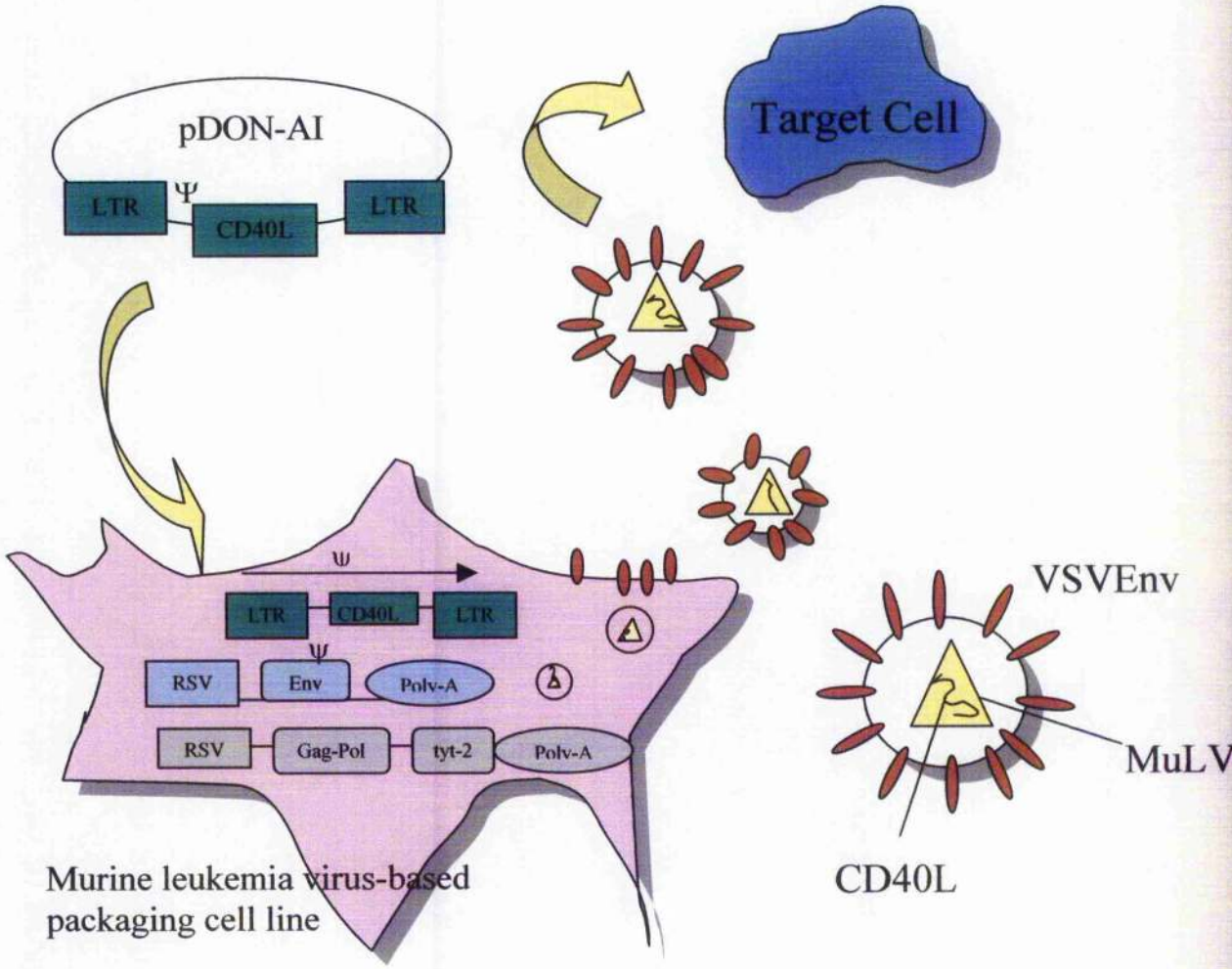
CLL PBMC were maintained for 68 days and at this time were still predominantly a blasting B-cell population. Previous studies (Kitada et al. 1999; Granziero et al. 2001) have demonstrated that the stimulation of B-CLL cells with CD40L may up-regulate inhibitor of apoptosis proteins. This could account for the maintenance of a neoplastic B-

cell population obtained from the CLL cat but not a normal B-cell population derived from a SPF cat.

After SPF cat or CLL cells had been cultured with CD40L-expressing cells or normal 3T3 cells, several different cell subsets were identified upon analysis by flow cytometry, including a B220/CD5 co-expressing cell population. B-cells can express the T-cell marker, CD5, upon activation and therefore a degree of expression of CD5 in B-cells stimulated with CD40L might be expected. However, this $B220^{+}/CD5^{+}$ immunophenotype was also detected in PBMC that were cultured with normal 3T3 cells or cultured alone. The $B220^{+}/CD5^{+}$ immunophenotype was more evident in the CLL PBMC than in the control SPF cat PBMC. It has been shown in human B-CLL that the typical immunophenotype is $CD19^{+}$, $CD20^{+}$, $CD21^{+}$, sIg and $CD5^{+}$ (Freedman et al. 1987). In the same study, normal B-cells were induced to express CD5 by activating with 12-0-tetradecanoylphorbal-β-acetate (TPA). In cattle, $CD5^{+}$ B-cells are found normally in peripheral blood and spleen, and the number of $CD5^{+}$ B cells was found to be increased in cattle infected with *Trypanosoma congolense* (Naessens and Williams 1992). This increase in $CD5^{+}$ B-cells could to be related to the excessive activation of the humoral component of the immune system by the Trypanosome.

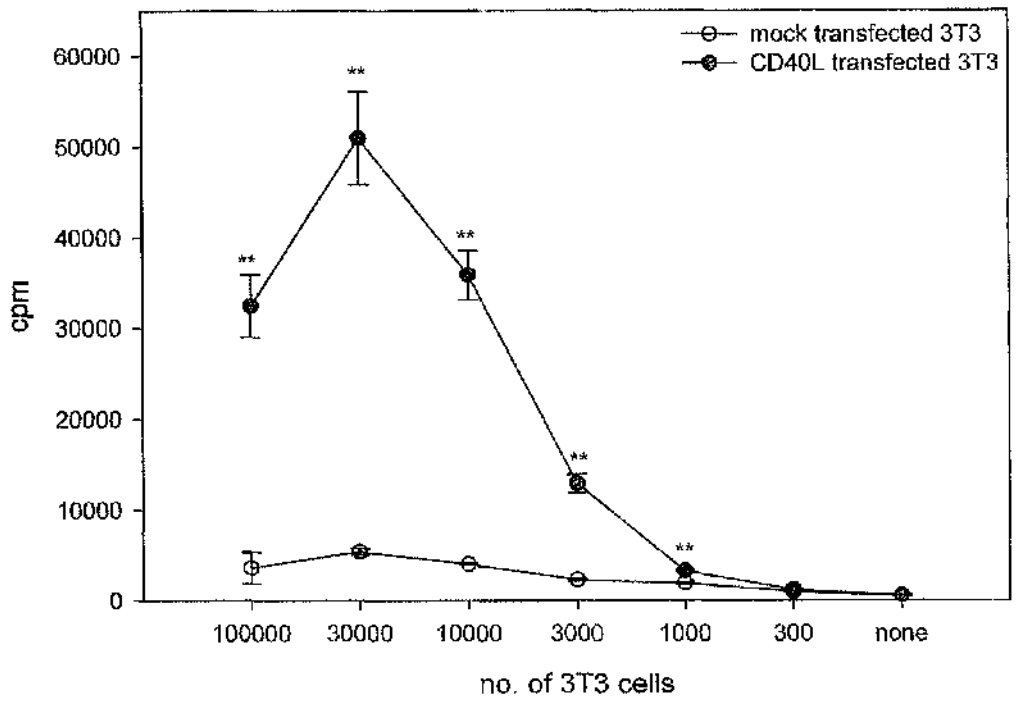
After prolonged culture with CD40L-expressing 3T3 cells and normal 3T3 cells, a degree of co-expression of CD4 and CD8 on T-cells was also demonstrated. In man, immature thymic T lymphocytes acquire both CD4 and CD8 molecules prior to the expression of surface CD3. With maturation, T-cells lose either the CD4 or CD8 antigen to gain a mature peripheral T-cell phenotype, either $CD4^{+}$ $CD8^{-}$ or $CD4^{-}$ $CD8^{+}$, associated with helper or cytotoxic T-cell functions respectively. The finding of $CD4^{bright}CD8^{dim}$ T-cells has been reported in swine, chickens, monkeys, rats and humans (as reviewed in Zuckermann 1999). However, the $CD4^{dim}CD8^{bright}$ immunophenotype has been recorded less often (Sullivan et al. 2001). Sullivan and co-workers showed that this dual expression resulted following activation of $CD8^{+}$ cells by co-stimulation with staphylococcal enterotoxin B and anti-CD3/CD28 antibodies. However, it may also result from prolonged culture of $CD8^{+}$ and $CD4^{+}$ cells. Further immunophenotypic studies will be required to determine whether the CD4/CD8 dual expressing cell subset that arose in cell populations cultured with CD40L were doublets (a CD4 expressing cell and a CD8 expressing cell joined together), or a distinct CD4 cell population that was induced to express CD8.

Figure 3.1. Production of 3T3 cells stably transfected with feline CD40 ligand



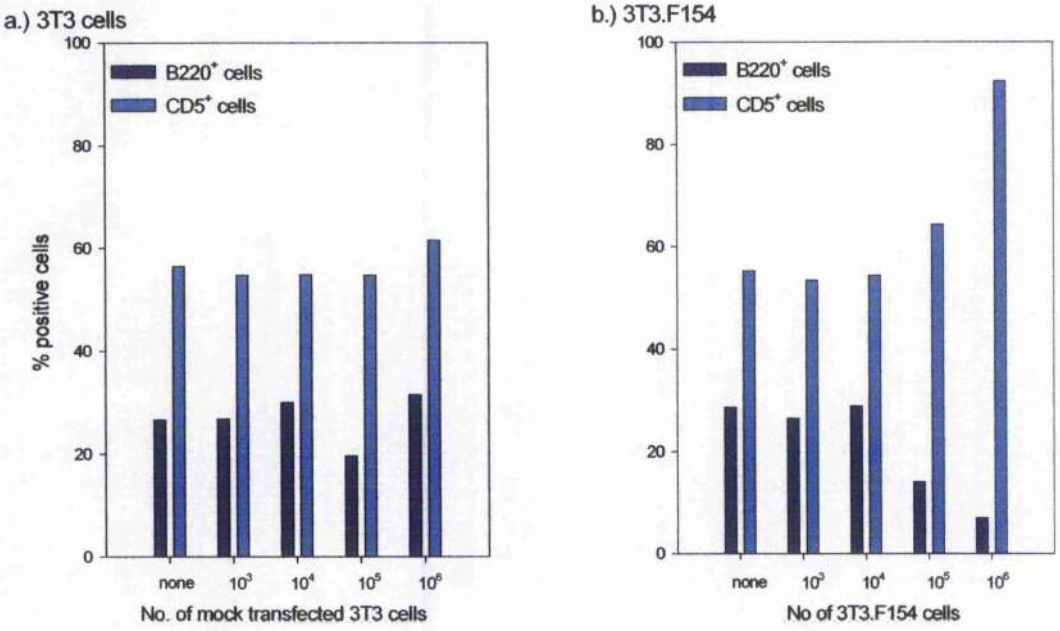
The pDON-AI vector contains Ψ (psi; the extended viral packaging signal), but not the structural genes necessary for viral particle formation and replication (*gag, pol* and *env*). Therefore, when this vector is transfected into the murine leukaemia virus-based packaging cell line containing those genes, RNA from the vector is packaged in an infectious but replication incompetent retrovirus particle. By infecting a target cell with the virus particles produced, the target gene can be integrated into the target cells chromosomal DNA. Modified from www.Stanford.edu/group/nolan/retroviral-systems/phx.html.

Figure 3.2. Proliferation of PBMC in response to 3T3 cells stably expressing feline CD40L (3T3.F154)



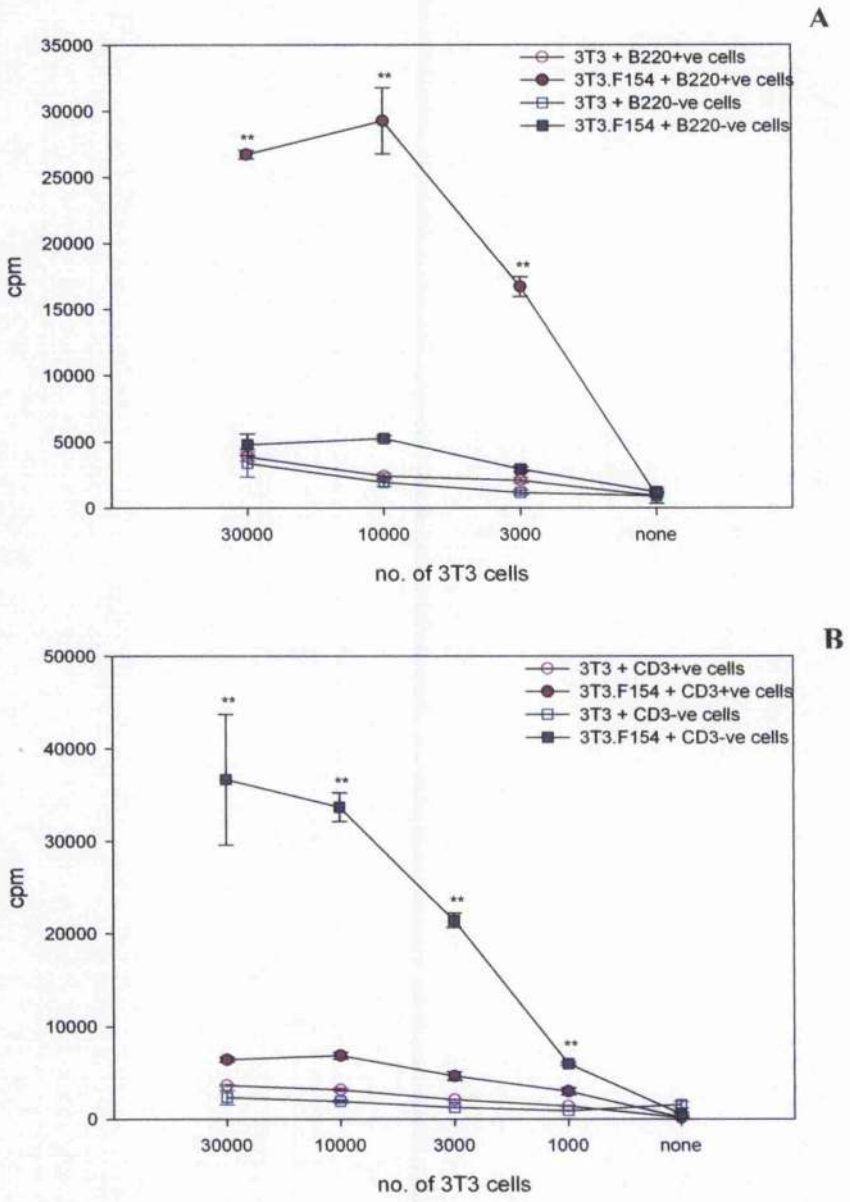
Proliferative response of feline PBMC induced by 3T3 cells expressing feline CD40 ligand (3T3.F154) (•), compared to mock transfected 3T3 cells (○). PBMC induced to proliferate to a maximum of 50958 +/- SEM 5126 counts per minute (cpm) by $3x10^4$ 3T3.F154 cells. ** The difference between the two groups is highly significant P=0.001, P=0.0008, P=0.003, P=0.0004 and P=0.001 respectively (Student's t-test).

Figure 3.3. Flow cytometric analysis of proliferating PBMC



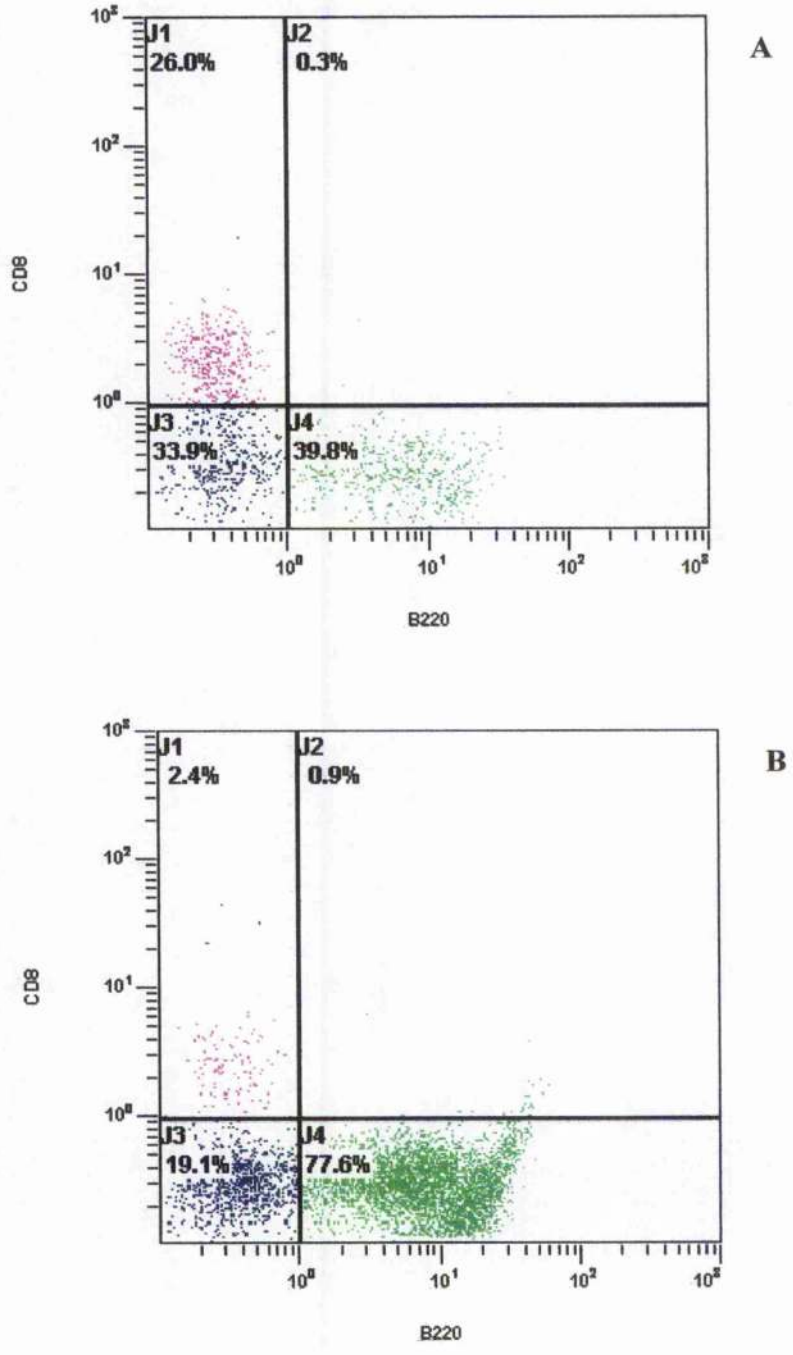
a)PBMC stimulated with mock transfected 3T3 cells or b) with CD40L transfected cells (3T3.F154). PBMC were stained with PE-conjugated anti-CD5 and FITC-conjugated anti-B220 antibodies.

Figure 3.4. Proliferation of PBMC cell fractions in response to CD40L



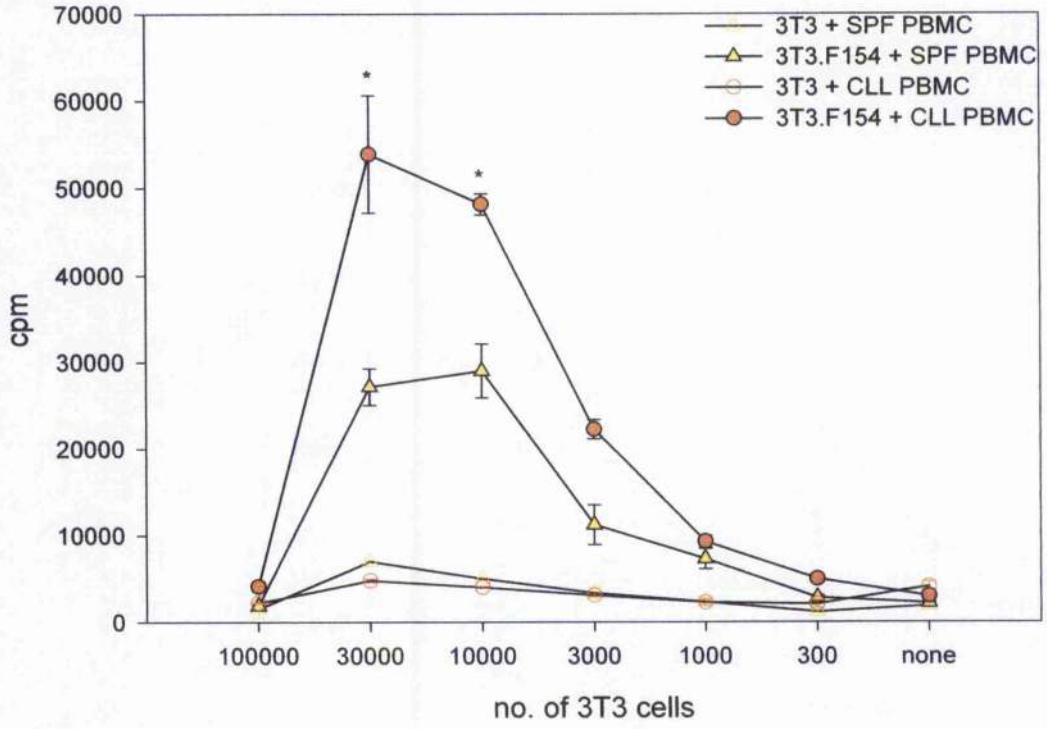
B220 enriched PBMC ($B220^+$) were induced to proliferate to a maximum of 29252 ± 2500 SEM cpm by 3T3.F154 cells (A). However, PBMC depleted of CD3 positive cells ($CD3^-$) were induced to proliferate to a maximum of 36662 ± 7049 SEM cpm by 3T3.F154 cells (B). ** When the proliferation of $B220^+$ cells in response to 3T3.F154 cells was compared with the proliferation of $B220^-$ cells in response to 3T3.F154 cells it was highly significant, P=0.00001, P=0.0006 and P=0.00005 respectively. Similarly the difference between $CD3^-$ cells proliferation and $CD3^+$ cells proliferation in response to 3T3.F154 cells was also highly significant, P=0.01, P=0.00007, P=0.00004 and P=0.002 respectively.

Figure 3.5. Flow cytometric analysis after addition of IL-4 to CLL PBMC co-cultured with CD40L (CLL.F154)



(A) CLL.F154 culture without IL-4. (B) CLL.F154 culture after the addition of IL-4 for 7 days. The addition of IL-4 increased the $B220^+$ cell population and decreased the $CD8^+$ cell population. Cells were stained with PE-conjugated anti-CD8 and cychrome-conjugated anti-B220 antibodies.

Figure 3.6. Comparison of CLL cat PBMC and SPF cat PBMC proliferation in response to CD40L



CLL cat PBMC and SPF cat PBMC were incubated with mock transfected 3T3 cells and 3T3.F154 cells. CLL PBMC were induced to proliferate to a maximum of 54 000 ± 6700 SEM cpm. * The difference in proliferation of CLL cat PBMC and SPF cat PBMC was highly significant at $3x10^4$ and 10^4 3T3.F154 cells, P=0.01 (Student's t-test).

Figure 3.7. FACS analysis of SPF and CLL PBMC co-cultured with CD40L

SPF and CLL cat PBMC cultured in RPMI and stimulated with CD40L (SPF.F154 and CLL.F154 respectively) were stained with FITC-conjugated anti-B220 and PE-conjugated anti CD5 antibodies after 7 days in culture. Stimulation of SPF and CLL PBMC with CD40L results in the production of lymphoblasts (BLASTS). The lymphoblasts of both SPF and CLL PBMC co-express B220 and CD5. The SPF.F154 lymphocytes (LYMPH) predominantly expressed CD5, however the CLL.F154 lymphocytes also co-expressed B220 and CD5.

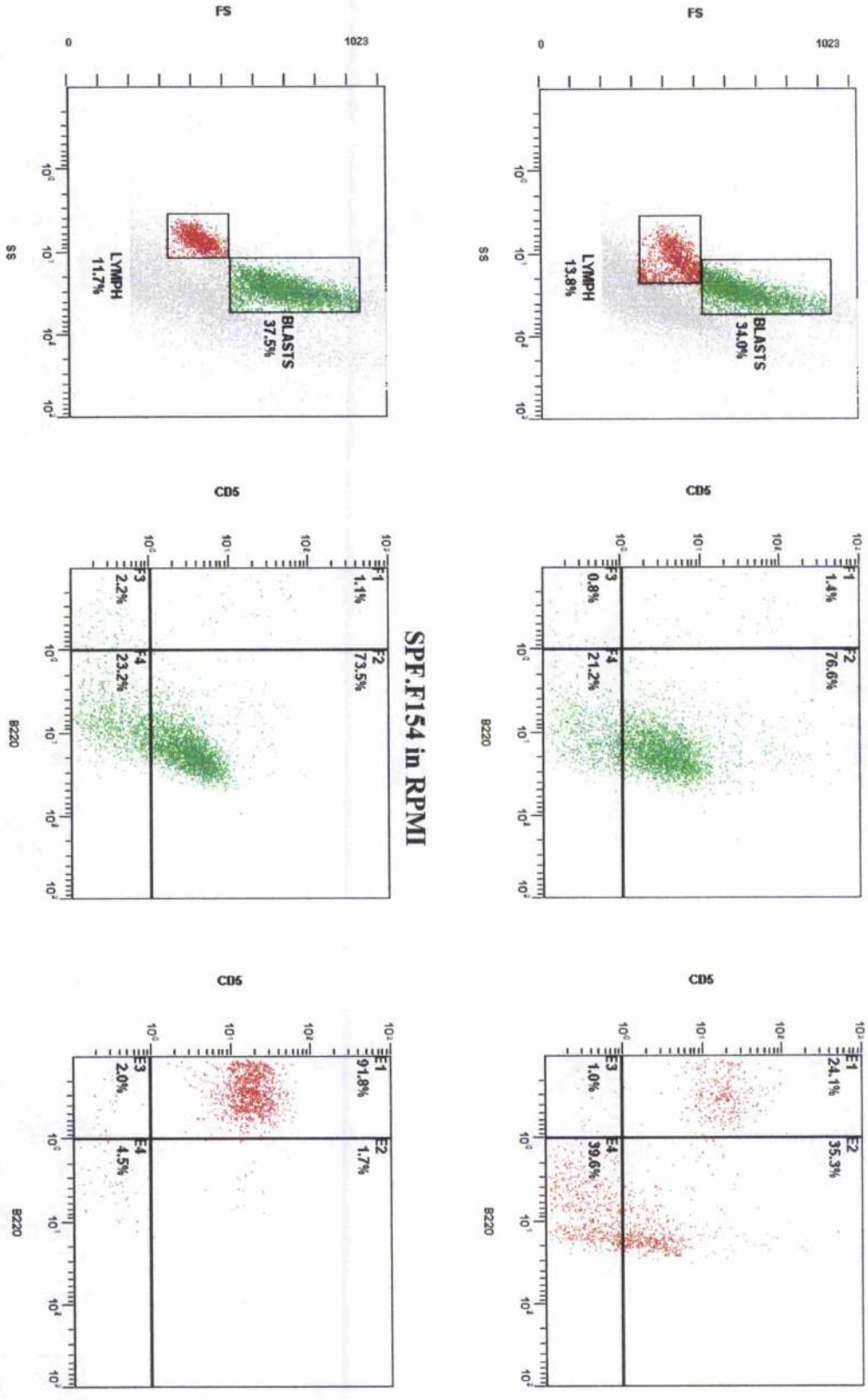
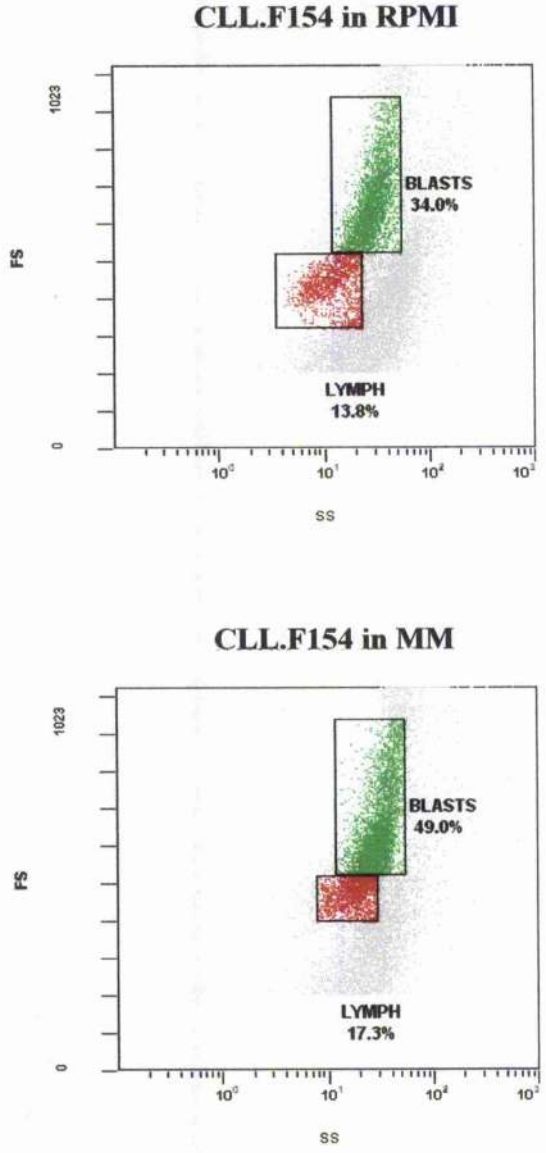
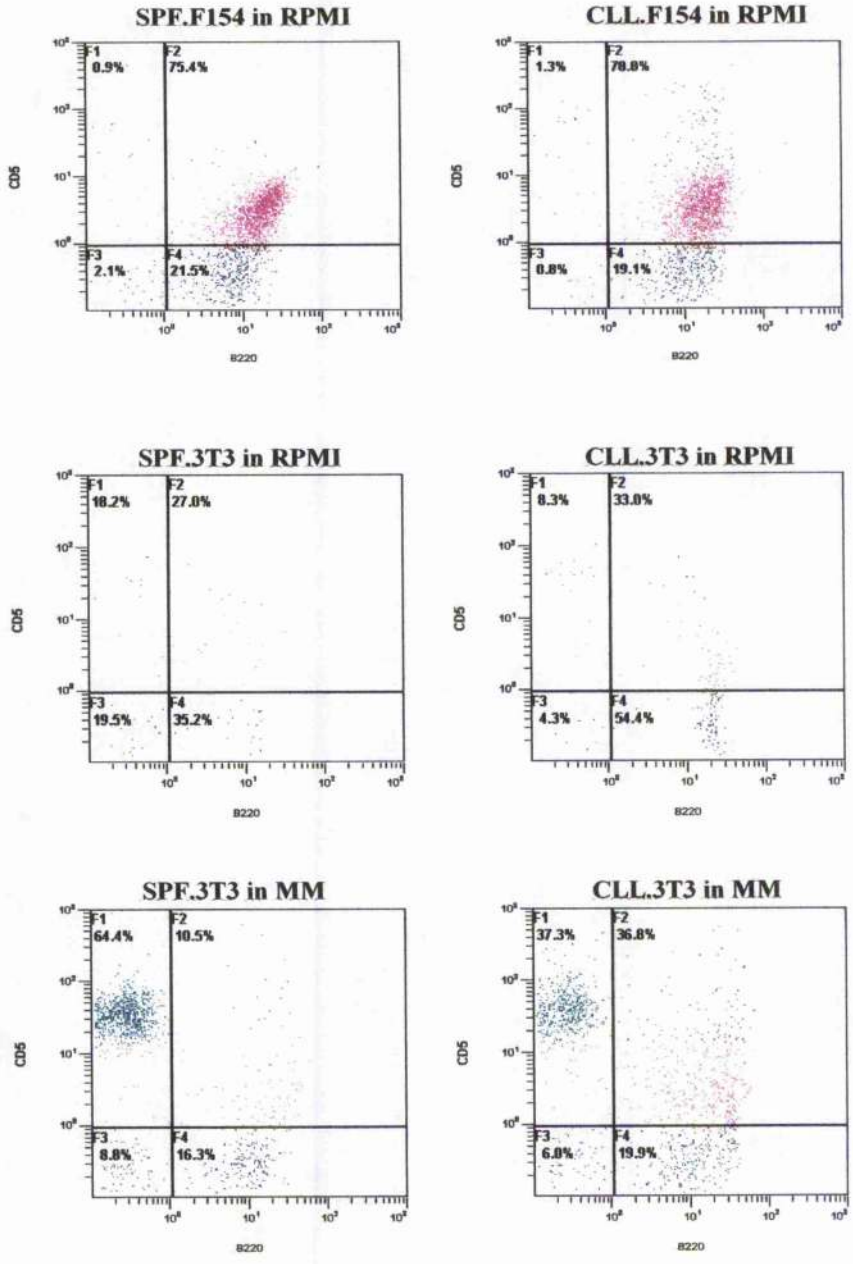

CLL.F154 in RPMI
SPF.F154 in RPMI
FS
SS
LYMPH
13.8%
BLASTS
34.0%
LYMPH
11.7%
BLASTS
37.5%
CD5
B220
E1
1.4%
E2
76.6%
E3
0.8%
E4
21.2%
E1
24.1%
E2
35.3%
E3
1.0%
E4
39.6%
F1
1.1%
F2
73.5%
F3
2.2%
F4
23.2%
E1
91.8%
E2
1.7%
E3
2.0%
E4
4.5%

Figure 3.8. FACS analysis of CLL PBMC co- cultured in RPMI and MM with CD40L (CLL.F154)



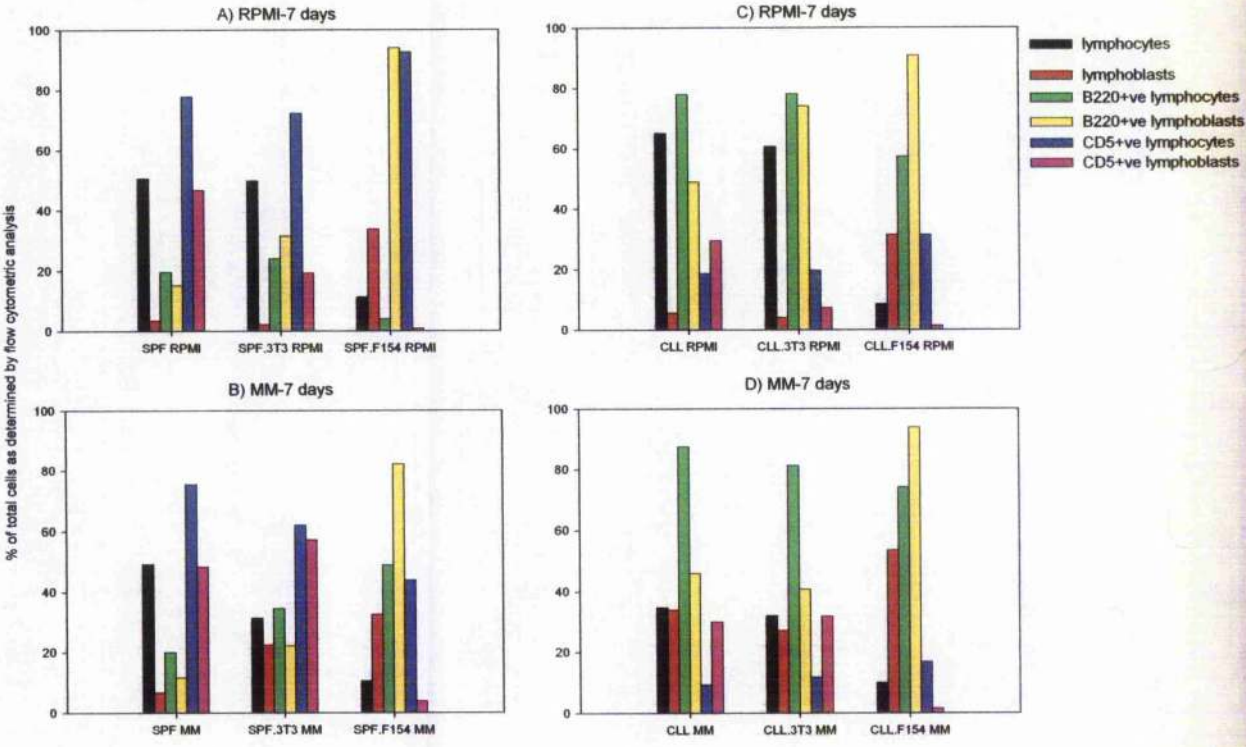
The dotplots demonstrate that the CLL.F154 cell population cultured in MM has more lymphocytes (LYMPH) and lymphoblasts (BLASTS), than the corresponding CLL.F154 cell population cultured in RPMI.

Figure 3.9. FACS analysis of SPF.F154, SPF.3T3, CLL.F154 and CLL.3T3 cultured in RPMI and MM



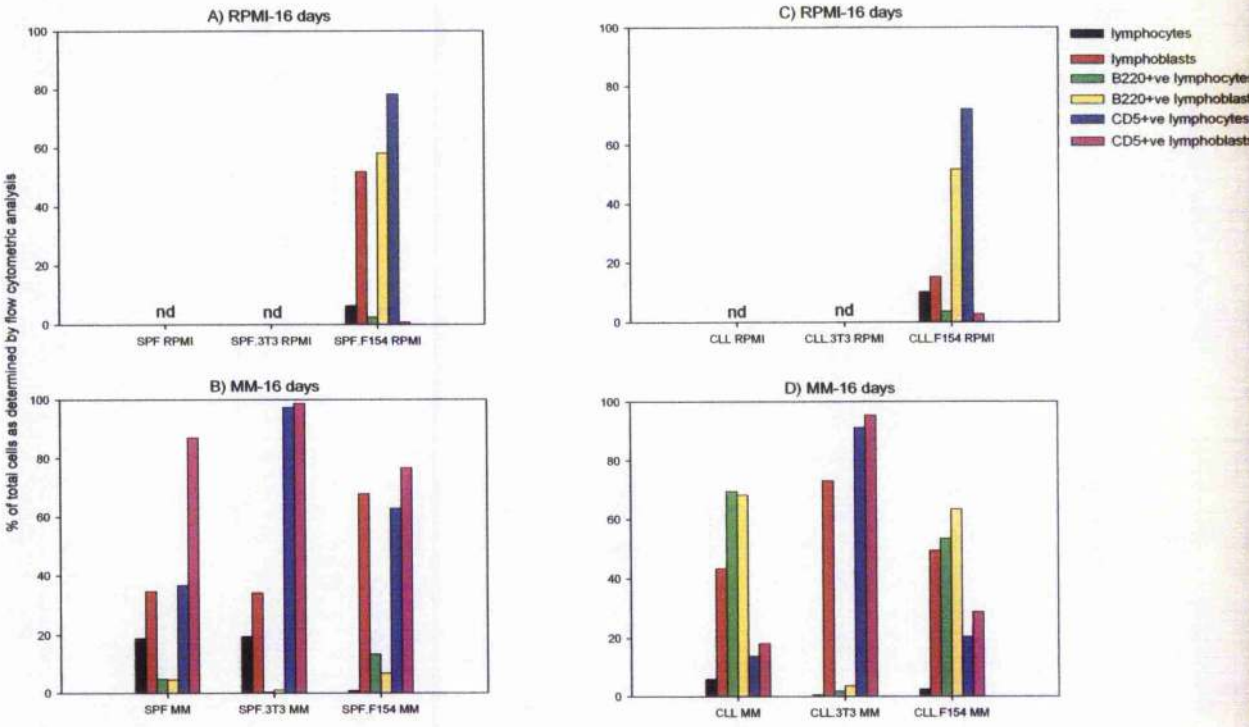
FACS analysis of lymphoblasts stained with PE-conjugated anti-CD5 and FITC-conjugated anti-B220 antibodies revealed co-expression of B220 and CD5. This co-expression is more evident in cultures stimulated with CD40L. SPF and CLL PBMC co-cultured with 3T3 in MM display an increased number of $CD5^+$ cells when compared with the corresponding cell populations cultured in RPMI.

Figure 3.10. Flow cytometric analysis of CLL and SPF PBMC cultures at 7 days



Cell populations were stained with PE-conjugated anti-CD5 and FITC-conjugated anti-B220 antibodies. SPF and CLL PBMC co-cultured with CD40L contained predominantly $B220^{+}$ lymphoblasts, however SPF PBMC cultures also had a large population of $CD5^{+}$ lymphocytes. Conversely the lymphocytes in CLL PBMC cultures expressed B220. MM promoted the growth of lymphoblasts, in SPF and CLL PBMC cultures.

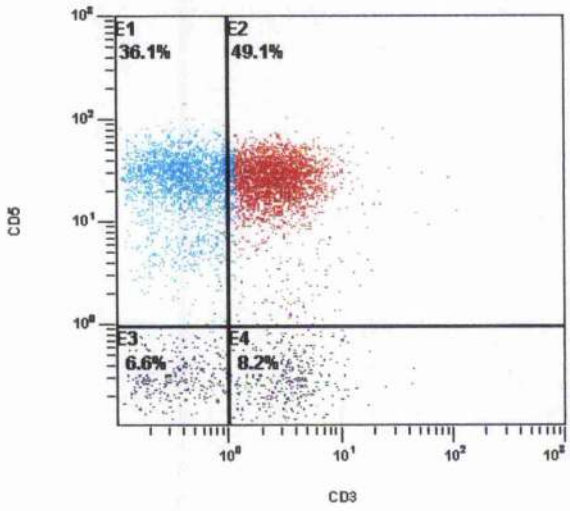
Figure 3.11. Flow cytometric analysis of CLL and SPF PBMC cultures at 16 days



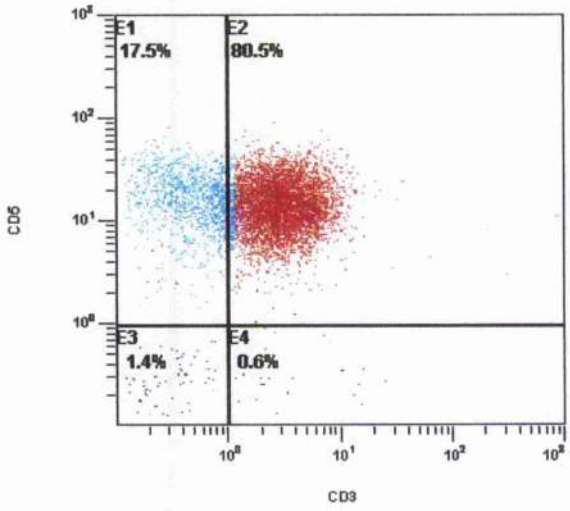
Cell populations were stained with PE-conjugated anti-CD5 and FITC-conjugated anti-B220 antibodies. After 16 days, the SPF.F154 cell population in RPMI remained similar to at 7 days. The CLL.F154 cell population in RPMI showed a decrease in $B220^+$ lymphocytes and an increase in $CD5^+$ lymphocytes. MM still supported the growth of lymphoblasts, however these were no longer predominantly $B220^+$ in SPF.F154 cells. CLL.3T3 cells were now predominantly a T-cell population. nd- not done, as cells were no longer viable.

Figure 3.12. FACS analysis of SPF PBMC after 16 days in culture

A



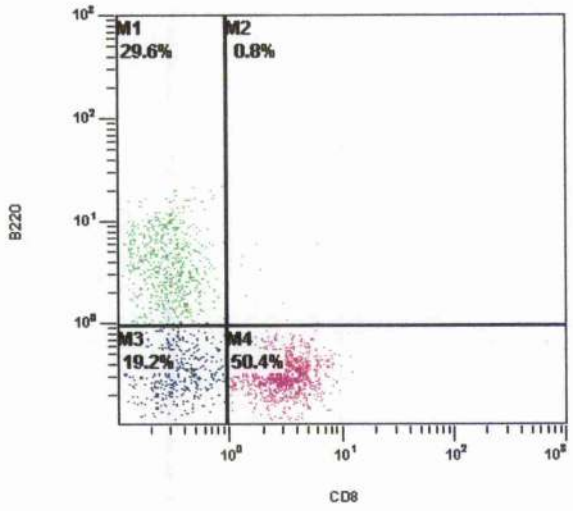
B



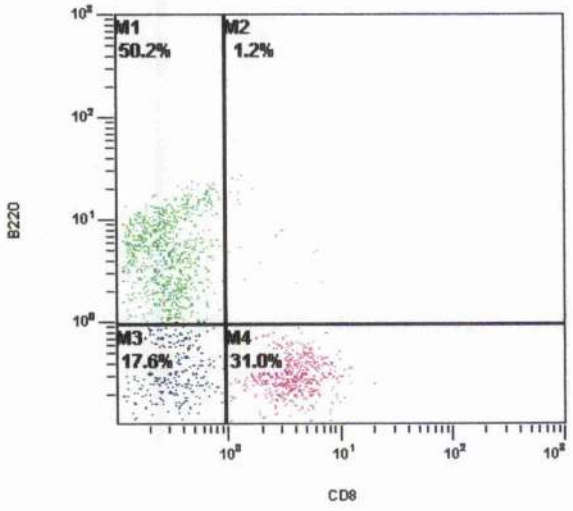
Lymphoblasts were stained with FITC-conjugated anti-CD3 and PE-conjugated anti-CD5 antibodies. (A) SPF PBMC cultured alone in MM. (B) SPF PBMC cultured with 3T3 cells in MM, showing a decrease in $CD5^+$ and $CD3^+$ individual cell populations, but an increase in the CD5/CD3 dual positive subset.

Figure 3.13. FACS analysis post-separation of CLL.F154 into B220 enriched and B220 depleted cell populations using MACS beads

A



B

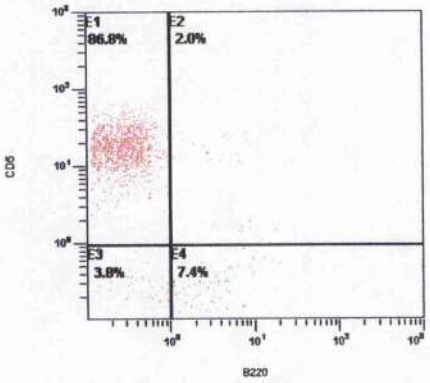


Cells were stained with PE-conjugated anti-CD8 and cychrome-conjugated anti-B220 antibodies. (A) B220-depleted population. (B) B220-enriched population. FACS analysis demonstrates that cell depletion using MACS beads does not result in a pure cell population.

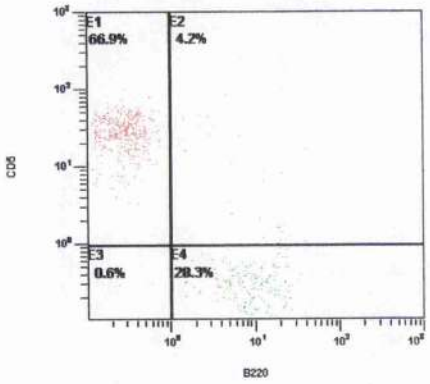
Figure 3.14. Dual expression of B220 and CD5 in SPF and CLL PBMC co-cultured with CD40L in RPMI and MM

Cells were stained with FITC-conjugated anti-B220 and PE-conjugated anti-CD5 antibodies. FACS analysis demonstrated a sub-population of $CD5^{+}$ B-cells in the SPF.F154 lymphoblasts and in the CLL.F154 lymphoblasts and a smaller population of $CD5^{+}$ B-cells in the CLL.F154 lymphocytes. MM promoted this dual expression in CLL.F154 cells.

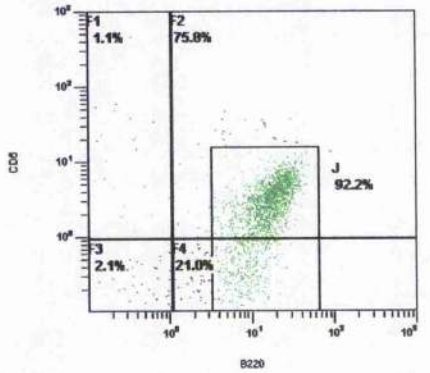
SPF.F154 lymphocytes in RPMI



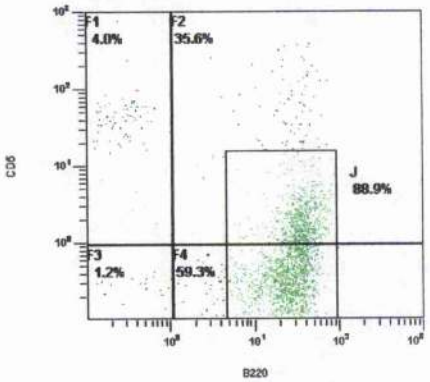
SPF.F154 lymphocytes in MM



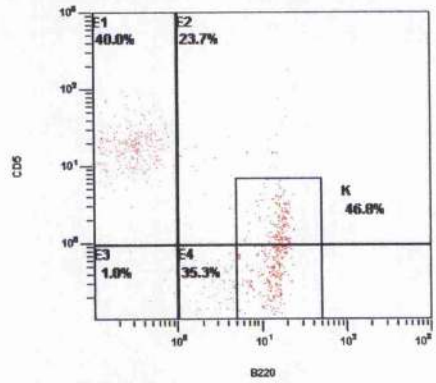
SPF.F154 lymphoblasts in RPMI



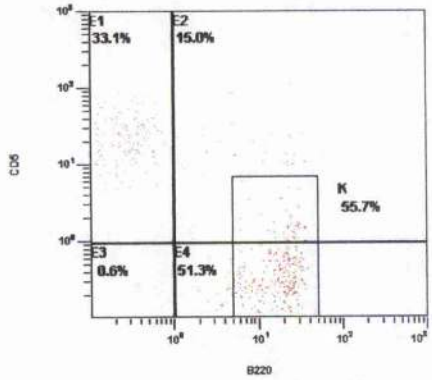
SPF.F154 lymphoblasts in MM



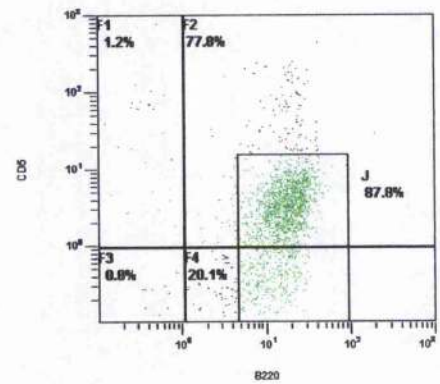
CLL.F154 lymphocytes in RPMI



CLL.F154 lymphocytes in MM



CLL.F154 lymphoblasts in RPMI



CLL.F154 lymphoblasts in MM

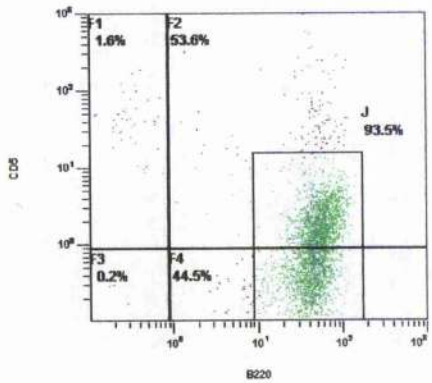
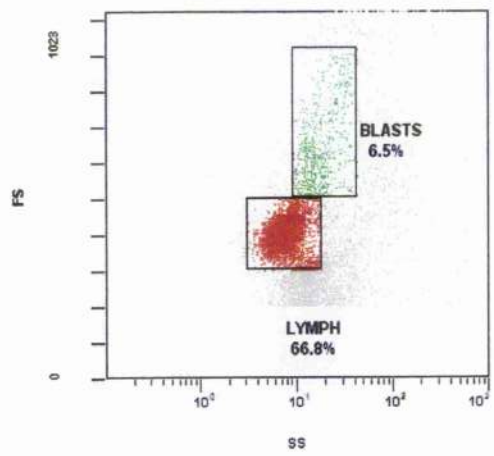
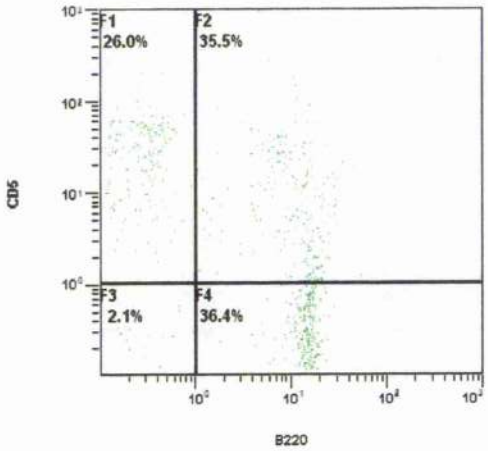


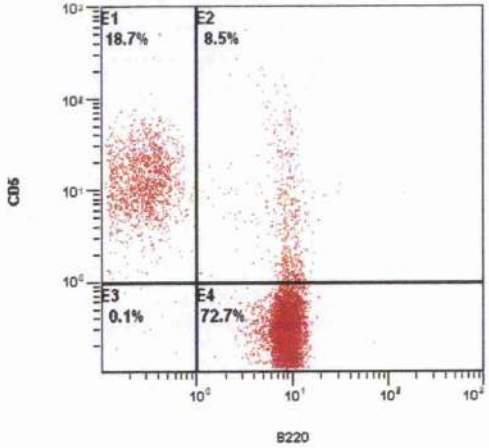
Figure 3.15. FACS analysis of CLL PBMC cultured alone in RPMI and MM

CLL PBMC were stained with FITC-conjugated B220 and PE-conjugated CD5 antibodies. The production of lymphoblasts (BLASTS) was increased by culturing in MM and the co-expression in lymphoblasts of B220 and CD5 was also increased by culturing in MM.

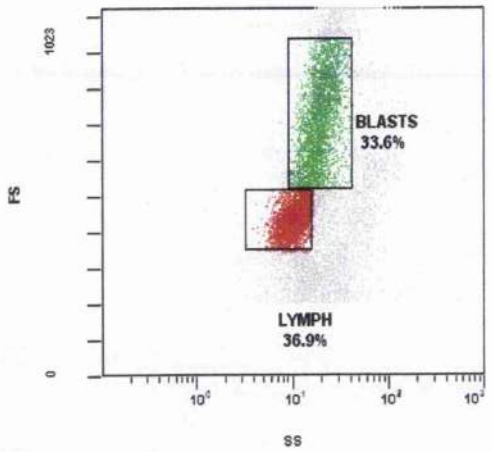
CLL PBMC in RPMI

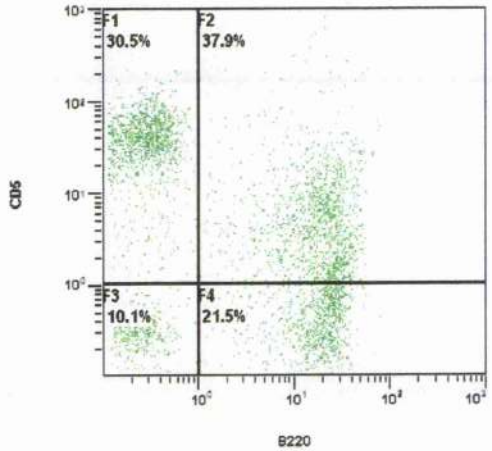






CLL PBMC in MM





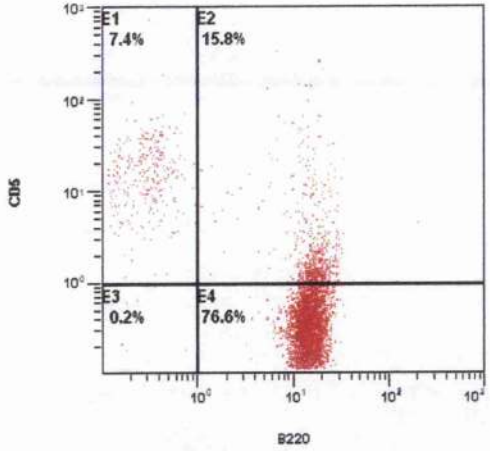
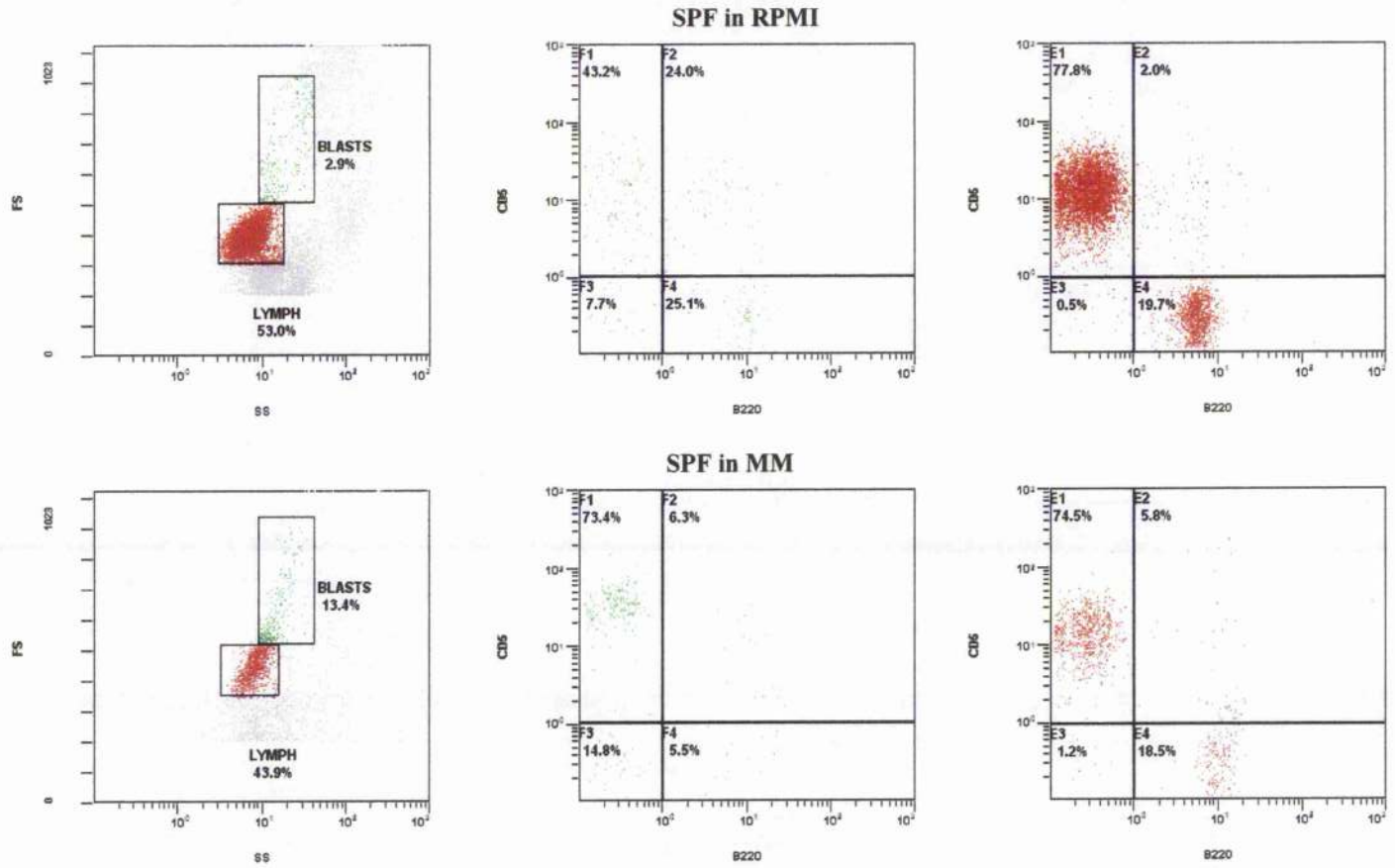
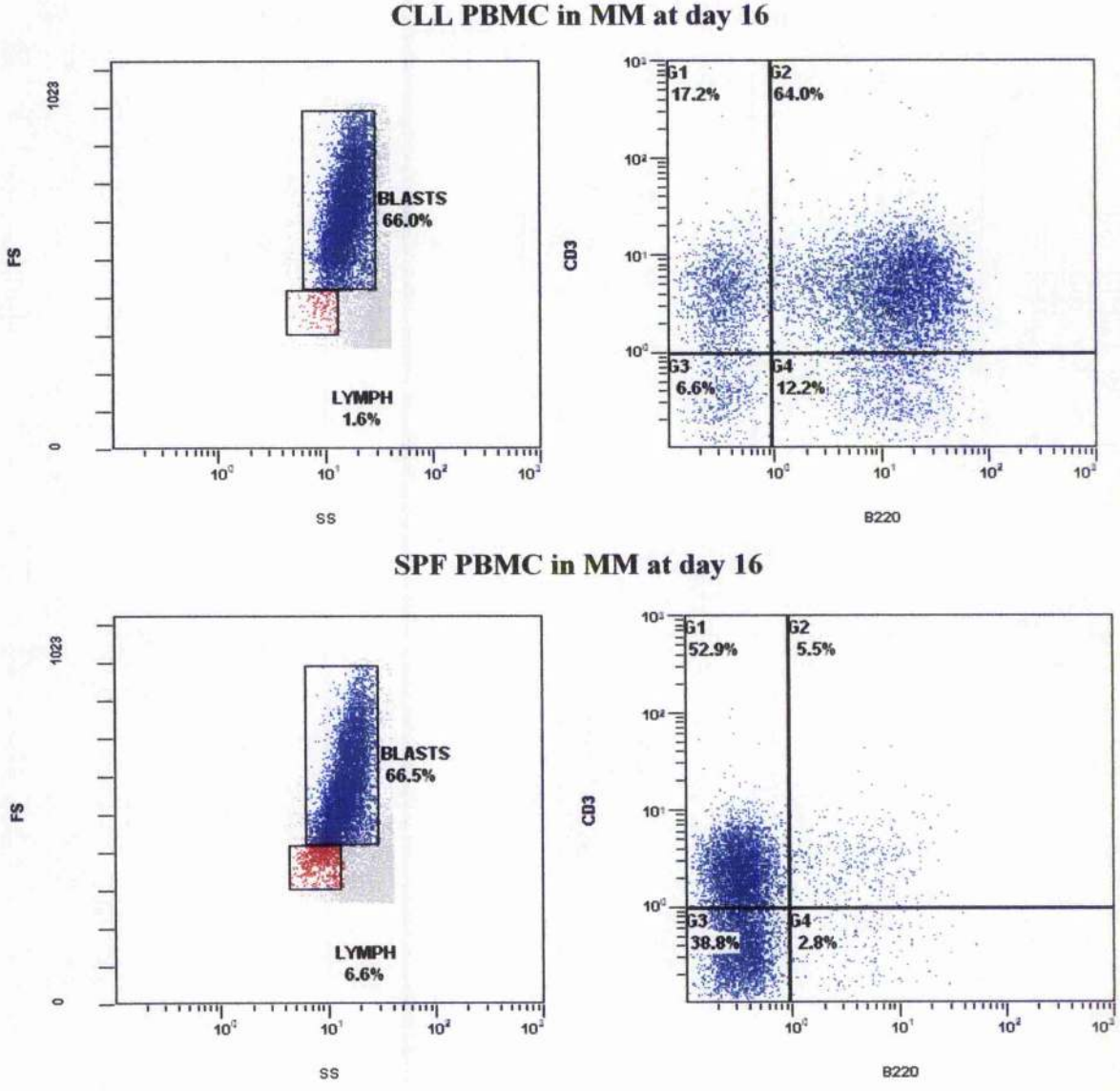


Figure 3.16. FACS analysis of SPF PBMC cultured alone in RPMI and MM



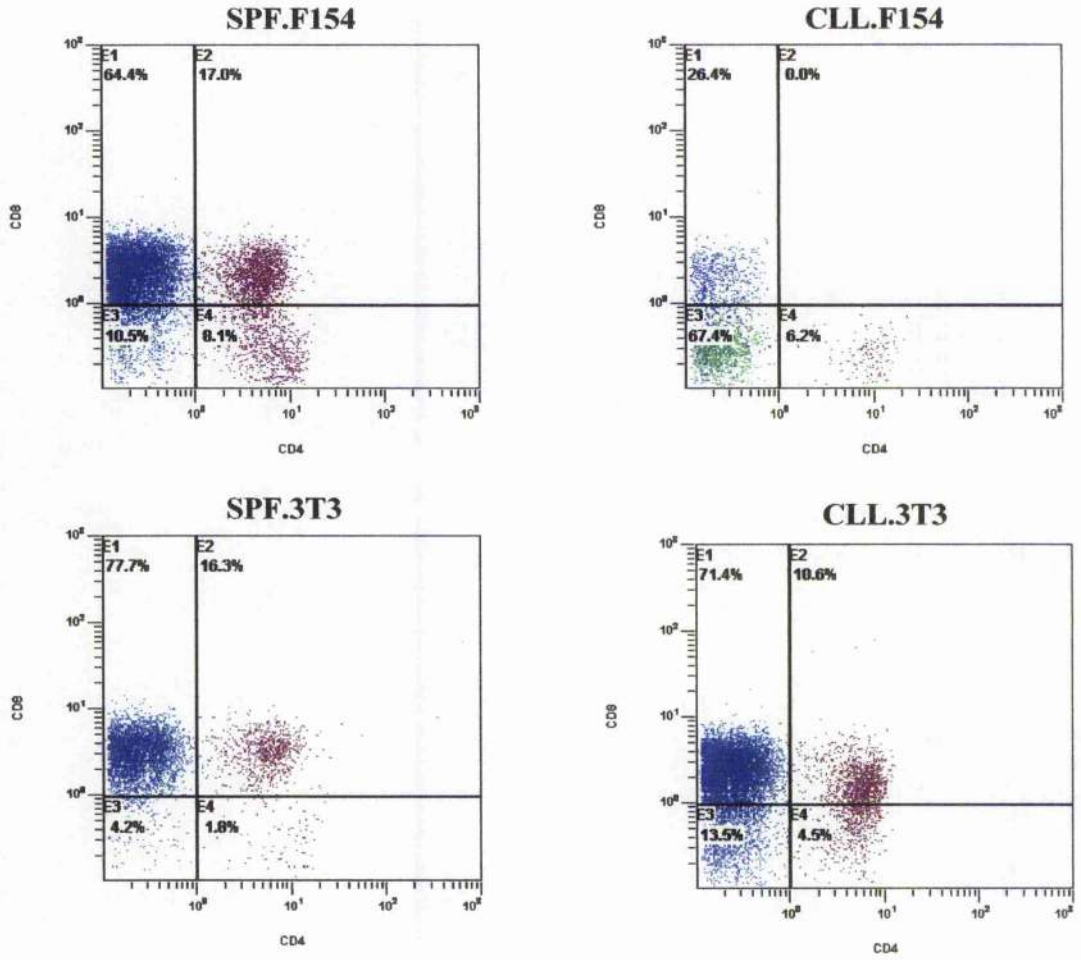
SPF PBMC were stained with FITC-conjugated B220 and PE-conjugated CD5 antibodies. The production of lymphoblasts (BLASTS) was increased by culturing in MM

Figure 3.17. FACS analysis of CLL and SPF PBMC cultured alone at day 16



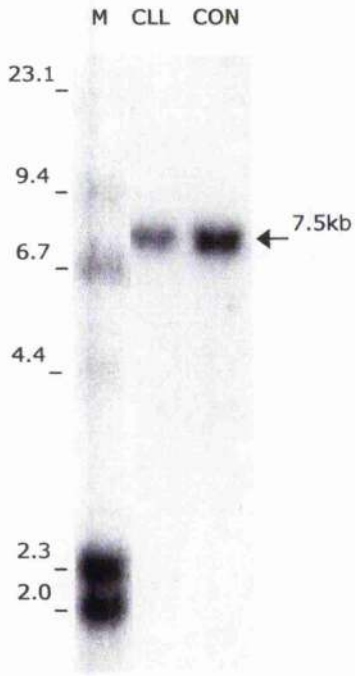
Cells were stained with FITC-conjugated anti-CD3 and cychrome-conjugated anti-B220 antibodies. FACS analysis revealed co-expression of CD3 and B220, which was more evident in the CLL PBMC culture.

Figure 3.18. FACS analysis of SPF and CLL PBMC co-cultured with CD40L and control 3T3 in MM



Cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies. FACS analysis revealed a sub-population of CD4/CD8 co-expressing cells, in the SPF.F154, SPF.3T3 and CLL.3T3 cell populations, but interestingly not in the CLL.F154 cell population.

Figure 3.19. Southern blot analysis of B-CLL lymphoblasts



Hind III digested DNA from the CLL cells (lane 1) or control PBMC (lane 2) was screened with a probe specific for immuno-globulin Cμ heavy chain. A 7.5kb band was detected in both lanes, indicative of immunoglobulin genes in the germline con-figuration. ^{M}λHind III marker

Table 3.1. CD21 expressing lymphocytes and lymphoblasts

Cell populations	% Lymphocytes	% Lymphoblasts	% CD21 expressing lymphocytes	%CD21 expressing lymphoblasts
SPF.F154 (RPMI)	8.4	40.1	1.9	93.5
CLL.F154 (RPMI)	5.7	35.8	56.5	96.6
SPF.F154 (MM)	4	50.2	5.5	75.7
CLL.F154 (MM)	8.9	49.3	73.7	95.2
SPF.3T3 (RPMI)	45.3	1.8	23.2	58.3
CLL.3T3 (RPMI)	65.9	1.7	61	64.1
SPF.3T3 (MM)	27.1	29.4	40	13.6
CLL.3T3 (MM)	37.6	21.7	71.4	17.1
SPF (RPMI)	48.4	2.1	12.1	31.6
CLL (RPMI)	59.1	3.3	18.8	25.5
SPF (MM)	33.9	13.1	29.9	14.7
CLL (MM)	41.8	24.9	74.3	20.9

FACS analysis of SPF and CLL cat PBMC at 7 days in culture. Cells were stained with FITC-conjugated anti-CD21 antibody.

Chapter Four

ENHANCING THE IMMUNE RESPONSE AGAINST FIV IN MICE WITH FELINE CD40L

4.1. Introduction

The realisation that naked DNA could be used to induce immune responses was almost accidental. Wolff et al. (1990) discovered that the injection of mice intramuscularly with naked DNA resulted in long-term reporter gene expression in transfected muscle fibers. It was subsequently shown that if the DNA plasmid encoded an antigenic protein, such as human growth hormone, immune responses could be induced in the mouse against the protein (Tang, DeVit, and Johnston 1992). The ability of proteins encoded by DNA plasmids to induce a protective host immune response was then established using an influenza virus model, again in the mouse (Ulmer et al. 1993). This study confirmed the potential for the use of naked DNA as a vaccine. Since then, the immune stimulating potential of injecting DNA plasmids encoding different pathogens into mice has been studied extensively. Pathogens have included HIV, hepatitis B virus, measles virus, bovine herpesvirus type 1, murine leukaemia virus and rabies virus (reviewed in Davis and McCluskie 1999).

In order to induce an effective immune response, several stimulatory signals are required. The primary signal is provided by the vaccine antigen itself and secondary signals are provided by co-stimulatory molecules or cytokines. Several novel approaches have been taken to provide such secondary signals in order to improve the efficacy of DNA vaccination. The immune stimulating capacity of DNA vaccines administered in conjunction with plasmids encoding co-stimulatory molecules has been studied in the mouse (Iwasaki et al. 1997; Davis et al. 1997; Kim et al. 1998). Many studies have been conducted more specifically with the co-stimulatory molecule, CD40L (Mendoza, Cantwell and Kipps 1997, Gurunathan et al. 1998, Ihata et al. 1999, Burger, Mendoza and Kipps 2001, Sin et al. 2001) .

In the cat, the potency of FIV DNA vaccines has been enhanced with the addition of plasmids encoding cytokines (Hosie et al. 1998; Boretti et al. 2000; Leutenegger et al. 2000; Hanlon et al. 2001; Dunham et al. 2002). DNA vaccination has been successful in

protecting cats against FIV challenge when FIV DNA plasmids have been administered with plasmids encoding cytokines, including IFN-γ (Hosie et al. 1998), IL-12 and IL-18 (Dunham et al. 2002). This, combined with the success of plasmids encoding co-stimulatory molecules inducing immune responses in the mouse model, warranted further research into the use of co-stimulatory molecules as novel adjuvants in DNA vaccination against FIV. In this study, the effect of immunising with the feline co-stimulatory molecule CD40L on the immune response to FIV was investigated in the mouse.

Female BALB/c mice were inoculated with feline CD40L DNA and FIV DNA together. Controls of either FIV or CD40L DNA alone and PBS alone were included. Because FIV is not infectious in mice, the aim of the study was not to challenge the immunised animals, but to examine the immune response against FIV that was elicited by the DNA vaccine. After three inoculations, serum samples were tested for anti-viral antibodies by Western blotting, ELISA and IF and splenocytes were assessed for the ability to proliferate in response to FIV p24 and WIV.

The ability of feline CD40L to bind the murine CD40 receptor has been previously demonstrated in proliferation assays in which murine PBMC were induced to proliferate in response to 3T3 cells stably expressing feline CD40L (Brown et al. 2002).

4.2. Materials and methods

4.2.1. Mice

Twelve female BALB/c mice, 6 to 8 weeks of age, were purchased from Harlan UK Ltd (Bicester, Oxford, UK) and maintained at the Diagnostics Scotland animal facility (Pentlands Science Park, Penicuik, UK) for the duration of the study. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

4.2.2. Plasmids and DNA preparation

The FIV and CD40L DNA plasmids used for inoculation in this study are described in Chapter 2.

4.2.3. Inoculation and blood sampling protocol

All inoculations and blood sampling were conducted at the animal facility of Diagnostics Scotland. Mice were randomly allocated into four groups of three mice. The mice were

inoculated at day 7, day 14 and day 21. Each inoculation consisted of one injection in the quadriceps muscle of the hind leg. Per inoculation, group 1 received 50μg of FIV DNA in 50μl of PBS, group 2 received 50μg of FIV DNA and 50μg of feline CD40L DNA in 50μl of PBS, group 3 received 50μg of feline CD40L DNA in 50μl of PBS and group 4 received 50μl of PBS only as a control.

Whole blood was collected into plain tubes at day 0 (pre-immune bleed), day 18 (test bleed) and day 28 (end of study bleed) to obtain serum samples. At the end of the study (day 28) spleens were collected aseptically.

4.2.4. Detection of anti-viral antibodies

Western blotting, ELISA to detect antibodies recognizing the immunodominant FIV TM peptide and FIV p24 and IF were conducted to determine the antibody responses made against FIV. See sections 2.1.6.1, 2.1.6.2 and 2.1.6.3 for a full description of these serological assays. Antibodies recognising FIV-infected CRFK cells were detected by an indirect IF technique using FITC sheep anti-mouse IgG (Diagnostics Scotland, Edinburgh, UK) secondary antibody.

4.2.4.1. Western blotting

Western blotting was conducted as described in Section 2.1.6.3. Blots were incubated with mouse serum. There were 3 samples per mouse, the first sample was taken prior to any DNA inoculations at day 0 (pre-immune), the second sample was taken after two DNA inoculations at day 18 (test) and the third sample was taken 7 days after the third and final DNA inoculation (day 21) at day 28 (end of study). The secondary antibody used for membranes incubated with mouse sera was a biotinylated anti-mouse IgG antibody (Vector Laboratories) used at a final concentration of 1μg/ml.

4.2.5. Detection of a cell-mediated immune response

A proliferation assay was employed as described in Section 2.1.7. to detect T-cell proliferation in response to either FIV p24 core protein or WIV. Cells were obtained from spleens collected aseptically at the end of study (day 28) by rupturing the splenic capsule and teasing the cells out with a scalpel blade. The resultant cell suspension was centrifuged over Ficoll at 2000rpm for 10 minutes and the cells were washed and counted and $2x10^5$ spleen cells were added to each well.

4.3. Results

4.3.1. Anti-viral antibodies detected by Western blotting

The serological responses against FIV, induced by inoculating mice with FIV DNA, FIV and CD40L DNA and CD40L DNA alone were assessed by IF, p24 and TM ELISAs and Western blotting. No fluorescence was detected by IF conducted with serum samples collected at the end of the study after 3 inoculations. Also the antibody titres for TM and p24 were zero when ELISAs were performed with serum samples also taken at the end of the study. However, as illustrated in Figure 4.1, antibodies were detected in some serum samples by Western blotting. Western blotting was conducted using all 3 serum samples collected at day 0 (pre-immune serum), day 18 after 2 inoculations (test serum) and day 28 after all 3 inoculations (end of study serum). Antibodies against the structural proteins of FIV, gp120 and p24 were evident in group 2 (FIV and CD40L DNA) only. This would suggest that co-immunisation with CD40L DNA had enhanced the humoral immune response against FIV DNA in the mice in group 2. No bands were evident in the pre-immune serum samples, indicating that this enhancement of the humoral immune response was directly attributable to DNA inoculation.

4.3.2. No cell-mediated responses induced by DNA inoculation

Proliferation assays were performed to detect cell-mediated responses induced against FIV following inoculation with the DNA plasmids. As shown in Figure 4.2, there was no marked proliferation of spleen cells in any of the groups, vaccinates or controls, when tested against WIV. However, there was a response against p24 in all of the groups tested, including the control group (PBS only), suggesting that this response was non-specific (see Figure 4.2). A positive experimental control of Con A was also included and splenocytes from all groups of mice proliferated in response to this mitogen. Therefore, the failure of any groups splenocytes to respond to WIV would be a true indicator that CD40L inoculation did not increase the immune response to WIV. The failure of this test to differentiate between proliferation of PBS control splenocytes from vaccinates splenocytes to FIV-p24, could be due to the splenocytes proliferating in response to the GST component of the FIV p24-GST fusion protein employed in this assay. However the same p24-GST fusion protein was used in experiments with feline PBMC and this non-

specificity was not apparent in those results. However, it is well known that results cannot be extrapolated from one species to another.

4.4. Discussion

In this study the immune responses to FIV in mice inoculated with FIV DNA and CD40L DNA together were determined. Because the response to the antigenic protein encoded by a DNA plasmid alone is often weak, this study sought to increase the immune response by inoculating the FIV DNA in the same syringe as the co-stimulatory molecule CD40L. This study demonstrates the immune stimulating capacity of feline CD40L in mice.

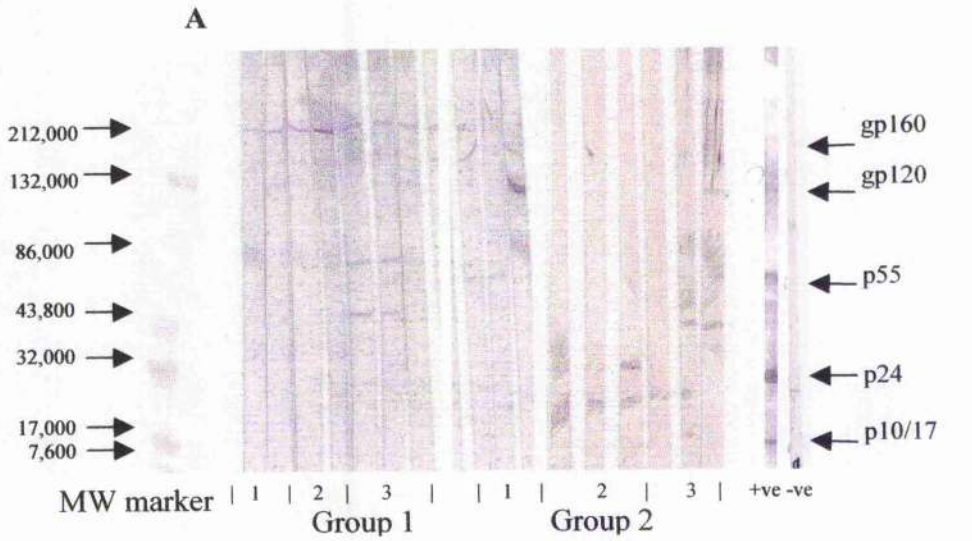
In agreement with previous studies (Gurunathan et al. 1998; Ihata et al. 1999; Burger, Mendoza, and Kipps 2001), the administration of the co-stimulatory molecule, CD40L in conjunction with FIV DNA did enhance the humoral immune response to FIV in all three mice in group 2 (FIV and CD40L DNA), as demonstrated by Western blotting. However, there was no evidence of enhancement of the cell-mediated response against FIV when spleen cells were incubated with either FIV p24 or WIV to test for activated $CD4^+$ antigen-specific T-cell proliferation. Helper T-cells play an important role in both humoral and cell-mediated immunity, via expansion of antigen-stimulated B-cells and $CD8^+$ T-cells. Previous studies (Gurunathan et al. 1998; Ihata et al. 1999; Burger, Mendoza, and Kipps 2001; Sin et al. 2001) have all shown an increase in cell-mediated immunity with the administration of CD40L. Sin et al. (2001) demonstrated that the protection induced in mice against a lethal dose of HSV was elicited by a DNA vaccine co-inoculated with CD40L DNA and was due to an increase in cellular immunity as demonstrated by T-cell proliferation and enhanced production of IL-2 and IFN-γ. Therefore, the immune response induced was predominantly a Th1 response. Th1 responses are thought to drive induction of cellular immunity, whereas Th2 immune responses preferentially drive humoral immunity. Interestingly, Sin et al. (2001) did not demonstrate an enhancement in humoral immunity. There was no increase in antibodies to HSV when serum was tested by ELISA. Sin et al. (2001) postulated that this could be due to the CD40L used in their study being membrane-anchored, thereby specifically targeting the cellular immune response, without enhancing the magnitude of the humoral immune response. However, the CD40L used in the present study was also membrane bound and yet appeared not to target the cellular immune response. Differences in the immune responses elicited between the different

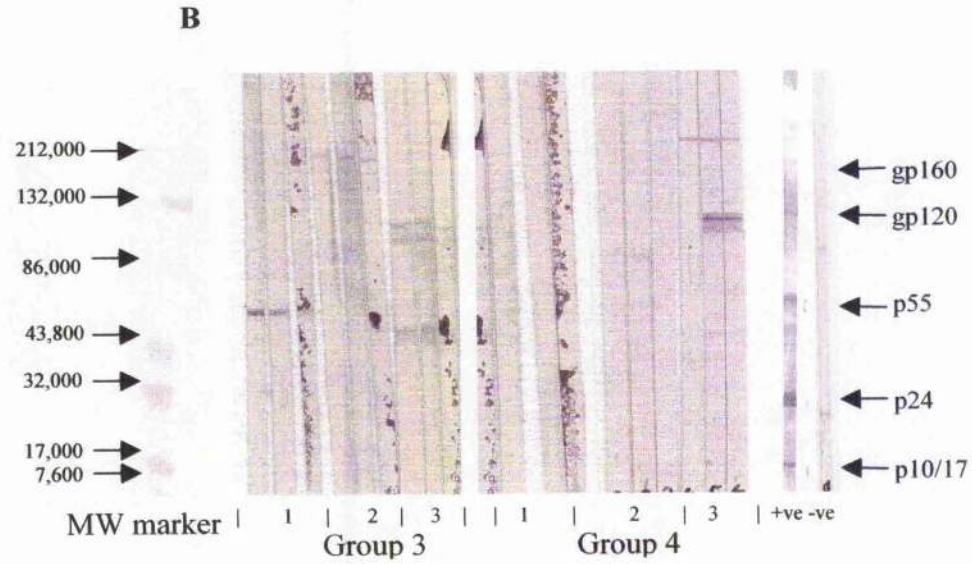
studies might be due to differences in volume of DNA injected, promotor used, site of injection or breed and age of mice.

In this study, a humoral response against FIV was elicited in mice inoculated with FIV and CD40L DNA together, whereas FIV DNA alone did not induce a humoral immune response as determined by ELISA, IF and Western blotting. A FIV-specific cell-mediated immune response was not detected in any of the mice inoculated with FIV DNA alone or FIV and CD40L DNA together, however the method used to detect cell-mediated responses requires further optimisation. Further studies may involve chromium release assays for the detection of CTLs.

Figure 4.1. Anti-viral serological responses

Antibodies against the structural proteins of FIV could be detected in mouse sera by Western blotting. (A) group 1 (FIV DNA only) and group 2 (FIV and CD40L DNA), (B) group 3 (CD40L DNA only) and group 4 (PBS control). The pre-immune, test and end of study serum samples were tested (lanes 1, 2 and 3 in each mouse respectively, except for mouse 1 and 2 in group 1, where there are no test serum samples). Specific bands were only evident in serum samples from the mice in group 2. Samples from mouse 1 and mouse 3 in group 2 demonstrated reactivity against gp120 in the end of study samples and the end of study sample from mouse 2 in group 2 appeared to demonstrate reactivity against a protein corresponding to p24. The reactivities observed in groups 1 and 3 could be non-specific as binding was evident in the pre-immune serum samples (which were collected prior to inoculation) and similarly bands in group 4 could be non-specific as bands were evident in the pre-immune serum samples and group 4 were inoculated with PBS. Known FIV +ve and –ve feline serum samples were used as controls.


A
212,000
132,000
86,000
43,800
32,000
17,000
7,600
gp160
gp120
p55
p24
p10/17
MW marker | 1 | 2 | 3 | | 1 | 2 | 3 | +ve -ve
Group 1
Group 2


B
212,000
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17,000
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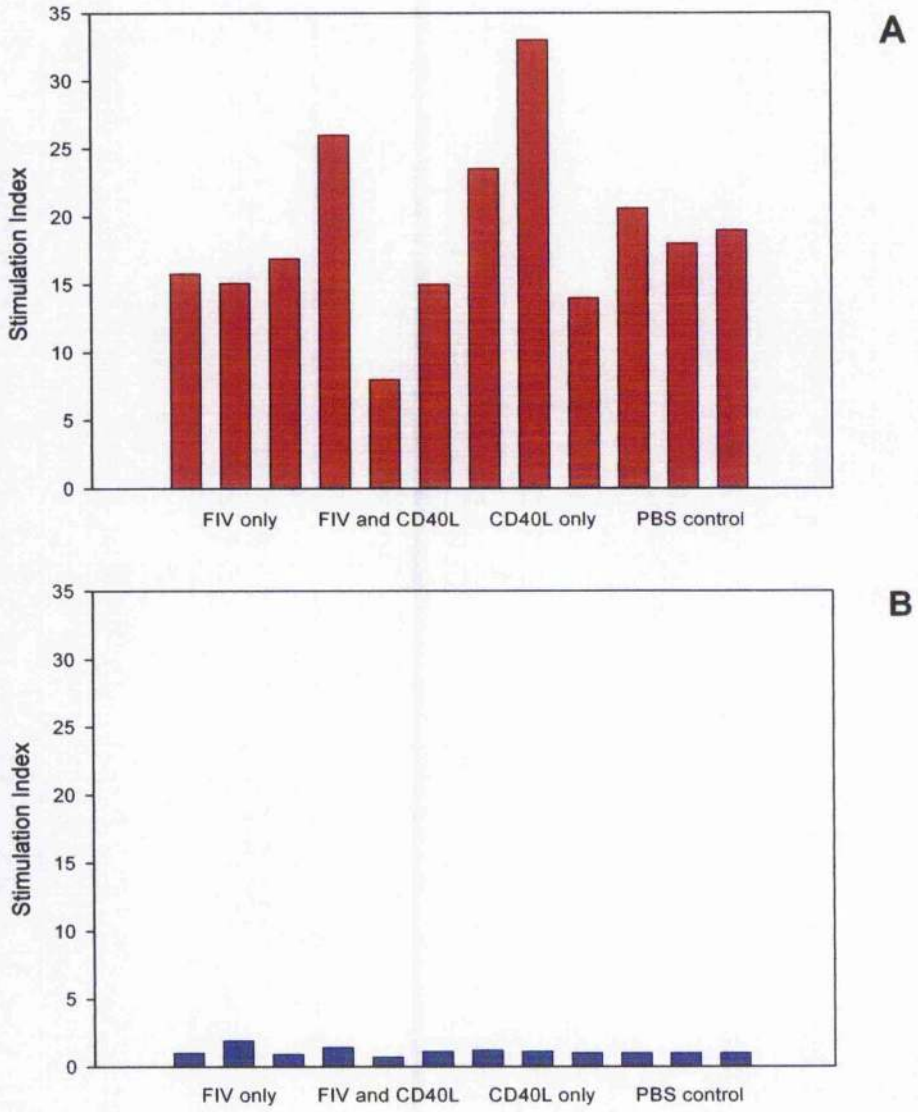
gp160
gp120
p55
p24
p10/17
MW marker | 1 | 2 | 3 | | 1 | 2 | 3 | +ve -ve
Group 3
Group 4

Figure 4.2. Proliferation of splenocytes in response to p24 and WIV



Mouse splenocytes obtained post mortem were incubated with FIV p24 and WIV to detect any cell-mediated immune response post vaccination. Graph (A) demonstrates a non-specific response to p24 (25μg/ml) as the PBS control vaccinates proliferated to high levels in parallel with the study vaccinates. Graph (B) demonstrates a failure of any of the vaccinates to respond to WIV. A SI of ≥2 was regarded as significant.

Chapter Five

EVALUATION OF FELINE CD40L AS AN ADJUVANT IN A DNA VACCINE AGAINST A VIRULENT ISOLATE OF FIV

5.1. Introduction

A number of vaccine trials have been successful in inducing protection against FIV infection, initially using WIV and inactivated IC vaccines (Yamamoto et al. 1991b; Yamamoto et al. 1993) and then DNA vaccines (Hosie et al. 1998; Lockridge et al. 2000; Boretti et al. 2000; Dunham et al. 2002). Protection has been restricted largely to challenge viruses of low virulence, such as FIV-PET and similar vaccine preparations did not protect against the more virulent primary FIV-GL8 (Hosie et al. 1992). It is likely that it will be necessary for a commercially available vaccine to confer protection against more virulent, and presumably more relevant primary isolates, such as FIV-GL8 and FIV-UT113 that have so far proved resistant to vaccine induced protection (Hesselink et al. 1999).

Both humoral and cell-mediated immunity have been implicated in protection associated with WIV vaccines (Hosie and Flynn 1996b) and therefore both are considered important in protecting against virulent strains of FIV. When cats immunised with FIV-PET WIV were challenged with FIV-GL8, although not protected from infection, suppression of viral load post challenge was seen (Hosie et al. 1995; Hosie et al. 2000). In contrast, when a similar study was conducted using DNA vaccines (Hosie et al. 2000), there was no suppression of viral load when cats immunised with a FIV-PET DNA vaccine were challenged with FIV-GL8. These findings suggested that the broader spectrum of immune responses elicited by the WIV vaccine was beneficial in controlling the FIV-GL8 challenge.

Such success as has been achieved with FIV DNA vaccines has been dependent on co-administration of plasmids encoding cytokines, which act as immune-stimulating adjuvants. Therefore, in this study the effect of immunising cats with a FIV DNA vaccine together with a co-stimulatory molecule, feline CD40L, to act as an adjuvant, was investigated. It was hoped that by inoculating with a plasmid encoding FIV-GL8 provirus together with a plasmid encoding feline CD40L, that CD40L would encourage both

cellular and humoral immune responses, which might provide protection against the FIV-GL8 challenge.

Prior to this study, the feline CD40L gene was cloned and 3T3 cells were transduced so that a cell line expressing CD40L on the cell surface was developed. In order for the 3T3 cells to stably express CD40L, they were infected with supernatant from the Phoenix-Eco ecotropic murine leukaemia virus-based packaging cell line (previously transfected with the pDON-AI retroviral vector with a feline CD40L insert, pDON-AI.F154). In Chapter 3, the establishment of the *in vitro* biological activity of the cloned feline CD40L using 3T3 cells stably transfected with CD40L was reported. However, this study required that feline CD40L be in a plasmid vector suitable for use as a DNA vaccine. The plasmid chosen was VR1012; the VR1012 plasmid has previously been shown to promote DNA expression in skeletal muscle, the preferred route of immunisation of DNA vaccines and the route used in this study (Hartikka et al. 1996). In order to assess the biological activity of the VR1012 plasmid with a CD40L insert (VR1012.F154) which would be used as the DNA vaccine, 3T3 cells were transiently transfected with VR1012.F154 and a proliferation assay was conducted using the methods described previously.

A FIV-GL8 DNA construct was then selected from a panel of FIV DNA constructs following a comparison of the expression of FIV structural proteins using Western blotting. Once the biological activity of the CD40L plasmid had been assessed and the FIV DNA construct selected, the required amounts of both DNA plasmids were prepared for vaccination and the immune responses elicited in cats by an FIV DNA vaccine with and without the inclusion of CD40L as a genetic adjuvant were studied. SPF cats were inoculated with FIV DNA alone, FIV DNA and CD40L DNA, CD40L DNA alone or PBS only as a control. To test the efficacy of immunisation, animals were challenged with the biological isolate of FIV-GL8 by i.p inoculation. Following challenge several parameters were monitored including; viral load, proviral load, CD4:CD8 ratio, $CD8\beta^{low}$ cell numbers and haematology to determine the effect, if any, of CD40L as an adjuvant.

5.2. Materials and methods

5.2.1. Production of the CD40L DNA vaccine

Please see Section 2.1.3.1.

5.2.2. Demonstration of the biological activity of the CD40L DNA vaccine, VR1012.F154

To assess the biological activity of the VR1012.F154 plasmid prior to use as a DNA vaccine, 3T3 cells were transiently transfected using the Superfect reagent (Qiagen) with VR1012.F154 or with empty plasmid (VR1012). Circular tissue culture plates 10cm in diameter (Becton Dickinson), were seeded with 10^6 3T3 cells and incubated overnight at 37°C in an atmosphere of 5% CO_2, to allow the 3T3 cells to establish themselves. The 3T3 cells were then rinsed in serum-free DMEM and incubated with 10μg of the plasmid DNA and 60μl Superfect reagent in 3 ml of serum-free DMEM for 3 hours at 37°C in an atmosphere of 5% CO_2. After this time, the cells were again rinsed in serum-free DMEM and then returned to the incubator in fresh complete DMEM for a further 2 days, at which point they were 90% confluent. To determine the efficiency of transfection, control plates of 3T3 cells were transfected with LacZ and subsequently stained with X-gal. The transiently transfected 3T3 cells were then used as target cells in proliferation assays, as described previously in section 2.1.4. Briefly, irradiated (3000 rads) VR1012.F154 cells and mock transfected 3T3 cells were added in decreasing numbers in triplicate wells of a 96-well flat bottom plate. SPF cat PBMC (10^5 in number) were added to each well and the plates were incubated at 37°C in an atmosphere of 5% CO_2 for 4 days, after which time 20μCi/well of ^{3}H-thymidine was added for the final 18 hours of incubation. The cells were harvested from the plates onto absorbent filter plates and the radioactivity incorporated into the cells was measured on a microplate scintillation counter.

5.2.3. Expression of FIV proteins by FIV DNA constructs

The expression of FIV proteins *in vitro* by FIV DNA constructs was investigated by transfecting 293T cells as described above and performing Western blotting with an anti-FIV cat serum, as described in section 2.1.6.3, to enable the selection of a construct suitable for use as a DNA vaccine. The DNA constructs investigated are listed in Table 5.1. Briefly, 293T cells were seeded at 1.5 x 10^5 cells/well in a 6-well poly-l-lysine coated tissue culture plate (Becton Dickinson) and transfected with 2μg of FIV plasmid DNA construct using the Superfect reagent (Qiagen). After 3 days, 1ml of culture fluid was removed from each well and tested for FIV p24 antigen, using a commercial FIV antigen ELISA (IDEXX), and RT activity was measured using a LentiRT assay (Cavidi Tech AB, Uppsala, Sweden). Also at 3 days post transfection, cell lysates were made from the

transfected 293T cells. RSB was added to the lysates, which were then boiled for 3 minutes and resolved on a 4-20% gradient Tris-HCl SDS-PAGE (Biorad). Transfer was performed onto a nitrocellulose membrane (Biorad), which was blocked overnight and then incubated for 2 hours at room temperature with FIV-positive cat serum or anti-Env monoclonal antibody (vpg71.2) (Willett et al. 1997a) diluted 1:10 with PBS supplemented with 1% non-fat milk powder and 0.5% Tween-20. Following incubation, the membranes stained with FIV-positive cat serum were washed and incubated for 1 hour with biotinylated protein A (ICN Pharmaceuticals Ltd.) at a concentration of 4μg/ml. The secondary antibody used for membranes incubated with vpg 71.2 was a biotinylated anti-mouse IgG antibody (Vector Laboratories) (1μg/ml). After incubating with the secondary antibodies the membranes were washed again and incubated for a further hour with streptavidin-alkaline phosphatase (Bio-Rad). Membranes were then washed and incubated in AP buffer for 10 minutes before bound antibody was detected using 10 ml AP buffer containing BCIP (Sigma) (50mg/ml) and NBT (Sigma) (50mg/ml).

5.2.4. Production of the FIV DNA vaccine

The CT5bG8MΔpol plasmid was selected from 5 FIV DNA constructs investigated for use as a FIV DNA vaccine. This plasmid consists of the 34TF10 (FIV PET) molecular clone of FIV (Talbott et al. 1989) with the U3 element within the 5' LTR deleted by fusing the human CMV immediate early gene promoter to the R repeat, forming the CT5 plasmid (Poeschla, Wong-Staal, and Looney 1998). CT5b is a modification of CT5 in which the SV40 T-antigen binding site is inserted downstream of viral sequences to permit T antigen-dependent plasmid amplification. The CT5b plasmid was kindly supplied by Dr E.M. Poeschla and was developed further by Dr B.J. Willett, at the University of Glasgow. The modified CT5b plasmid was derived by digesting CT5b with *Pac1* and *Nde* enzymes (New England Biolabs (UK) Ltd., Hitchin, Herts, UK) to remove the *pol* and *env* genes of 34TF10, which were replaced by the *pol* and *env* genes of the GL8 molecular clone of FIV (Hosie et al. 2000) (CT5bG8M). A 397bp deletion in *pol* in CT5bG8MΔpol renders this plasmid replication defective and hence non-infective. Dr. B.J Willett, University of Glasgow, kindly supplied the CT5bG8MΔpol plasmid.

Plasmid DNA was purified using double caesium chloride-ethidium bromide gradient centrifugation, butanol extraction and ethanol precipitation (see section 2.1.3.2).

5.2.5. Immunisation of cats

A group of fifteen, 11-week-old SPF cats was obtained from a certified breeder and housed by Biological Services, University of Glasgow. Animal care and husbandry was conducted under the Codes of Practice published by the Home Office. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act (1986).

The cats were randomly allocated into three groups of four (groups 1-3) and one group of three cats (group 4). Each group was vaccinated at 0, 3 and 6 weeks. Each vaccination consisted of 4 individual injections (one in each of the gastrocnemius muscles and one in each of the quadriceps muscles of the hind limb). Group 1 received 100μg of FIV DNA in 200μl of PBS per injection. Group 2 received 100μg of FIV DNA and 100μg of feline CD40L DNA in 200μl of PBS per injection. Group 3 received 100μg of feline CD40L DNA in 200μl of PBS per injection and group 4 received 4 x 200μl injections of PBS only as a control. Cats were challenged 3 weeks after the third vaccination, at week 9, by i.p injection of 10 ID_{50} of the biological isolate of FIV-GL8, which had been previously titrated *in vivo*.

5.2.6. Isolation of FIV

Peripheral venous blood samples were collected into EDTA tubes at 3, 6, 10 and 13 weeks after challenge for isolation of FIV. PBMC were obtained by overlaying Ficoll-Hypaque with whole blood diluted 1 in 4 in serum-free RPMI and centrifuging at 2000rpm for 10 minutes. In T25 cell culture flasks (Corning), 10^6 PBMC were co-cultured with 10^6 Mya-1 cells. The media were refreshed on day 4 and culture fluids were tested at day 7 by FIV p24 antigen ELISA (IDEXX). Positive cultures were discarded and negative cultures were maintained for a further seven days, when culture fluids were tested again.

5.2.7. Measurement of viral and proviral loads

Plasma samples and PBMC pellets from whole blood collected on the day of challenge and 3, 6, 10, 13 and 15 weeks post challenge were analysed by real time PCR to determine viral and proviral loads. Peripheral lymph node (PLN) and mesenteric lymph node (MLN), as well as spleen and thymus were collected aseptically at post mortem examination (15 weeks post challenge) into RPMI containing 100 IU/ml penicillin and 100μg/ml streptomycin. The tissues were teased apart with scalpel blades and the resultant cell

populations were centrifuged at 1000rpm for 5 minutes. Spleen cells were centrifuged over Ficoll to remove red blood cells and therefore obtain splenocytes. These cell pellets were analysed for FIV proviral loads. FIV proviral load was measured by quantitative real-time PCR (Leutenegger et al. 1999). Viral loads were measured in plasma samples by quantitative reverse transcriptase-PCR (Klein et al. 2001). Dr. Dieter Klein conducted viral and proviral load measurements at The University of Vienna.

5.2.8. Quantitative virus isolations

MLN cells obtained at necropsy were added in decreasing numbers (10^4, $3x10^3$, 10^3, $3x10^2$, 10^2, 30, 10, 3, 1 and none) to a 96-well tissue culture plate (Becton Dickinson) and co-cultivated, in 8 replicate wells, with 10^5 Mya-1 cells/well. The plates were incubated at 37°C in an atmosphere of 5% CO_2 for 14 days and half of the medium was removed and replenished every 4 days with complete RPMI supplemented with IL-2. Culture fluids were collected on day 14 and tested for FIV p24 by ELISA. The 50% endpoint (the dilution of MLN cells at which 50% of the wells containing MLN and Mya-1 cells were positive for FIV p24) was calculated using the method of Reed and Muench (1938).

5.2.9. Analysis of cell-mediated immune responses

A proliferation assay as described in Section 2.1.7. was employed to detect lymphocyte proliferation in response to either FIV p24 core protein or WIV. PBMC collected post 1st vaccination, post 2nd vaccination or on the d.o.c were added to the proliferation assays.

5.2.10. Serology

5.2.10.1. Immunofluorescence

Antibodies recognising FIV-infected CRFK cells were detected by an indirect IF technique as described in section 2.1.6.2. FITC goat anti-feline IgG secondary antibody was used to detect feline antibodies (prepared by Mr. T. Dunsford, Retrovirus Research Laboratory, University of Glasgow).

5.2.10.2. Flow cytometry

Flow cytometry was also used to detect FIV anti-Env antibodies in plasma collected on the d.o.c. Thus, 10^6 Mya-1 cells infected with FIV-GL8 were removed from culture and incubated with plasma samples for 30 minutes at 4°C in Falcon 2054 tubes. After washing

twice in PBA the cells were incubated with FITC-conjugated goat anti-feline IgG for a further 30 minutes at 4°C. The cells were then washed twice in PBA, resuspended in 1ml of PBA and analysed on an EPICS Elite flow cytometer (Beckman-Coulter) using the EXPO analysis software package. For each sample 10000 events were collected.

5.2.10.3. Western blotting

Plasma samples collected prior to the 1st vaccination and up to 15 weeks post challenge were assayed for anti-FIV antibodies by Western blotting, as described in Section 2.1.6.3. Briefly, a cell lysate was prepared from Mya-1 cells infected with FIV-GL8 and resolved on a 4-20% Tris-HCl SDS-PAGE (Biorad). Proteins were transferred to a nitrocellulose membrane (Biorad), which was blocked overnight. The membrane was cut into 2mm strips, which were incubated with each of 9 plasma samples and a positive and a negative control serum for two hours at room temperature. Staining was conducted using biotinylated protein A (ICN) as the secondary antibody.

5.2.10.4. ELISA

A peptide-based ELISA was employed to determine titres of antibodies recognising an immunodominant epitope in the TM glycoprotein (CNQNQFFCK), using the method described previously in section 2.1.6.1. Antibodies recognising the FIV p24 core protein were also measured by ELISA. TM and p24 ELISAs were conducted by Matt Golder, Companion Animal Diagnostics, University of Glasgow.

5.2.10.5. Virus neutralising antibody activity

Plasma samples collected on the d.o.c and 6 and 13 weeks post challenge were tested for VNA. Eight-fold dilutions of plasma were set up in quadruplicate in a 96-well flat-bottomed tissue culture plate (Falcon). The biological isolate of FIV-GL8 was diluted to 10 $TCID_{50}$/ml and 50µl was added to each well containing plasma samples. Plasma samples and virus were incubated for 1 hour at 4°C. After incubation, 10^5 Mya-1 cells in 100µl of complete RPMI were added to each well. The plates were incubated at 37°C in an atmosphere of 5% CO_2 for 7 days. After 4 days, 100µl of culture fluid was removed, discarded and replaced with fresh medium. At 7 days, 20µl of culture fluid was removed and RT activity was measured. The dilution of plasma at which 50% of RT activity was neutralized was calculated using the method of Reed and Muench (1938).

5.2.11. Analysis of lymphocyte subpopulations by flow cytometry

EDTA blood samples, 1ml in volume, were subjected to ammonium chloride lysis to obtain peripheral blood leucocytes. Thus, 1ml of 0.4% paraformaldehyde in PBS was added to 1ml of whole blood and incubated at room temperature for no more than 4 minutes. To this suspension, 25ml of pre-warmed (37°C) ammonium chloride/Tris (0.88% ammonium chloride/0.01M Tris-HCL pH 7.4) was added to lyse the red blood cells and the suspension was then centrifuged at 1000rpm for 5 minutes. The resultant leucocyte cell pellet was washed in PBA and resuspended in 200µl of PBA. The leucocytes were divided into 4 groups and labelled with antibodies by incubating for 30 minutes at 4°C with 5µl of antibody. Group 1 was labelled with FITC-conjugated mouse anti-feline CD4 (Vpg 34, Serotec) and PE-conjugated mouse anti-feline CD8αβ (Vpg 9, Serotec) antibodies. Group 2 was labelled with FITC-conjugated anti-feline CD3 (NZMI, Dr. Y. Nishimura, University of Tokyo) and PE-conjugated mouse anti-feline CD4 (Fe 17, Southern Biotechnology) antibodies. Group 3 was labelled with FITC-conjugated anti-feline CD3 (NZMI, Dr. Y. Nishimura) and PE-conjugated mouse anti-feline CD8αβ (Vpg 9, Serotec) antibodies and Group 4 was labelled with FITC-conjugated anti-feline CD8α (12A3, kindly provided by Dr. M. Shimojima, University of Tokyo) and PE-conjugated anti-feline CD8β (FT2, Southern Biotechnology) antibodies. After incubation, the cells were washed twice with PBA, resuspended in a final volume of 1ml of PBA and analysed on an EPICS Elite flow cytometer (Beckman-Coulter) using the EXPO analysis software package. Lymphocytes were "live-gated" on the basis of size and granularity and 5000 events were collected for each sample with the exception of group 4 for which 10000 events were collected because of the small number of gated cells.

5.2.12. Haematology

Peripheral venous blood samples (200µl in volume) were collected into EDTA at 6 and 3 weeks prior to challenge, on the d.o.c, and 3, 6, 10, 13 and 15 weeks post challenge and were subjected to haematological analysis. The haematological parameters analysed were red blood cell count (RBC), haemaglobin (Hb), haematocrit (HCT), mean cell volume (MCV), mean cell haemaglobin (MCH), mean cell haemaglobin concentration (MCHC), white blood cell count (WBC), and counts of neutrophil, lymphocyte, monocyte, eosinophil and basophil numbers.

5.3. Results

5.3.1. VR1012.F154 induces proliferation of feline PBMC *in vitro*

Prior to its use as an adjuvant in vivo, the activity of the feline CD40L DNA intended for use in the vaccine inoculum was assessed in vitro using proliferation assays. 3T3 cells were transiently transfected with CD40L DNA (VR1012.F154) and then used as target cells for proliferation of feline PBMC. Thus, 10^5 PBMC from an SPF cat was incubated with irradiated 3T3 cells, either transiently expressing CD40L or mock transfected. As shown in Figure 5.1, feline PBMC were induced to proliferate to a maximum value of 7028 ± 664 SEM cpm by VR1012.F154, confirming the biological activity of the CD40L DNA construct. The proliferation of PBMC induced by 3T3 cells expressing CD40L at 10^5, $3x10^4$ and 10^4 3T3 cells was highly significant and significant at $3x10^3$ 3T3 cells, when compared with the proliferation of PBMC induced by mock transfected 3T3 cells at these numbers (Student's t-test).

5.3.2. Selection of a FIV DNA construct for vaccination

To investigate the effect of CD40L as a DNA adjuvant, a FIV DNA construct suitable for use as a DNA vaccine was first selected. 293T cells were transfected with FIV DNA constructs including the CT5bG8MΔpol plasmid, which was eventually selected for use as a FIV DNA vaccine. At 3 days post-transfection, cell lysates were made and Western blotting was performed to examine the proteins that were expressed. Env expression from the DNA constructs was compared on the basis of staining with anti-Env monoclonal antibody vpg 71.2 and pooled FIV-positive cat serum. Strong expression of the envelope protein was deemed to be of value as it is recognised to contain B-cell epitopes (Lombardi et al. 1993) important in the humoral immune response. It has also been shown that the induction of Env-specific CTL as part of the cellular immune response is an important outcome of vaccination (Flynn et al. 1996). Furthermore, in a previous study of HIV vaccination in chimpanzees, protection from HIV-1 infection was associated with the generation of antibodies directed against the third variable domain (V3) of the HIV-1 gp120 Env glycoprotein (Emini et al. 1992). The CT5bG8MΔpol plasmid was selected for use as a DNA vaccine as Western blotting confirmed strong expression of FIV proteins in vitro, especially the Env proteins TM and SU, as shown in Figure 5.2.

At 3 days post-transfection, culture fluids were tested for FIV p24 and RT activity. The CT5bG8MΔpol plasmid was positive for p24, but negative for RT activity due to the 397bp deletion in *pol*, thus confirming that the plasmid was replication incompetent and therefore safe for use as an immunogen.

5.3.3. Virus was isolated from all of the cats post challenge

In the light of these findings, a vaccine was prepared comprising the CT5bG8MΔpol plasmid and cats were inoculated with CT5bG8MΔpol alone, CT5bG8MΔpol with CD40L, CD40L alone and PBS. Cats were given three doses of vaccine and then challenged with 10 ID_{50} FIV-GL8 i.p. To determine whether the DNA vaccine induced protection against the challenge, virus isolation was conducted post challenge. At weeks 3, 6, 10 and 13 post challenge, PBMC were isolated from peripheral venous blood samples and 10^6 PBMC were co-cultivated with 10^6 Mya-1 cells. After 7 and 14 days culture fluids were tested for FIV p24 core protein by ELISA. At week 3 post-challenge, all cultures were postive for FIV p24, however, 2/4 cats in group 1 (A723 and A724) and 1/4 cats in group 2 (A728) remained negative until day 14 whereas the other cultures were positive for p24 by day 7 in culture, suggesting that the cats which yielded positive cultures later had lower viral loads. At week 6, 1/4 cats in group 1 (A723) remained negative for p24 until day 14 in culture, however all other cats were positive at day 7. At weeks 10 and 13 post-challenge all cultures were positive by day 7, indicating that inoculation with the DNA vaccine, with or without CD40L did not induce sterilising immunity against the homologous FIV-GL8 challenge.

5.3.4. CD40L adjuvanted FIV DNA vaccine reduces viral loads

To monitor the progression of infection and to determine whether feline CD40L DNA immunisation, while not having induced sterilising immunity, may have led to decreased viral and proviral loads post challenge, viral and proviral loads were measured in each cat at intervals from the d.o.c to 15 weeks after challenge (see Table 5.2). Plasma viral RNA loads have been shown previously to be a good indicator of disease progression (Diehl et al. 1996; Goto et al. 2002). Plasma viral loads were highest overall in group 3 (CD40L DNA only), and lowest in group 2 (FIV + CD40L DNA), as shown in Figure 5.3. These results indicate that immunisation with the combination of FIV and CD40L DNA may have led to decreased viral loads, whereas CD40L DNA alone may have enhanced

infection, since the viral loads in group 3 were greater than those of the controls. In particular, Cat A731 (group 3), had a very high peak viral load of $> 10^5$ virions/ml of plasma.

The proviral loads reflected the viral loads, being highest in the cats in group 3, as shown in Figure 5.3. Interestingly, the proviral loads were lowest in the control group, group 4 (PBS). The proviral loads measured in the cats in group 2 were most similar to those of the control cats, indicating that the combination of CD40L with FIV DNA did not lead to the increased level of infection detected in a proportion of the cats inoculated with either FIV or CD40L DNA alone. In group 3, cat A729 developed a very high peak proviral load of 182914 provirus copies/10^6 cells.

Proviral loads in tissues collected post mortem demonstrated a similar pattern to PBMC proviral loads. Overall, group 3 had the highest loads in all tissues sampled (PLN, MLN, spleen and thymus) except for PLN where loads were broadly similar amongst the groups, although the loads in group 1 were slightly higher, as shown in Figure 5.4. Viral loads did not correlate with proviral loads in individual cats, consistent with previous findings (Pistello et al. 1999). In agreement with the virus isolation findings (section 5.3.3), the viral loads of group 3 and group 4 were higher at week 3 post-challenge than those of groups 1 and 2. Viral loads also peaked earlier in groups 3 and 4 than in groups 1 and 2 (see Table 5.2.). Therefore, virus was more readily detected in groups 3 and 4 by virus isolation. No virus or provirus could be detected in d.o.c samples, which also confirmed that the FIV DNA construct was non-infective.

5.3.5. Low viral levels were detected in MLN

Infectious viral burdens were quantitated by conducting quantitative virus isolations (QVI) with cells from MLN collected post mortem at 15 weeks post challenge. Lower levels of virus were detected in group 2 (FIV and CD40L DNA) and group 4 (PBS) than in groups 1 (FIV DNA) and 3 (CD40L DNA), see Figure 5.5. The virus levels detected in MLN by QVI correlated in some (but not all) cats with MLN proviral loads at week 15 post-challenge. When viral levels in MLN were compared with PBMC proviral loads, cats 722 and 729 (groups 1 and 3 respectively) displayed the highest infectious virus levels in MLN and also had the highest PBMC proviral loads in their respective groups.

mediated responses against FIV before challenge. These studies were also conducted in an attempt to establish whether the reduced viral loads in group 2 (FIV and CD40L DNA) could be associated with cell-mediated immunity. PBMC collected after the first, second and third vaccinations were recovered from liquid nitrogen and set up in 96-well plates with either p24 antigen or WIV. PBMC were shown to proliferate in response to both p24 and WIV, as shown in Figure 5.6. In group 1, (FIV DNA only), cats A723 and A724 displayed significant proliferative responses to p24 post first vaccination. Both of these cats had low viral and proviral loads, whereas the other 2 cats in the group which did not respond well developed higher viral and proviral loads post challenge. This result is consistent with a role for cell-mediated immunity in controlling the challenge virus. In group 2, no cat responded to p24 and WIV following the first vaccination, although 2/4 responded after the second vaccination. In this group all of the cats except A726 had low viral loads and 2/4 had low proviral loads. These data are consistent with CD40L acting as an adjuvant to enhance the cell-mediated immune response. In Group 3 (CD40L only), 2/4 cats responded to p24 and 1/4 to WIV following the first and second vaccination. Interestingly, two of the responders in this group, A729 and A730, had the highest responses to 25μg/ml of p24 (SI of 7.5 and 4.5 respectively) of all the animals tested. In this group, the cats that responded well to p24 developed high viral and proviral loads post challenge and a high level of virus was detected in MLN cells (Figure 5.5). Therefore, although CD40L had increased the immune response, the response may not have been sufficiently specific to prevent or even control infection post challenge and may even have resulted in enhancement of infection in these cats. Moreover, the single cat that did not respond to p24 and WIV in group 3 (A732) had remarkably low viral and proviral loads post challenge, further demonstrating that CD40L immunisation could have led to enhancement rather than inducing protective immune responses. The results were unexpected after the third vaccination as only one animal responded to p24 (A724) and no animal responded to WIV. The control group, group 4, displayed no significant responses to p24 or WIV at any of the time-points assessed.

5.3.7. No anti-viral antibodies were induced by vaccination

After vaccination and before challenge, no FIV-specific antibodies could be detected by flow cytometry, Western blotting, ELISA or IF. The ELISA to detect antibodies recognising the immunodominant TM peptide or p24 antigen confirmed that there were no

anti-viral antibodies prior to infection (Table 5.3), with the exception of two animals (A726 and A732) that had very low antibody titres (positive results detected at plasma dilutions of 1:10) to the TM peptide before challenge. However, this result was not confirmed by any other method and is likely to have been non-specific, since A732 was seronegative 3 weeks later. No antibodies directed against FIV-GL8 infected Mya-1 cells were detected by flow cytometry.

5.3.8. Seroconversion following challenge

Western blotting was performed using plasma samples collected prior to the first vaccination to 15 weeks post challenge to detect anti-viral antibody production against the major structural proteins of FIV (TM, SU, CA, MA and NC) and confirmed that no anti-viral antibodies had been induced in any of the cats before challenge (Table 5.4).

Anti-FIV antibodies developed in the cats following challenge, and analysis of sequential samples enabled comparisons of the humoral response between groups to be made (Table 5.4). In group 1, 3/4 cats developed antibodies by 3 weeks after challenge, whereas the control cats remained seronegative. These results are consistent with either an anamnestic response following challenge of the cats immunised with FIV DNA, or enhanced viral replication leading to a high antigenic challenge. Cat A722 developed antibodies to both CA and SU by 3 weeks post-challenge. In group 2, only 2/4 cats developed antibodies at 3 weeks post-challenge (see Figure 5.7). Therefore, there was no evidence that CD40L enhanced the humoral immune response to FIV. In group 2, cat A726 had the greatest antibody response at 3 weeks post-challenge, interestingly, this cat also developed the highest viral and proviral loads of the group. In groups 3 and 4, there was no evidence of antibody production against the main structural proteins of FIV until 6 weeks post-challenge. A similar trend was also evident in the p24 and TM ELISAs (Table 5.3), where 2/4 cats in groups 1 and 2 had developed antibodies to p24 by the 3rd week post challenge and 4/4 cats in group 1 and 3/4 cats in group 2 had developed antibodies to the TM peptide 3 weeks after challenge. However, antibodies to p24 and the TM peptide were not detected until 6 weeks post challenge in groups 3 and 4, except for cat A731 which had a very low anti-TM peptide titre at 3 weeks post challenge.

5.3.9. Virus neutralizing antibody activity

The virus neutralising activity of plasma collected on the d.o.c and at 6 and 13 weeks post challenge was assessed in Mya-1 cells. None of the d.o.c samples had detectable VNA, consistent with previous studies (Cuisinier et al. 1997; Hosie et al. 1998). At 6 weeks after challenge, cats A721 and A722 from group 1 (FIV DNA only) had the highest VNA of their group, as shown in Figures 5.8 and 5.9, which correlates with their high viral loads at 6 weeks post-challenge. In comparison, cats A723 and A724 from the same group had very low VNA and also very low viral loads at 6 weeks post-challenge. These results are consistent with the induction of increased VNA in response to increased virus replication. A similar pattern is evident in group 2 (FIV and CD40L DNA). However, at 6 weeks post-challenge in groups 3 and 4, the results for individual cats do not correlate with viral loads.

At 13 weeks after challenge, the VNA had increased in all plasma samples tested (Figure 5.9). The degree of neutralising activity did not correlate with viral loads in any of the groups and the viral loads in all groups were lower at 13 weeks than at 6 weeks post-challenge. Therefore, it is tempting to speculate that the increased virus neutralising activity could be controlling viral replication at this time point.

5.3.10. CD4:CD8 ratio inversion was more marked in the control groups

Flow cytometric analysis of lymphocyte subpopulations was performed on the d.o.c to acquire baseline data and at 3, 6, 10, 13 and 15 weeks post-challenge to monitor the progression of infection. Common findings in FIV infection are an increase in the $CD8\beta^{low}$ lymphocyte subpopulation (Willett et al. 1993; Shimojima et al. 1998; Orandle et al. 2000) and a decrease in the $CD4^{+}$ lymphocyte subpopulation (Willett et al. 1991; Torten et al. 1991; Hoffmann-Fezer et al. 1992), which ultimately results in an inversion of the CD4:CD8 ratio (Torten et al. 1991; Lehmann et al. 1992). Although this trend was evident in all of the groups following challenge, the increase in the $CD8\beta^{low}$ subpopulation was more marked in group 3 (CD40L DNA only), as shown in Figure 5.10. This result is consistent with this group having an enhanced infection. Also groups 3 and 4 (PBS control) had the greatest inversion of the CD4:CD8 ratio, in that the mean for group 3 decreased from 2.6 to 1.2 (a difference of 1.4) and the mean for group 4 decreased from 2.9 to 1.3 (a difference of 1.6) see Figure 5.11. In contrast the means for groups 1 and 2

decreased by 0.6 and 1.1 respectively. Cat A731 in group 3 had the greatest increase in the $CD8\beta^{low}$ subpopulation from 3 to 6 weeks post-challenge, which correlated with this cat having the highest viral load of its group at 3 weeks post-challenge. In group 4, cat A734 had the most marked increase in $CD8\beta^{low}$ subpopulation; while it did not have the highest viral load of this group, it had the highest proviral load.

5.3.11. Neutropenia was detected post challenge

Routine haematology was carried out on blood samples from all cats at 6 and 3 weeks pre-challenge, on the day of challenge and 3, 6, 10, 13 and 15 weeks post-challenge. As shown in Appendix A., all parameters were within normal limits in all cats before challenge. The only parameter in which abnormalities were detected following challenge was the neutrophil count, as illustrated in Figure 5.12. All cats suffered from a mild neutropenia, which was most evident at 6 and 10 weeks post-challenge. However, in some cats although lower than before challenge, the neutrophil count did not decrease to outwith the normal range ($2.5\text{-}12.5 \times 10^9/l$). There was no correlation between neutropenia in individual cats and the type of vaccine administered. By 15 weeks after challenge, all neutrophil counts were again within the normal range. In the primary phase of FIV infection, a transient neutropenia is a common finding (Shelton, Linenberger, and Abkowitz 1991; Hofmann-Lehmann et al. 1995) and normal neutrophil functions are depressed (Yamamoto et al. 1988; Hanlon et al. 1993).

5.4. Discussion

This study demonstrated that CD40L has the potential to act as a useful adjuvant in a DNA vaccine, since cats co-inoculated with CD40L and FIV DNA developed lower viral and proviral loads post-challenge, compared to cats inoculated with FIV DNA alone. However, the difference in viral and proviral loads between these two groups was not significant. Statistical analysis between groups with such small numbers can prove problematic, as there are a limited number of tests that can be performed. Immunisation with CD40L DNA alone appeared to have a detrimental effect on FIV infection, as cats that were inoculated with CD40L DNA developed higher viral and proviral loads post-challenge, compared to control cats.

Evaluation of the FIV DNA vaccine prior to inoculation demonstrated that the major structural proteins (including Env) were expressed well in vitro. However, the level of

expression was unknown in vivo. Similarly, the biological activity of CD40L was demonstrated in vitro, but the activity in vivo was not known. Furthermore, because the study lasted for 24 weeks and was designed to enable the efficacy of the DNA vaccine to be established by challenging with virus, we were unable to determine the exact location of the DNA directly after inoculation. Therefore, there was no way of knowing whether the inoculated DNA had gone directly into muscle or between muscle bellies. This would presumably have an impact on the results of the study and possibly explain inter-group differences. Other factors that influence the distribution of DNA post vaccination include the volume in which the DNA is inoculated, pre-injection of muscles with substances such as sucrose and bupivicaine, and also the promoter used (Davis, Whalen, and Demeneix 1993; Fomsgaard et al. 1998). Pre-injecting the muscles with sucrose and bupivicaine results in damage of the muscle cells, causing immune cells to migrate to the area of damage and therefore enhancing the immune response to the DNA vaccine (Matzinger 2002). In addition, whether the DNA constructs remained in the muscle after inoculation or whether there was migration to the local lymph nodes was unknown. The iliac lymph nodes that would have drained the site of inoculation were collected at necropsy for future investigation by PCR to detect DNA constructs. Previous studies have demonstrated that plasmid DNA can remain in muscle for many months after inoculation (Wolff et al. 1992) but can also migrate to the local lymph nodes very rapidly (Winegar et al. 1996; Dupuis et al. 2000).

After challenging the vaccinated cats, there was evidence of enhancement of infection in the group that received CD40L DNA alone (group 3). This group had higher viral and proviral loads than group 4 (PBS only), the control group. In addition, some cats in the group which received FIV DNA only (group 1), also developed higher viral and proviral loads than the control group, demonstrating that the FIV DNA alone vaccine may have also induced a degree of enhancement of infection. However, the viral and proviral loads in groups 1 and 3 were not statistically significantly higher than the control groups. These results are consistent with those of a previous vaccine trial, in which cats inoculated with FIV Env DNA developed high viral loads much faster than the control cats post challenge (Richardson et al. 1997). Enhancement of FIV infection has been encountered in other vaccine trials, with immunogens including FIV envelope DNA, FIV p24 ISCOMS, FIV IC and envelope subunit vaccines (Hosie et al. 1992; Siebelink et al. 1995b; Richardson et al. 1997; Karlas et al. 1999; Giannecchini et al. 2002). It has been proposed that lymphoid

cell activation following immunisation with virus or non-virus specific antigens may increase the number of target cells available for infection by the challenge virus (Richardson et al. 2002). Further analysis was conducted to characterise potential modes of enhancement in this vaccine study, as described in Chapter 6.

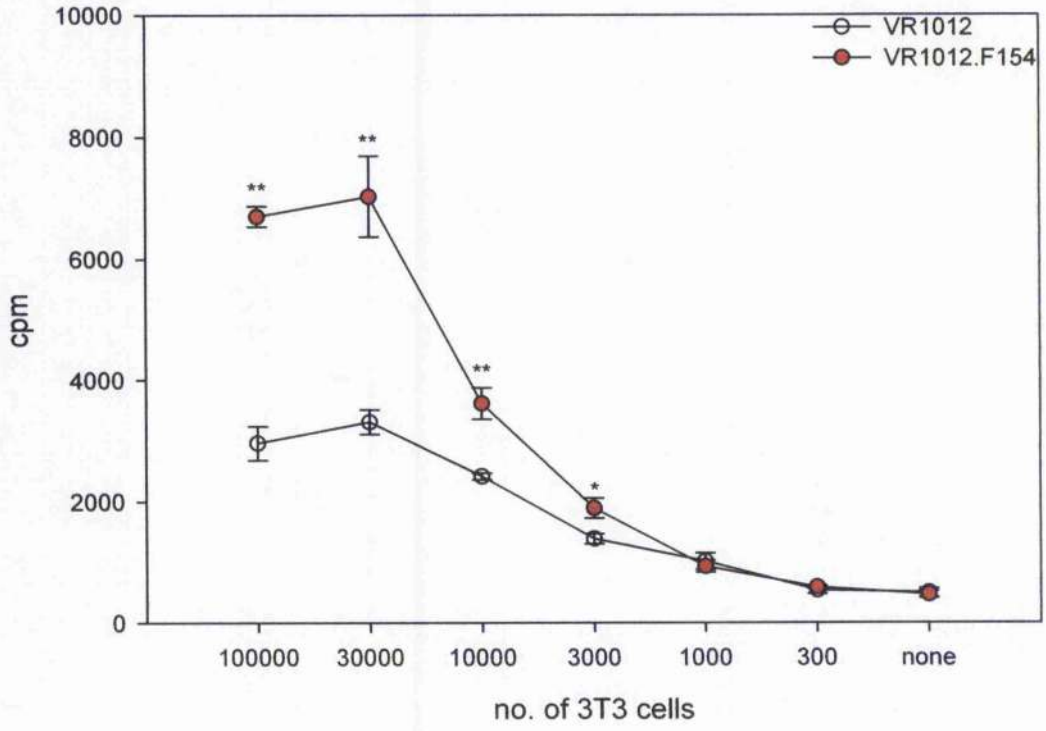
The mechanism by which CD40L modifies the immune response to DNA vaccination is of great interest. CD40L has been shown in previous studies to enhance the cellular and humoral immune response to gene immunization (Mendoza, Cantwell, and Kipps 1997). Low antibody titres were recorded in two of the cats against the TM peptide before challenge, however cats in groups 1 and 2 developed strong serological responses after challenge, these responses were consistent with the increased levels of viral replication evident from the high viral and proviral loads that developed post challenge. At present we are not able to discern what proportion of these serological responses are attributable to either antigen stimulation as a result of viral replication or memory cells primed by DNA immunisation against FIV before challenge. Previous work has shown that it is not unusual for a DNA vaccine not to induce anti-viral antibodies prior to challenge (Richardson et al. 1997; Hosie et al. 1998; Dunham et al 2002) and that DNA vaccines can still induce protection in the absence of detectable anti-viral antibodies. The methods used in this study to test for anti-viral antibodies have proven to be highly sensitive in previous studies (Hosie and Jarrett 1990)

As CD40L is also involved in the development of cell-mediated immunity, it was important to study the cell-mediated immune responses developed in this trial. Preliminary findings indicate that the cats in group 2 (immunised with FIV and CD40L DNA) developed strong cell-mediated immunity to WIV and p24 following vaccination, since increased proliferative responses were noted against WIV and p24. As previous studies have indicated that protection may be associated with cell-mediated immunity, (Hosie and Flynn1996; Flynn et al. 1996), it is tempting to speculate that the cell-mediated response in the cats in group 2 could be associated with the lower viral and proviral loads that developed post challenge. Further studies into the cell-mediated responses induced by FIV and CD40L vaccination are warranted and may involve demonstrating CTL activity with Chromium release assays and measuring IFN γ production by ELISPOT.

In conclusion, the results of this vaccine trial demonstrate that the use of CD40L as an effective adjuvant in a vaccine against FIV requires further optimisation. This study also

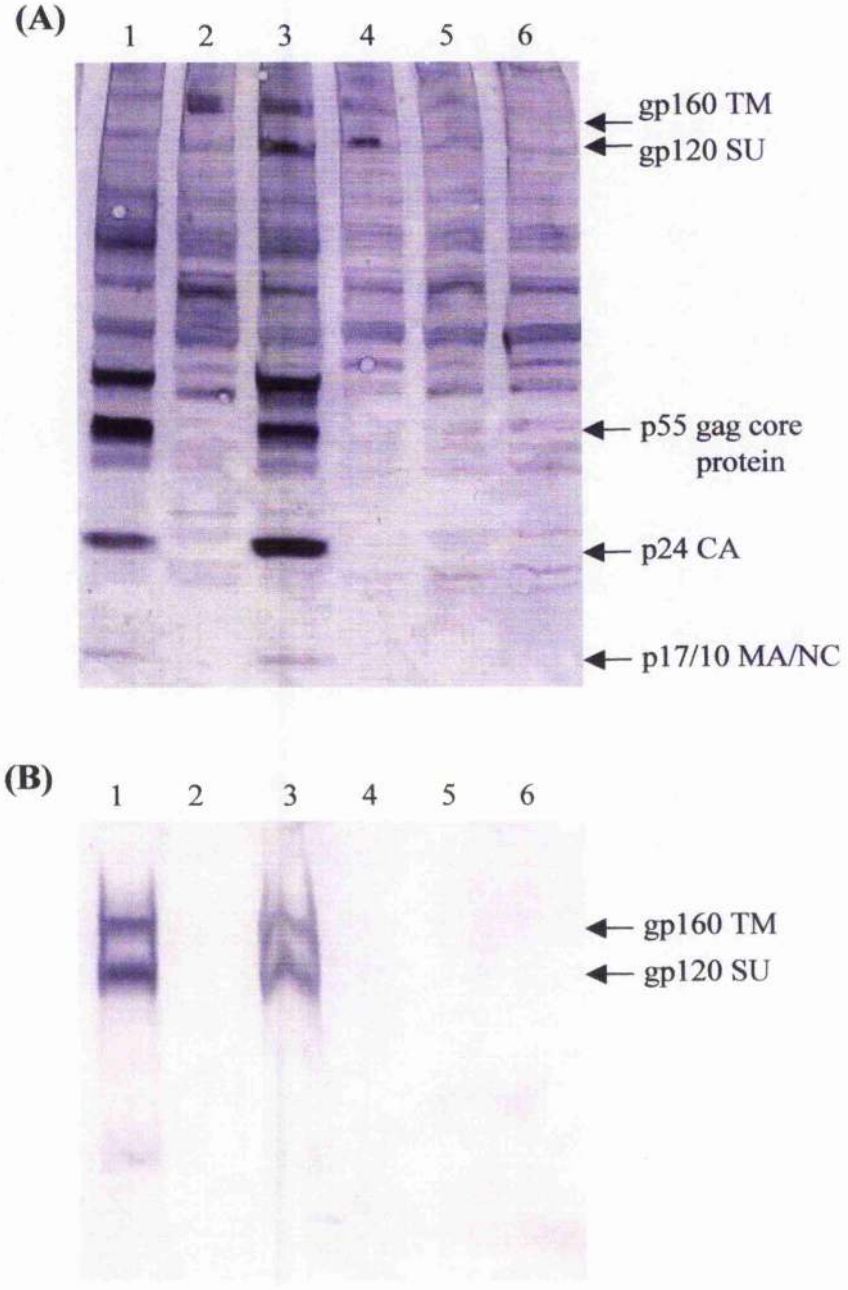
demonstrates the need for further analysis to define protective and enhancing immune responses elicited by immunisation, so that more effective FIV vaccines may be developed. Furthermore, it may be possible to employ the lessons learnt from the FIV system to contribute to the development of effective vaccine strategies against other lentiviruses including HIV.

Figure 5.1. Biological activity of feline CD40 ligand DNA construct (VR1012.F154)



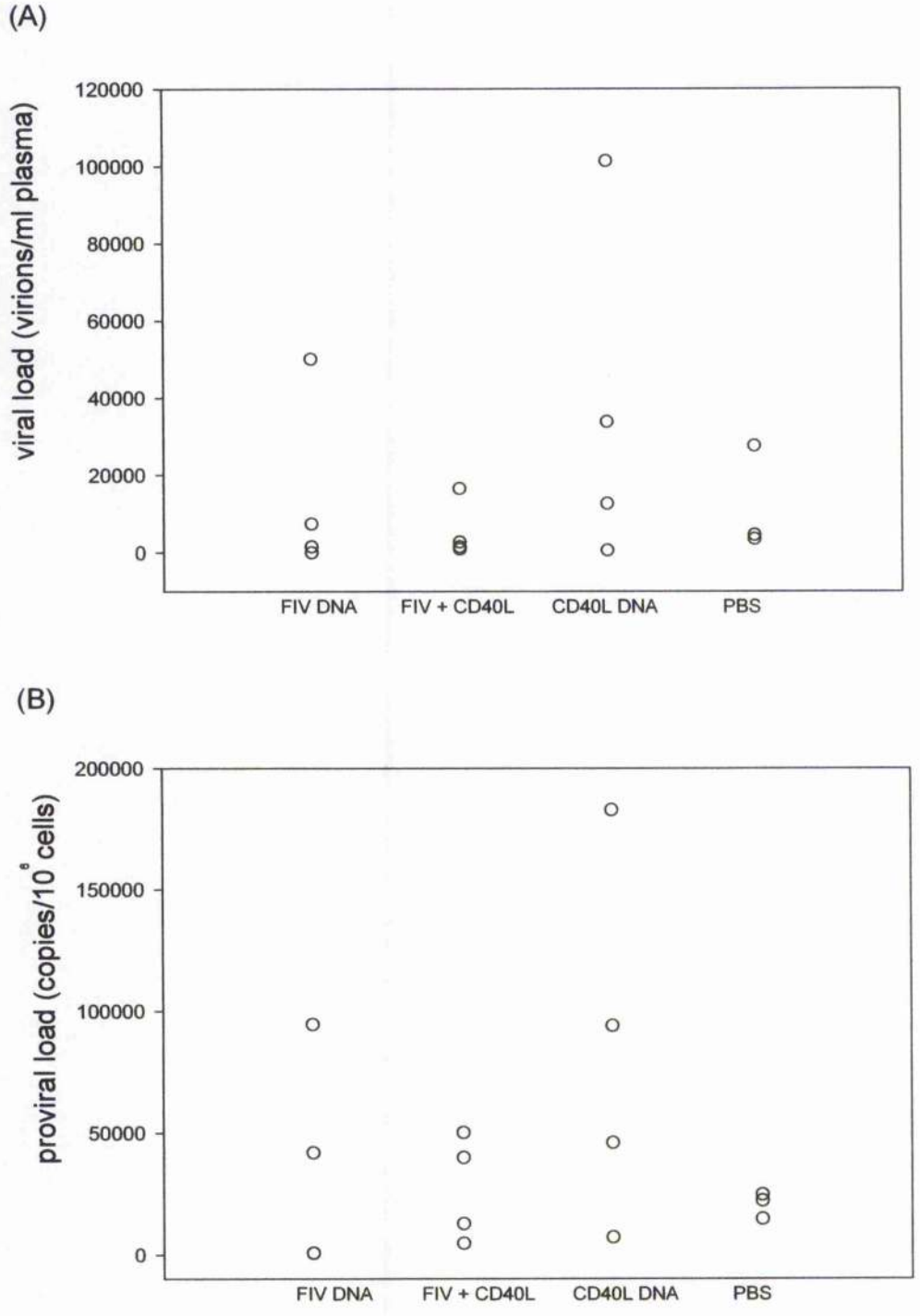
Proliferative response of feline PBMC induced by 3T3 cells expressing feline CD40 ligand (VR1012.F154) (•), compared to mock transfected 3T3 cells (VR1012) (○). ** The difference between the two groups is highly significant P=0.0003, P=0.005 and P=0.01, and * significant P=0.05 respectively (Student's t-test).

Figure 5.2. Comparison of FIV protein expression of FIV DNA constructs *in vitro*



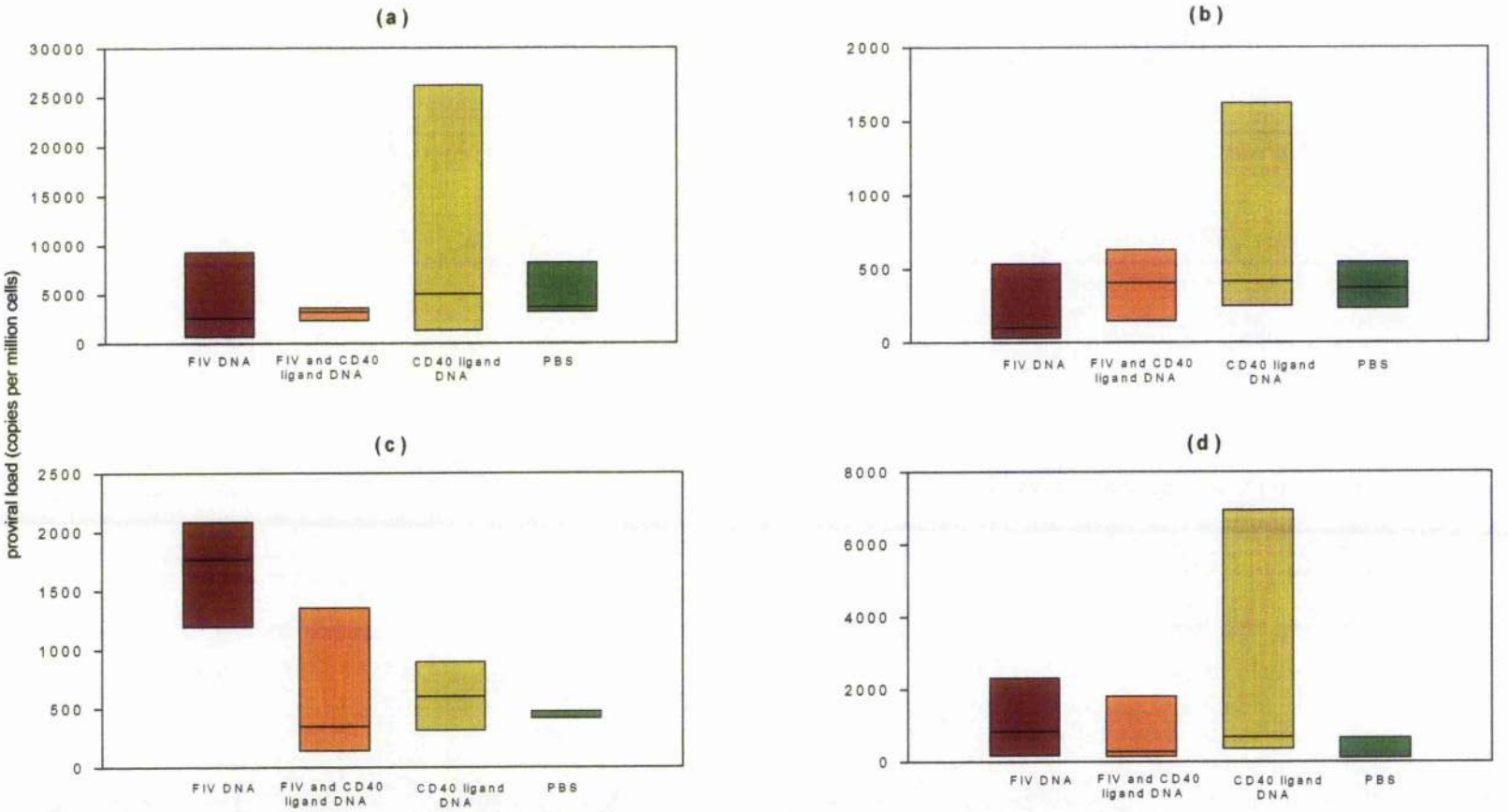
(A) Western blots were stained with pooled FIV+ve serum and (B) monoclonal anti-Env antibody, vpg 71.2. 1, CT5bG8MΔpol; 2, GL8ΔIN; 3, CF1G8M; 4, G8M control; 5, GL8ΔRT and 6, GL8YI. See Table 5.1 for a full description of the FIV DNA constructs.

Figure 5.3. Peak viral and proviral loads post challenge



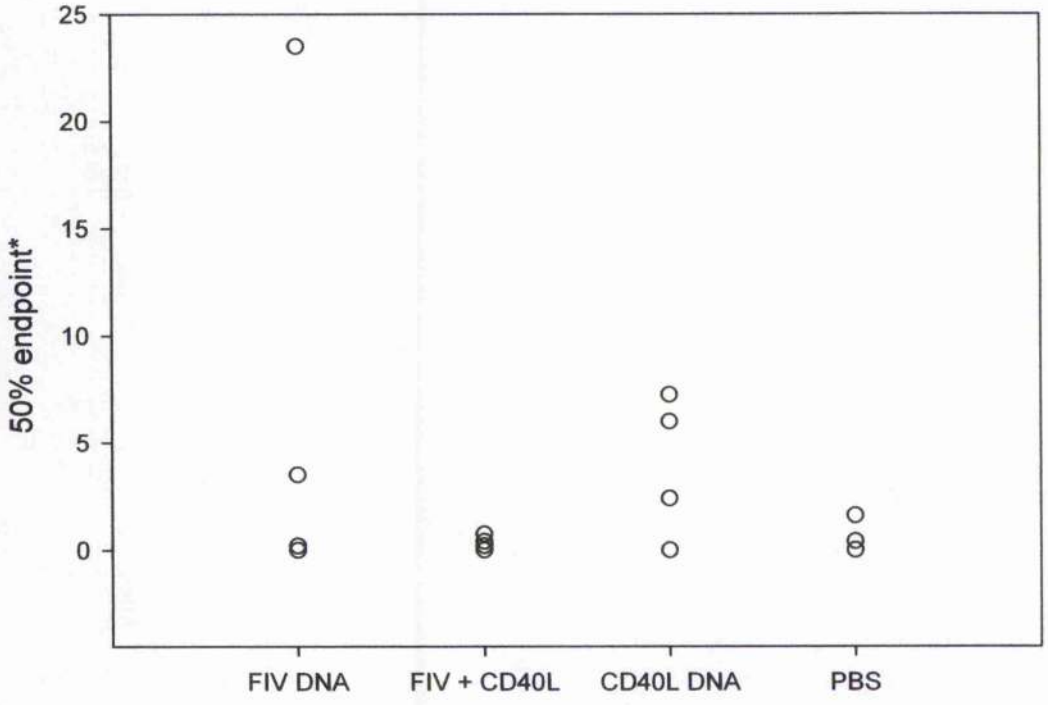
(A) Peak viral load of individual cats in each of the vaccination groups. Viral loads expressed as virions/ml of plasma. (B) Peak proviral load of individual cats in each of the vaccination groups. Proviral loads are expressed as copies/10^6 cells. See Table 5.2 for the time points of peak loads of individual cats.

Figure 5.4. Tissue proviral loads at week 15 post challenge



Group median and 10th and 90th percentiles of tissue proviral loads expressed as copies/10^6 cells, in (a) thymocytes, (b) mesenteric lymph node cells, (c) peripheral lymph node cells and (d) splenocytes.

Figure 5.5. Mesenteric lymph node cell quantitative virus isolations



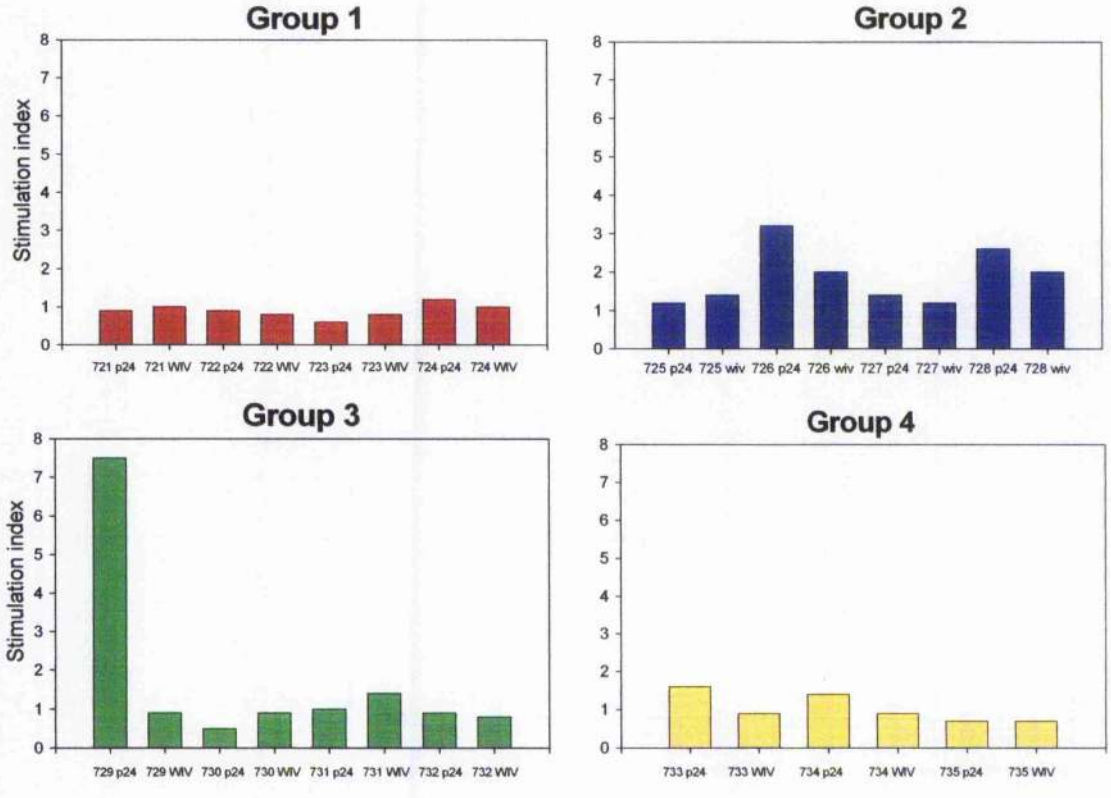
Quantitative virus isolations were performed using cells from MLN collected at the end of study (15 weeks after challenge). * The dilution of MLN cells at which 50% of the MLN and Mya-1 cell wells were infected with FIV was calculated using the method by Reed and Muench (1938) (50% endpoint).

Figure 5.6. Stimulation indices

(a)

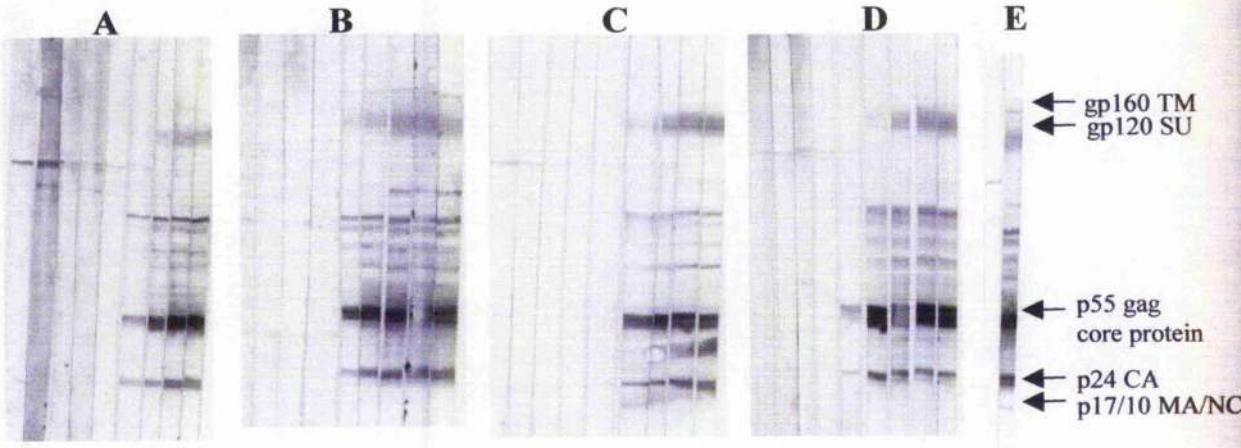
Group	Lymphoproliferative response					
	Post 1st vacn		Post 2nd vacn		Post 3rd vacn	
	p24	WIV	p24	WIV	p24	WIV
FIV DNA	2/4	0/4	0/4	0/4	1/4	0/4
FIV + CD40L DNA	0/4	0/4	2/4	2/4	0/4	0/4
CD40L DNA	1/4	0/4	1/4	0/4	0/4	0/4
PBS	0/3	0/3	0/3	0/3	0/3	0/3

(b)



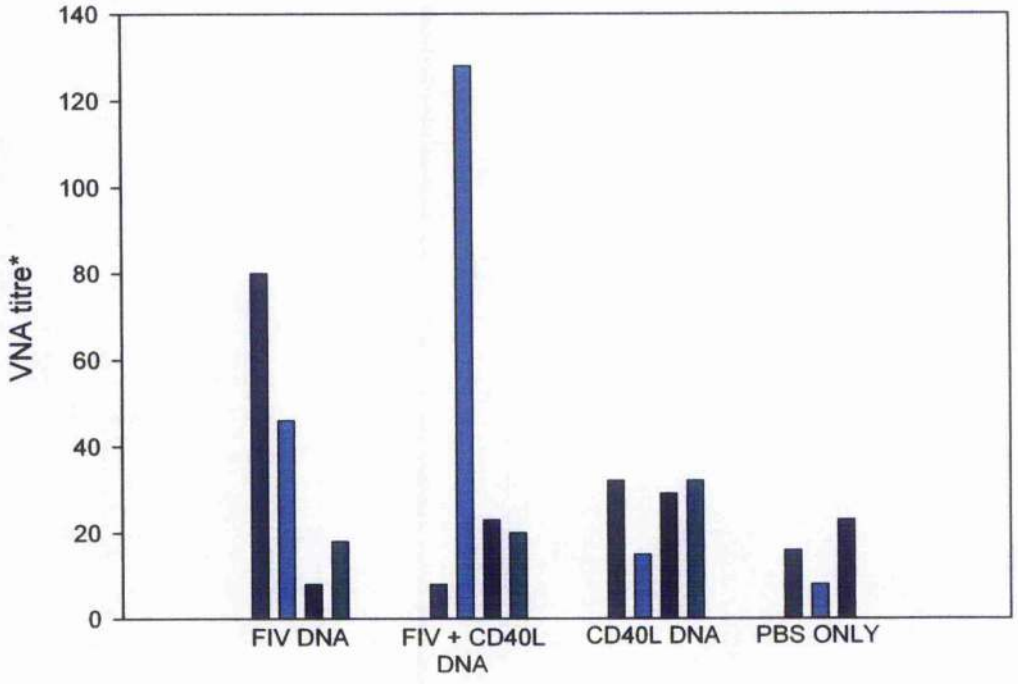
(a) Lymphoproliferative responses of post 1st, 2nd, and 3rd vaccination PBMC after incubation with 25μg/ml of FIV p24 or WIV. The stimulation index (SI) = experimental count/spontaneous count. A SI of ≥ 2 was regarded as significant. Results are presented as the number of animals with a SI of ≥ 2/the total number of animals in each group. (b) Stimulation indices for all cats after the 2nd vaccination.

Figure 5.7. Group 2 (FIV and CD40L DNA vaccine) Western blots



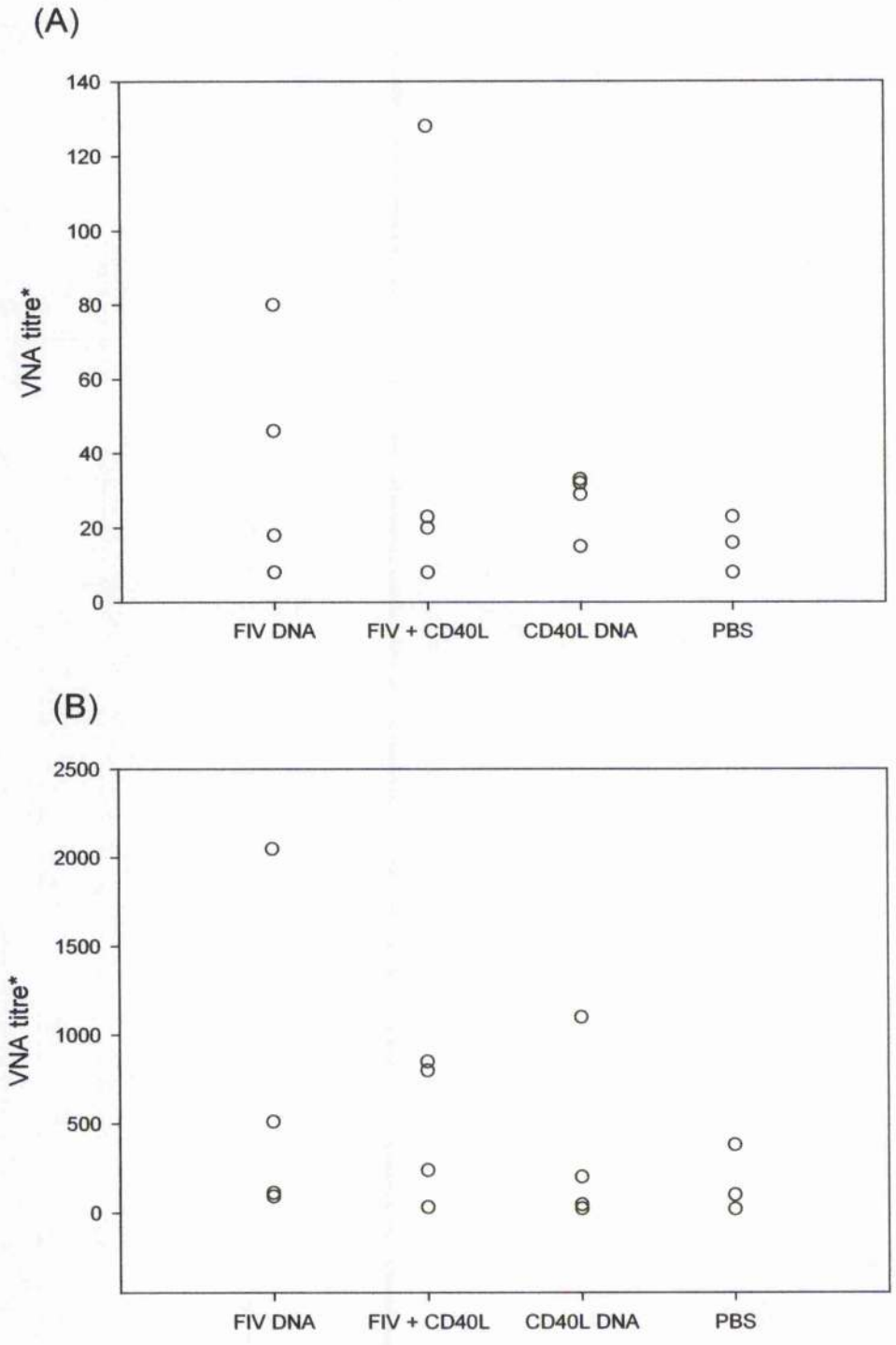
Plasma samples from –9w, -6w, -3w, 0w, 3w, 6w, 10w, 13w and 15w post challenge were tested for anti-FIV antibodies by Western blotting. a) cat number 725, b) cat number 726, c) cat number 727, d) cat number 728, e) -ve and +ve controls respectively.

Figure 5.8. VNA activity at 6 weeks post challenge



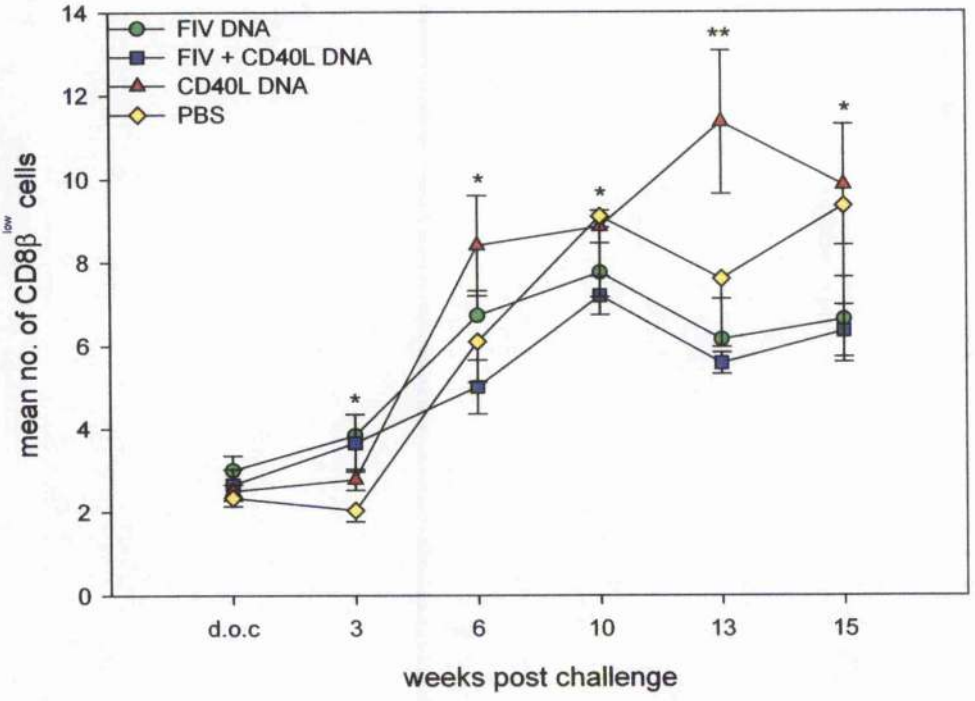
VNA of each cat in the vaccinated groups at 6 weeks post challenge. * The dilution of plasma at which 50% of the virus was neutralised, calculated using the method by Reed and Muench (1938).

Figure 5.9. Virus neutralising antibody titres



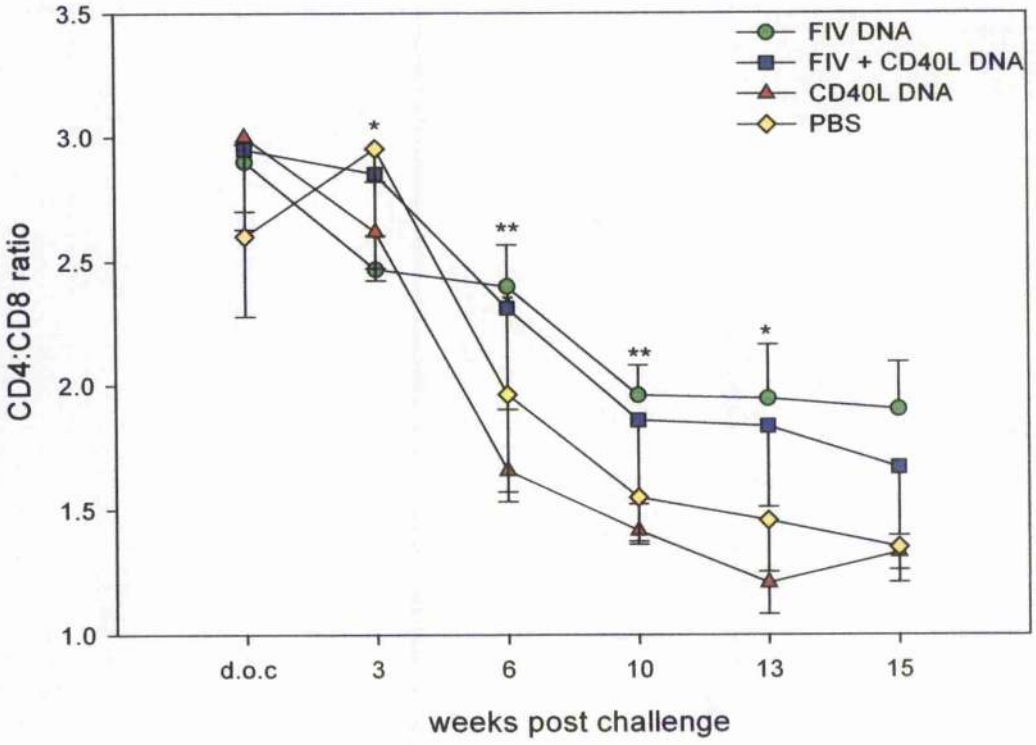
(A) VNA of each cat in the vaccinated groups at 6 weeks post challenge. (B) VNA of each cat in the vaccinated groups at 13 weeks post challenge. * The dilution of plasma at which 50% of the virus was neutralised, calculated using the method by Reed and Muench (1938).

Figure 5.10. CD8β^{low} group means



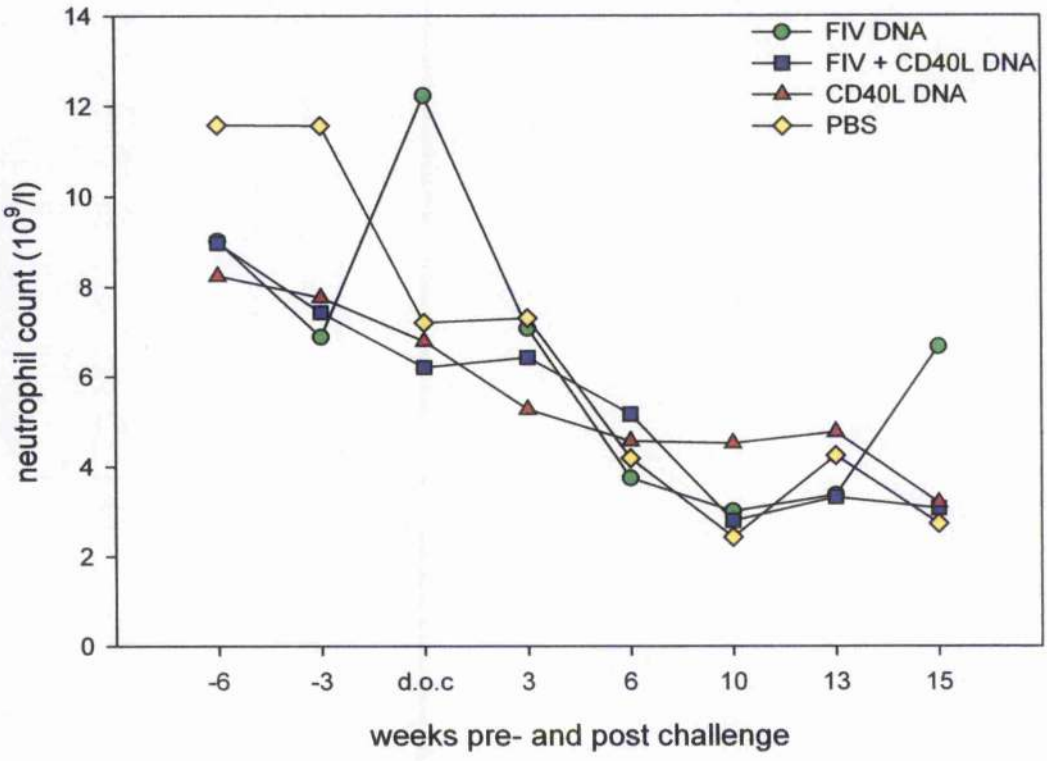
CD8β^{low} percentages obtained from flow cytometric analysis of stained lymphocyte populations. Values expressed as mean ± SE. * and ** Student t-test results are shown in Table 5.5.

Figure 5.11. CD4:CD8 ratio group means



$CD4^+$ and $CD8^+$ percentages obtained from flow cytometric analysis of stained lymphocyte populations. Values expressed as mean ± SE. * and ** see Table 5.5 for Student t-test values.

Figure 5.12. Neutrophil count group means



Mean neutrophil count for each group from 6 weeks prior to challenge to 15 weeks after challenge. Neutrophil counts expressed as neutrophils x 10^9/l, normal range (2.5-12.5x10^9/l).

Table 5.1. FIV DNA constructs investigated for use as a DNA vaccine

	DNA construct	Description
1	GL8ΔRT	GL8 molecular clone with a 99 bp deletion in reverse transcriptase (RT) (Hosie et al. 1998)
2	GL8ΔIN	GL8 molecular clone with an in-frame deletion in the integrase gene (IN) (Dunham et al. 2002)
3	GL8YI	GL8 molecular clone with a deletion in the endocytosis signal (Hosie et al. 2004)
4	CT5bG8MΔpol	PET/GL8 chimera with a deletion in the pol gene (Poeschla, Wong-Staal, and Looney 1998)
5	CF1G8M	PET/GL8 chimera with deletions in the LTRs (Poeschla, Wong-Staal, and Looney 1998)

DNA constructs 1 and 2 were kindly supplied by Dr. S. P. Dunham, University of Glasgow. Construct 3 was kindly supplied by Dr. M. J. Hosie also University of Glasgow and constructs 4 and 5 were kindly supplied by Dr. B. J. Willett, again University of Glasgow.

Table 5.2. Viral and proviral loads

Cat number	Vaccine	Viral load Time post-challenge (weeks)						Proviral load Time post-challenge (weeks)					
		0	3	6	10	13	15	0	3	6	10	13	15
A721	CT5b	0	2822	**7356**	3603	68	362	0	2718	**41913**	19284	4534	2495
A722	CT5b	0	6596	**50082**	23091	0	1343	0	30609	**94487**	11077	400	1802
A723	CT5b	0	0	0	529	0	**1551**	0	0	512	**794**	470	87
A724	CT5b	0	0	0	0	0	0	0	**666**	0	0	0	8
A725	CT5b + CD40L	0	0	453	**959**	347	0	0	**12935**	3068	861	38	80
A726	CT5b + CD40L	0	3390	**16456**	4728	929	0	0	20604	**50199**	26556	54	5174
A727	CT5b + CD40L	0	**2701**	0	2281	0	356	0	10600	**39883**	8217	783	580
A728	CT5b + CD40L	0	0	0	**1144**	0	0	0	3658	4041	**4807**	68	106
A729	CD40L	0	**33821**	5941	31915	819	1228	0	38165	**182914**	84503	11728	12191
A730	CD40L	0	**12612**	5016	905	0	0	0	7299	**93937**	7532	70	78
A731	CD40L	0	**101369**	24468	2787	305	0	0	5219	**45970**	5101	196	44
A732	CD40L	0	**603**	214	182	0	0	0	1099	**7293**	4683	392	18
A733	PBS	0	**27525**	2007	1516	0	1552	0	1591	**22223**	4620	798	440
A734	PBS	0	**3847**	259	230	0	528	0	5112	**24683**	4287	856	349
A735	PBS	0	**4256**	1170	0	0	0	0	4311	**14831**	1456	19	104

Plasma samples and PBMC cell pellets were collected on d.o.c and 3, 6, 10, 13 and 15 weeks post challenge for analysis of viral and proviral loads respectively. Viral loads are expressed as virions/ml of plasma and proviral loads are expressed as proviral copies/10^6 cells. Peak viral and proviral loads for each cat are indicated in bold.

Table 5.3. p24 and TM antibody titres

Cat number	Vaccine	P24 antibody titre Time post-challenge (weeks)						TM antibody titre Time post-challenge (weeks)					
		0	3	6	10	13	15	0	3	6	10	13	15
A721	CT5b	0	40	640	**10240**	10240	10240	0	2560	40960	40960	**163840**	10240
A722	CT5b	0	40	**160**	160	160	160	0	2560	10240	10240	**40960**	10240
A723	CT5b	0	0	40	**640**	640	640	0	40	10240	**40960**	40960	10240
A724	CT5b	0	0	160	160	160	**640**	0	160	**10240**	2560	2560	604
A725	CT5b + CD40L	0	0	10	**40**	40	40	0	0	**640**	640	640	640
A726	CT5b + CD40L	0	**160**	160	160	40	160	10*	**640**	640	160	160	160
A727	CT5b + CD40L	0	0	**160**	40	40	40	0	160	2560	2560	**10240**	10240
A728	CT5b + CD40L	0	10	**640**	160	160	160	0	640	**2560**	640	640	640
A729	CD40L	0	0	**40**	40	40	40	0	0	10240	10240	**163840**	40960
A730	CD40L	0	0	40	**160**	160	160	0	0	**10240**	2560	10240	10240
A731	CD40L	0	0	40	**160**	160	160	0	10	10240	10240	10240	**40960**
A732	CD40L	0	0	40	**160**	160	160	10*	0	10240	10240	40960	**163840**
A733	PBS	0	0	10	**40**	10	10	0	0	**10240**	10240	10240	10240
A734	PBS	0	0	40	40	**160**	160	0	0	**10240**	2560	2560	2560
A735	PBS	0	0	10	**40**	40	40	0	0	2560	2560	10240	**10240**

Plasma samples were collected on d.o.c and 3, 6, 10, 13 and 15 weeks post challenge for analysis of p24 and TM antibody titres by ELISA. Peak values are indicated in bold. * This result was deemed non-specific as it was not confirmed by Western blotting and also cat A732 has a negative titre 3 weeks later.

Table 5.4. Western blot analysis of plasma samples, from 9 weeks pre-challenge to 15 weeks post challenge

(A)

Vaccine Administered	Weeks pre and post challenge (week 0)								
	-9	-6	-3	0	3	6	10	13	15
FIV DNA	0/4	0/4	0/4	0/4	3/4	4/4	4/4	4/4	4/4
FIV + CD40L DNA	0/4	0/4	0/4	0/4	2/4	4/4	4/4	4/4	4/4
CD40L DNA	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4	4/4
PBS	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4	4/4

(B)

Week 3 post challenge	Antibodies induced to :-			
	Gp160/120	P55	P24	P17/10
FIV DNA				
A721	-	+	++	+
A722	+	-	++	+
A723	-	-	-	-
A724	-	-	+	-
FIV + CD40L DNA				
A725	-	-	-	-
A726	+	+	++	+
A727	-	-	-	-
A728	-	-	+	+

(A) The number of cats that had an antibody response, over the number of cats in each group at certain time points as demonstrated by Western blotting. (B) of the cats that did mount an antibody response, the response to each FIV protein differed. At 3 weeks post challenge, not all cats in each group had an antibody response. + indicates a band was observed on the Western blots, ++ denotes a stronger band and therefore, a stronger response.

Table 5.5. Student t-test values from comparisons of CD8β^{low} group means and CD4:CD8 ratio group means at 3, 6, 10, 13 and 15w p.c.

	CD8β^{low}					CD4:CD8 ratio				
t-test	3w	6w	10w	13w	15w	3w	6w	10w	13w	15w
Group1 against2	0.8	0.09	0.6	0.5	0.8	0.4	0.8	0.7	0.7	0.5
Group1 against3	0.1	0.2	0.3	0.03*	0.1	0.4	0.01**	0.005**	0.02*	0.1
Group1 against4	0.03*	0.5	0.5	0.4	0.3	0.05*	0.3	0.1	0.1	0.1
Group2 against3	0.2	0.04*	0.03*	0.01**	0.05*	0.6	0.1	0.2	0.1	0.4
Group2 against4	0.1	0.3	0.3	0.2	0.2	0.8	0.5	0.5	0.4	0.4
Group3 against4	0.1	0.2	0.8	0.1	0.9	0.1	0.4	0.4	0.3	0.9

Interestingly in the CD8β^{low} comparisons most of the significant results are between group 2 (FIV and CD40L DNA) and 3 (CD40L DNA alone) and not group 2 and 4, (PBS only), this demonstrates the positive effect of CD40L as an adjuvant to a FIV DNA vaccine and the negative effect of CD40L injected alone. However, in the CD4:CD8 ratio comparisons a greater response to infection was seen with the FIV DNA alone vaccine as more significant results were gained between group 1 (FIV DNA alone) and group 3. *t-test value significant ($P \leq 0.05$) and ** highly significant ($P \leq 0.01$).

Chapter Six

INVESTIGATION OF ENHANCEMENT OF FIV INFECTION AFTER VACCINATION

6.1. Introduction

In the vaccine trial conducted in cats to assess the efficacy of a CD40L adjuvanted FIV DNA vaccine (Chapter 5), a degree of enhancement of FIV infection was observed in animals that had received feline CD40L DNA or FIV DNA alone. As 2/4 cats in the CD40L only group had viral loads higher than the cats in the PBS control group and 1/4 of the cats in the FIV DNA only group had viral loads higher than the PBS controls. Furthermore, 3/4 cats in the CD40L DNA only group and 2/4 cats in the FIV DNA only group had proviral loads higher than the PBS control group. However, 2/4 cats in the FIV and CD40L DNA group also had proviral loads higher than the PBS controls. Enhancement of FIV infection after challenge has been observed in many FIV vaccine trials conducted previously, including DNA vaccines, recombinant virus vaccines, IC vaccines and subunit vaccines (Hosie et al. 1992; Osterhaus et al. 1996; Richardson et al. 1997; Flynn et al. 1997; Karlas et al. 1999).

Enhancement of infection has also been seen in other diseases caused by retroviruses including the oncornavirus, feline leukaemia virus (Pedersen et al. 1986) and other lentiviruses including HIV and SIV infection (reviewed in Robinson and Mitchell 1990). The enhancement of HIV infection has given cause for concern and led to caution in approaches to the development of a vaccine against HIV. Other diseases where enhancement has been observed are feline infectious peritonitis caused by a coronavirus (Hohdatsu et al. 1991), yellow fever, dengue and West Nile virus, all flaviviruses (Fagbami et al. 1988; Halstead 1988).

One mechanism of enhancement is known as antibody-dependent enhancement (ADE) of viral infection. ADE of viral infection is of concern because of its suspected role in producing enhanced viraemias, shorter incubation times from exposure to infection and development of disease, and vaccination failure. Two distinct types of infection-enhancing antibodies have been identified. One type utilises Fc receptors found on monocytes and macrophages, while a second type requires complement and complement receptors

(reviewed in Montefiori 1993). McKeating et al. (1990) demonstrated that Fc receptors on fibroblasts could bind and internalise HIV-1 in the presence of sub-neutralising amounts of antibody to HIV. However in the experiments of Yamamoto et al. (1991b), the presence of VNA, correlated with protective immunity rather than with enhancement of infection. Furthermore, this immunity could be transferred to naïve animals by passive transfer of plasma from protected, immunised animals (Hohdatsu et al. 1993). Therefore, there is clearly a balance to be reached between enhancing and protecting immune responses.

Another postulated mechanism of enhancement involves lymphoid activation and has been demonstrated in HIV (Douek et al. 2002) and FIV infections (Richardson et al. 2002). Douek et al. (2002) demonstrated that HIV-specific $CD4^+$ T-cells are preferentially infected in vivo, as HIV-specific memory $CD4^+$ T-cells in infected individuals contained more HIV viral DNA than other memory $CD4^+$ T-cells at all stages of HIV disease as well as following viral rebound after interruptions in anti-retroviral therapy. Richardson et al. (2002) also demonstrated in FIV infection that rapid dissemination of virus *in vivo* was associated with the presence of Th responses prior to challenge, after vaccination with a FIV Env DNA vaccine. These responses were linked to increased susceptibility of lymphocytes to ex vivo FIV infection.

Another interesting factor that must be considered in the apparent enhancement of FIV infection in cats inoculated with CD40L DNA alone, is the finding that CD40/CD40L interactions are required for the progression of retrovirus-induced murine AIDS (MAIDS) (Green et al. 1996; Green, Noelle, and Green 1998), a disease that causes splenomegaly, lymphadenopathy, hypergammaglobulinaemia and an immunodeficiency syndrome with pathology in susceptible mice similar to that seen in AIDS. Furthermore CD40L has also been implicated in enhanced HIV infection. In a recent study, CD40L-stimulated macrophages were found to be more susceptible to HIV-1 entry (Bergamini et al. 2002), as incubating macrophages with soluble CD40L for 7 days increased the number of CD4 and CCR5 antibody-binding sites and the percentage of CD4 and CCR5-expressing cells. Infection of CD40L-stimulated macrophages with HIV-1 resulted in a marked increase of proviral DNA with respect to controls, as demonstrated by PCR. In addition Fong et al. (2002) demonstrated that productive infection of plasmacytoid dendritic cells with HIV-1 was triggered by CD40 ligation. Also in another study, CD40-CD40L interactions were linked with the increased susceptibility of B-lymphocytes to HIV-1 infection (Moir et al.

1999). Therefore, it is tempting to speculate that inoculation with CD40L DNA alone may have increased the susceptibility to FIV infection.

Enhancement of FIV infection in cats vaccinated with CD40L DNA alone may have resulted from an increased number or susceptibility of target cells or enhancing antibodies. In order to investigate further the protecting and enhancing immune responses elicited by the FIV and CD40L DNA vaccinations, PBMC susceptibility assays were performed. These assays determined whether the lymphocytes of cats in which infections were enhanced after challenge were more susceptible to infection before challenge. The percentage of lymphocytes in PBMC was assessed by flow cytometry and the number of PBMC that contained $2x10^5$ lymphocytes was incubated with FIV-GL8. After incubation, cells were serially diluted and co-cultured with Mya-1 cells to determine the proportion of infected cells. These results were examined for correlations with either neutralising antibody activity or cell-mediated immune responses elicited after vaccination to determine if enhancement could be attributed to either the humoral or cellular immune responses induced by immunisation.

6.2. Materials and methods

6.2.1. Susceptibility of PBMC to FIV challenge

PBMC from all cats in the vaccine trial described in Chapter 5 were isolated on the d.o.c following three inoculations and were stored in liquid nitrogen using a controlled rate freezer, Planer Kryo 10 Series II (PLANER plc). Following recovery from liquid nitrogen, PBMC were counted and 10^6 PBMC were prepared for flow cytometry to assess lymphocyte numbers in order to ensure that equivalent numbers of lymphocytes from each cat were added to the susceptibility assays. PBMC were washed twice in PBA and after centrifugation at 1000rpm for 5 minutes, the resultant cell pellet was resuspended in 1ml of PBA. Samples were analysed on an EPICS Elite flow cytometer (Beckman-Coulter) using the EXPO 32 ADC analysis software package (Beckman-Coulter). 5000 events were collected for each sample and lymphocyte numbers were enumerated based on size and granularity. Subsequently the number of PBMC that contained $2x10^5$ lymphocytes was incubated with 500µl of FIV-GL8 (3000 ID_{50}, approximately 10^4 $TCID_{50}$) in a Falcon 2054 tube for 1 hour at 37°C in an atmosphere of 5% CO_2. After incubation, cells were washed three times in PBS and added in decreasing numbers (10^4, $3x10^3$, 10^3, $3x10^2$, 10^2, 30, 10, 3,

1 and none) to a 96-well tissue culture plate (Falcon). Cells were then co-cultivated in 8 replicate wells, with 10^5 Mya-1 cells/well, to determine the proportion of PBMC that contained infectious virus. Plates were incubated at 37°C in an atmosphere of 5% CO_2 for 7 days (medium was replenished at day 4) at which time culture fluids were tested for FIV p24 antigen by ELISA (IDEXX). Results are expressed as the endpoint dilution at which 50% of the wells were infected and were calculated using the method described by Reed and Muench (1938).

To further investigate the relationship between the susceptibility of lymphocytes to FIV infection and the outcome following vaccination, further susceptibility assays were conducted using pre-vaccination PBMC from the three cats with the highest endpoint dilutions and therefore the most susceptible lymphocytes of all the cats to FIV infection (A721, A726 and A733) and from the three cats with the lowest endpoint dilutions and therefore the least susceptible lymphocytes of all the cats to FIV infection (A724, A728 and A731) as determined by the d.o.c PBMC susceptibility assays. In this way differences in susceptibility between pre-immunisation and post-immunisation PBMC could be assessed directly.

PBMC comprise a heterogenous cell population and therefore, to ascertain whether a more defined cell population was more or less susceptible to FIV infection, the above assay was also performed with d.o.c PBMC that had either been enriched or depleted of $CD4^+$ cells following incubation with a mouse anti-feline CD4 antibody (vpg34, Serotec) and goat anti-mouse IgG antibody-coated magnetic beads (MACS beads, Miltenyi Biotec), as described in section 3.2.2.

6.3. Results

6.3.1. Lymphocytes from cats vaccinated with CD40L DNA alone are less susceptible to FIV infection

The results from this study indicate that enhancement is not associated with increased activation of the cellular immune system, but with some other factor. Thus, the PBMC from the cats in group 3 that received CD40L DNA only and displayed a degree of enhancement of FIV infection after challenge did not demonstrate an increased susceptibility to FIV infection, when day of challenge lymphocytes were incubated with

FIV-GL8 and co-cultured with Mya-1 cells (see Table 6.1). However, the cats in group 3 did demonstrate significant stimulation indices, when PBMC collected after the first or second vaccinations were incubated with p24 and WIV consistent with increased activation of the cell-mediated immune system compared with the other groups. There was some correlation in cats between high viral and proviral loads in groups 1 (FIV DNA only) and group 2 (FIV and CD40L DNA) and lymphocyte susceptibility to FIV infection, in that the lymphocytes from cats that had high viral or proviral loads in these groups (A721, 722 and 726) were more susceptible to FIV infection after vaccination, as shown in Table 6.1. This cannot be explained completely by vaccination having increased the cell-mediated response to FIV, however, as cat A733 in the control group (PBS only) also demonstrated a correlation between viral load and PBMC susceptibility and did not demonstrate an increased response to p24 and WIV. A single cat, cat A726 from group 2, demonstrated increased lymphoid activation, susceptibility to FIV infection and high viral and proviral loads post challenge.

6.3.2. Vaccination has a role in increased susceptibility to FIV infection

The susceptibility assays demonstrated that vaccination against FIV, with a FIV DNA vaccine with or without CD40L as an adjuvant, did indeed affect the susceptibility of lymphocytes to FIV infection. The susceptibility of pre-vaccination lymphocytes from the three cats with the greatest susceptibility to FIV infection (A721, A726 and A733) and the three cats with the least susceptibility to FIV infection (A724, A728 and A731) after three vaccinations (as determined by d.o.c susceptibility assays) was tested, (see Table 6.1). It was found that in cats where there was an increase in susceptibility to FIV infection post-vaccination, this increase in FIV infection was not evident in lymphocytes collected prior to vaccination. Furthermore, in cats where there was no evidence of increased FIV infection of lymphocytes after vaccination, there was also no evidence of a greater susceptibility to FIV infection in lymphocytes collected prior to vaccination.

6.3.3. $CD4^+$ enriched cells are not more susceptible to FIV infection

To assess susceptibility of a defined cell population to FIV and as $CD4^+$ T-cells are targets for FIV infection, susceptibility assays were conducted with CD4-enriched and CD4-

depleted PBMC to determine the effect on susceptibility to FIV infection. As shown in Figure 6.1, there was no correlation observed between the cell population and the extent of FIV infection in the four cats tested. In cats A722 and A726, the CD4-depleted cell populations were more susceptible to FIV infection, whereas in cats A727 and A733, the CD4-enriched cells were more susceptible. Therefore, no correlation was evident between the expression of the CD4 molecule and susceptibility to FIV infection. However, seperation of cells using MACS beads does not result in a pure cell population as demonstrated in Chapter 3. Thus, the cell populations being compared may still have been heterogenous.

6.3.4. Correlation between VNA and enhanced infection?

The enhanced FIV infection observed in cats inoculated with CD40L alone could be explained by sub-neutralising levels of antibody. Thus, as shown in Figure 5.8, the cats in group 3 that received CD40L DNA alone, all had low levels of VNA activity, as determined in the experiment described in chapter 5. However, cat A726, from group 2 (FIV and CD40L DNA) that developed the highest VNA titre of its group, also had the highest peak viral and proviral load of its group (see Table 6.1 and Figure 5.8), suggesting that VNA alone were not responsible for controlling the level of viral replication post challenge. Furthermore, other cats in group 2 (A725, A727 and A728) with some of the lowest viral and proviral loads of the study also had some of the lowest VNA titres.

6.4. Discussion

The results of this study do not reveal a clear mode of enhancement of FIV infection in the DNA vaccine trial conducted with and without CD40L as an adjuvant. Previous studies have demonstrated VNA activity (Siebelink et al. 1995b), CD40-CD40L interactions (Green et al. 1996) and lymphoid activation (Richardson et al. 2002) as modes of enhancement of infection of MAIDS and FIV. In the vaccine trial conducted whereby cats were inoculated with FIV DNA, FIV and CD40L DNA, CD40L DNA, or PBS, a degree of enhancement was observed in cats that were inoculated with FIV DNA alone (group 1) and CD40L DNA alone (group 3). Thus, viral loads in 2/4 and proviral loads in 3/4 cats in group 3 were greater than the PBS controls. However, the level of enhancement was less marked in the FIV DNA alone vaccine group, since viral and proviral loads were greater in only 2/4 cats compared to the PBS controls.

In contrast to the findings reported by Richardson et al. (2002), the enhancement observed in this study could not be accounted for by increased lymphoid activation following vaccination. Cats in the CD40L only DNA group did demonstrate increased cell-mediated immune responses when post vaccination PBMC were incubated with FIV p24 and WIV, however, when day of challenge PBMC were incubated with FIV-GL8 there was no increase in susceptibility to infection. These conflicting results could be accounted for by the fact that in this study, PBMC numbers were adjusted to take into account the differences in percentages of lymphocytes among different cats, whereas in the lymphocyte susceptibility experiments performed by Richardson et al. the same total numbers of PBMC were used. Another factor to take into consideration is the numbers of other cell types that may play a role in susceptibility, such as monocytes. Therefore, although increased activation of the cell-mediated immune system cannot account for this increase in susceptibility to FIV infection, other undefined immunological events following vaccination can.

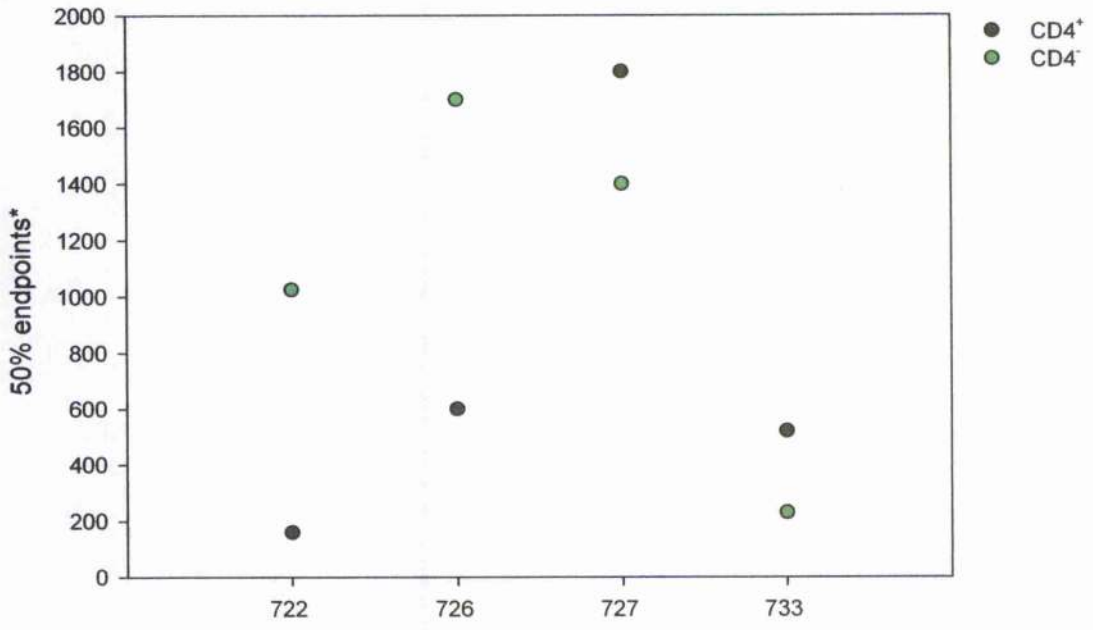
The possibility that VNA titres could account for the enhancement of FIV infection was also investigated. Cats in the CD40L DNA only group did develop low levels of VNA activity at 6 weeks post challenge, however, cat A726 in group 2 (FIV and CD40L DNA), had high VNA levels and high viral and proviral loads. The high VNA levels in this cat measured 6 weeks p.c could be a direct result of increased viral replication after challenge or may reflect that immunisation primed a VNA response. No VNA were detected in any of the groups in day of challenge plasma samples.

At present, no reliable systems are available to quantify or even detect FIV-enhancing antibodies in vitro. Therefore, this mechanism of enhancement cannot be ruled out. In this study VNA assays were performed using Mya-1 cells, further studies will be required to determine whether VNA antibodies were present in day of challenge plasma but were undetectable by this method. Siebelink et al. (1995b) demonstrated VNA activity in CrFK VNA assays and not in the feline lymphocyte assays conducted, however, the lymphocyte assay should be considered more relevant in terms of protective immunity against FIV infection, as feline lymphocytes are the natural targets for FIV infection. In a recent study, Giannecchini et al. (2001) demonstrated a masking of VNA phenomenon. Whilst studying the immune correlates of protection involved in a fixed-infected-cell vaccine, VNA were not thought to play an important role. However, further studies revealed that VNA were

indeed present and that failure to detect VNA initially was due to the presence of vaccine-induced antibodies to cellular antigens and that these antibodies could be removed by adsorption with selected feline cells. This phenomenon may be of relevance with respect to DNA vaccines.

Further studies will also be required to determine whether CD40-CD40L interactions do indeed increase susceptibility of cells to FIV infection as demonstrated previously in HIV-1 infection (Moir et al. 1999; Bergamini et al. 2002; Fong et al. 2002). It is interesting that in this study, the greatest level of enhancement was observed in cats immunised with CD40L DNA alone since previous studies have linked CD40-CD40L interactions to the development of MAIDS (Green et al. 1996; Green, Noelle and Green 1998). Green et al. (1996) demonstrated that a monoclonal antibody to CD40L inhibited the splenomegaly, hypergammaglobulinaemia and immunodeficiency disease state associated with MAIDS. In this study, mice infected with LP-BM5, an isolate of murine retroviruses were treated with anti-CD40L antibody (therefore preventing CD40L from interacting with CD40 on B-cells). This resulted in the symptoms of MAIDS being inhibited. Therefore, it is tempting to speculate that the increased viral and proviral loads in cats vaccinated with CD40L DNA may be due to increased CD40-CD40L interactions enhancing the disease process. Unfortunately until the mechanisms of enhancement of infection are fully understood, this phenomenon is a potentially fatal stumbling block in the continuing search for vaccines against infection with FIV and HIV.

Figure 6.1. CD4 enriched and CD4 depleted cell susceptibility assays



PBMC collected on the day of challenge from the cats in the FIV DNA vaccine trial were separated into CD4-enriched and CD4-depleted cell populations using magnetic antigen coated beads. Cat numbers A722, A726, A727 and A733 were selected as a larger number of PBMC had been collected for these cats. *Results are expressed as the endpoint dilution of PBMC at which 50% of the wells were infected with FIV and were calculated using the method by Reed and Muench (1938).

Table 6.1. Pre-vaccination and day of challenge PBMC susceptibility to FIV infection

	susceptibility		outcome post challenge	
cat number	pre-vacn	d.o.c	peak VL	peak PVL
group 1				
A721	9	160	7356	41913
A722	nd	47.5	50082	94487
A723*	nd	21.5	1551	794
A724*	7.5	6	0	666
group 2				
A725	nd	13.25	959	12935
A726**	8	92	16456	50199
A727	nd	23	2701	39883
A728**	13.5	7.5	1144	4807
group 3				
A729*	nd	8.5	33821	182914
A730*	nd	18	12612	93937
A731	5	5	101369	45970
A732	nd	17	603	7293
group 4				
A733	5	134	27525	22223
A734	nd	27	3847	24683
A735	nd	25.5	4256	14831

Lymphocytes from cats prior to immunisation (pre-vacn) or following 3 immunisations and prior to challenge (d.o.c) were compared for susceptibility to infection with FIV-GL8 *in vitro*. These results are compared with the peak viral load (VL) and proviral loads (PVL) measured during the 15 weeks post challenge. VL are expressed as virions/ml of plasma and PVL are expressed as proviral copies/10^6 cells. nd : not determined. * significant stimulation index (≥2) to either p24 or WIV, ** significant stimulation index (>2) to both p24 and WIV.

Chapter Seven

GENERAL DISCUSSION

7.1. Aims of this study

The main aim of this study was to test the effect of using the feline homologue of the co-stimulatory molecule CD40L as an adjuvant in a DNA vaccine against FIV infection. As a first step towards that goal, the biological activity of feline CD40L was demonstrated. Subsequently the properties of feline CD40L *in vitro* were investigated by monitoring the effect of co-cultivating feline PBMC with cells expressing CD40L and examining the resulting cell phenotypes by flow cytometry. *In vivo* studies with CD40L in both mice and cats were performed and finally the apparent phenomenon of enhancement of FIV infection following immunisation was investigated.

7.2. Feline CD40L is biologically active

When testing the biological efficacy of feline CD40L *in vitro*, 3T3 cells were either stably or transiently transfected with feline CD40L DNA, so that CD40L was then expressed on the cell surface of the 3T3 cells similar to the membrane-bound expression of CD40L on T-cells *in vivo*. The 3T3 cells expressing CD40L were then used as target cells in proliferation assays. As described in Chapter 3, feline PBMC were shown to proliferate in response to feline CD40L. The biological activity of feline CD40L was further demonstrated when the above mentioned target 3T3 cells expressing CD40L were used as a feeder layer. A B-CLL cell line, derived from a blood sample taken from a cat that had been admitted to Glasgow University Veterinary Hospital with a suspected CLL, was maintained using the 3T3 cells expressing feline CD40L, whilst a control feeder layer consisting of mock-transfected 3T3 cells was unable to maintain the neoplastic B-cells.

7.2.1. Further studies

The activity of membrane bound CD40L was investigated, however it would be interesting to test the activity of soluble feline CD40L both *in vitro* and *in vivo*. Hollenbaugh et al. (1992) and Mazzei et al. (1995) demonstrated the biological activity of soluble CD40L and other studies with soluble CD40L have been conducted. Khanna et al. (1997) demonstrated that soluble CD40L-treated Burkitts lymphoma (BL) cells consistently processed endogenously synthesised viral antigens for recognition by virus-specific CTLs.

BL is an Epstein-Barr virus-associated tumour and BL cells are known to escape CTL surveillance by down-regulating transporter for antigen presentation and surface MHC expression. However, this study showed that CD40L treatment of tumour cells might be used in conjunction with other immunotherapeutic protocols to prevent this down-regulation and thereby aid the immune system to fight the disease.

Future work could also be conducted to investigate the maintenance of a normal feline B-cell line using CD40L as a feeder layer and also using soluble CD40L, as in this study the neoplastic cell line only was maintained. Further investigations to determine the cytokines that are required to maintain a normal B-cell line are warranted.

7.3. CD40L could be used as an adjuvant in a DNA vaccine

Once the activity of feline CD40L had been established *in vitro*, studies were conducted *in vivo*. Mice were inoculated with CD40L DNA alone, FIV DNA alone and CD40L and FIV DNA together, to determine whether CD40L enhanced the humoral and cellular immune responses to FIV. In a similar study cats were inoculated with the same groups of DNA, however in an extension to the above protocol, the outcome of an i.p challenge with the homologous strain of FIV was investigated in addition to the humoral and cellular immune responses induced by DNA vaccination.

CD40L DNA inoculation appeared to induce humoral responses in mice as measured by Western blotting, however this was not the case in the feline vaccine trial, where no antibodies were detected following immunisation and prior to challenge. In the murine study the method used to detect a cellular immune response gave non-specific results, whereas the same method gave valid results in the feline study and, as described in Chapter 5, demonstrated that CD40L enhanced the cellular arm of the immune response against FIV. After the cats were challenged i.p with FIV-GL8, the cats were monitored for evidence of FIV infection by virus isolation and viral and proviral loads were measured. In addition, flow cytometry was employed to monitor CD4:CD8 ratios and $CD8\beta^{low}$ cell number counts. These results indicated that CD40L DNA, when inoculated in conjunction with FIV DNA, did act as an adjuvant to the FIV vaccine in some cats, as overall the cats in group 2 (FIV and CD40L DNA) developed lower peak viral and proviral loads than the cats in groups 1 and 3. Furthermore, at 6, 10, 13 and 15 weeks p.c, the mean of the

$CD8\beta^{low}$ cell numbers in cats in group 2 was lower than the mean of the cats in the other vaccine groups.

7.3.1. Further studies

In the mouse study the cellular immune response was measured using proliferation assays, however these proved to be non-specific. The results in the feline study were valid; however, further studies are required to demonstrate cellular immune responses in the mouse against FIV and to increase the data describing cellular immunity in the cat. One such study may be the conduction of CTL assays. The CTL assay has been used in previous FIV vaccine studies to demonstrate cellular immune responses induced in response to FIV vaccination (Flynn et al. 1995a; Flynn et al. 1996; Pu et al. 1999; Leutenegger et al. 2000). In brief, autologous skin fibroblast target cells collected prior to vaccination are labelled with sodium ^{51}chromate and then infected with recombinant vaccinia virus expressing either the *gag* or *env* gene of FIV. Fibroblast target cells are then incubated with effector lymphocytes derived from PBMC from vaccinates prior to challenge. After 4 hours, supernatants are removed and the sodium ^{51}chromate activity is measured. Cytotoxicity can also be measured by a lactate dehydrogenase (LDH) assay, whereby LDH a stable cytosolic enzyme that is released upon cell lysis is measured (Beatty et al. 1996; Lockridge et al. 2000) and by measuring the mitochondrial membrane potential, a reduction in which is an early step during CTL-mediated cell death (Hu and Kipps 1999; Burger, Mendoza, and Kipps2001). Burger, Mendoza, and Kipps (2001) also conducted studies with mice splenocytes to determine cellular immune responses. Splenocytes were separated into $CD8^+$ cells using flow cytometry and stimulated with the antigen (β-gal) used in the inoculations, flow cytometry was again used to assess numbers and therefore, detect any response to β-gal. Other methods to demonstrate cell-mediated immune responses are IL-2 assays, conducted using an IL-2 dependent CTL-L cell-line (Richardson et al. 2002) and IFN-γ measurement, conducted by detecting the level of anti-viral activity against vesicular stomatitis virus on a feline embryo cell-line (McCullough et al. 1986; Pu et al. 1999). IFN-γ production can also be measured using ELISPOT (Sirriyah et al. 2004). Sirriyah et al. (2004) demonstrated that equivalent numbers of $CD4^+$ and $CD8^+$ T-cells produced IFN-γ in naïve cats, however, in FIV infected cats the number of $CD8^+$ T-cells that produced IFN-γ was twice the number of IFN-γ producing $CD4^+$ T-cells.

Some of the results from the FIV vaccine trial were inconsistent. As shown in Chapter 5, a cat with a high antibody titre could still have a high level of FIV infection, as indicated by viral load measurements. Further studies to distinguish between a) an anamnestic immune response generated from a previous vaccination and b) an immune response induced in response to biological infection would enable greater interpretation of vaccine trial data. These studies could involve including a marker sequence or gene product in the vaccine and then testing for specific antibodies, or testing for antibodies against a gene product that is not included in the vaccine, but that is found in the biological challenge. Thus, in the ΔRT vaccine, if antibodies to RT were detected this would be indicative of an immune response to challenge and not an anamnestic response generated by vaccination.

Another common finding in vaccine trial results is inter-group variation. This could be due to the DNA inoculation site. When injecting DNA into the quadriceps and gastrocnemius muscles of the hind limb, unless the animal is examined immediately post mortem, there is no way of knowing whether the DNA has gone into the muscle belly or in-between muscle bellies. Previous studies (McCluskie et al. 1999; Dupuis et al. 2000) have shown that the vaccination site can play a large part in the ensuing immune response. Dupuis et al. (2000) demonstrated that only muscle cells expressed a transgene even though there was uptake of DNA from non-muscle cells, when DNA was injected into the tibialis anterior muscle of mice. McCluskie et al. (1999) conducted a large study, whereby mice and rhesus monkeys were inoculated in many different sites with a hepatitis B surface antigen (HbsAg)-encoding plasmid. This study demonstrated that out of eight sites (i.p, i.d, i.v, i.m, intraperineal, subcutaneous, sublingual and vaginal wall) of DNA inoculation in mice, anti-HbsAg antibodies were generated in only five sites of DNA inoculation. Furthermore, the highest levels of antibody were detected in mice injected i.m or i.v. In the monkey, two sites were inoculated (i.m and i.d) and both induced anti-HbsAg antibodies.

Another factor affecting individual results is the i.p challenge. If performed incorrectly, the challenge dose could conceivably be inoculated into the bladder or large intestine, presumably resulting in a lower challenge dose or a failed injection. Also, should we really be challenging with virus using methods more akin to natural challenge? In the wild FIV is transmitted via the saliva, through biting, since FIV is most common in intact adult males. Therefore, to simulate this, would an i.m or i.d challenge be a more effective method of testing vaccines for use in the field or does the i.p challenge represent a more

stringent test for the vaccine (Rigby et al. 1997)? Rigby et al. (1997) compared the FIV infections that resulted after either an i.p or an i.m inoculation of virus reconstituted from the, F14 molecular clone of FIV-PET. It was demonstrated that there was a higher viral load in cats inoculated i.p than those inoculated i.m, despite the fact that the virus concentration in the i.m inoculum was 10 times higher than the i.p inoculum. This would suggest that viral challenge via the i.m route is relatively inefficient at establishing infection. An i.d challenge would presumably be efficient at establishing infection, as the skin is rich in professional APC's that would carry the virus from the skin to the local lymph nodes. Perhaps the lack of APC's in muscle causes the slower rate of infection. In addition, as FIV is an invaluable animal model for HIV, should vaccine efficacy be tested by challenging using methods more akin to the natural transmission of HIV? Pistello et al. (2003) remarkably demonstrated protection against a FIV-GL8 i.p challenge when SPF cats were vaccinated with a live-attenuated FIV-PET vaccine, however when challenged mucosally via the vagina the vaccine did not induce sterilising immunity but the FIV-GL8 replication that ocurred in the vaccinated cats was much less than in the unvaccinated cats. This study provides encouragement for the development of live-attenuated FIV and HIV vaccines and allays some of the fears with regard to a live-attenuated HIV vaccine, as no vaccinated cat displayed any symptoms of disease derived from the vaccine strain of FIV.

Vaccine efficacy can be tested by different routes of challenge, as described above, and also by using different types of challenge inocula. The different types of inocula include: 1) early passaged inoculum grown in primary PBMC; 2) molecularly cloned inoculum; 3) in vivo-derived inoculum such as plasma from FIV-infected cats and 4) contact challenges to stimulate natural conditions such as housing vaccinates with FIV-infected cats (reviewed in Uhl et al. 2002). In vivo-derived inoculum is similar to natural challenge as it contains quasi-species of FIV. These different types of challenge inocula and routes of transmission should be considered when planning future FIV vaccine trials, to optimise vaccine efficacy evaluation. In field-testing is likely to become important in future FIV vaccine trials and results may have an impact on HIV vaccine efficacy evaluation.

Finally, as with other diseases, another factor to consider is whether individual cats have different thresholds for infection. Animals, like humans, have different susceptibilities to infection depending on environment, diet and the development of their immune systems. Most of these factors are overcome with SPF animals that are kept in similar environments

and have the same diets, however, an animal born with a poorly developed immune system could be more susceptible to infection, regardless of being vaccinated with a vaccine shown previously to induce protection. Moreover, an animal with a high threshold to infection may falsely demonstrate that a vaccine has induced protection.

7.4. Causes of enhancement of FIV infection still undetermined

In the feline vaccine trial described in this thesis, vaccination with FIV DNA alone and CD40L DNA alone appeared to result in enhancement of FIV infection after challenge in some cats, as 2/4 cats in the CD40L only group had viral loads higher than the PBS control group cats and 1/4 of the cats in the FIV DNA only group had viral loads higher than cats immunised with PBS as a control. Furthermore, 3/4 cats in the CD40L DNA only group and 2/4 cats in the FIV DNA only group developed proviral loads higher than the PBS control group cats. The investigation into this enhancement is described in Chapter 6. In this study PBMC isolated after all three vaccinations but prior to FIV challenge were incubated with FIV-GL8. Assays were then conducted to compare the susceptibilities of these cells to FIV infection. No links could be proven between susceptibility of PBMC to FIV infection and high viral loads post challenge. However, these studies did demonstrate that vaccination did indeed have an effect on susceptibility to infection, as when PBMC collected pre- and post- vaccinations were compared it was evident that the pre-vaccination PBMC were less susceptible to FIV infection.

7.4.1. Further studies

Richardson et al. (2002) demonstrated a link between lymphoid activation prior to challenge and enhancement of FIV infection post vaccination. Similar methods were employed to those described in Chapter 6 in this study, to detect an increased susceptibility of lymphocytes to FIV infection, with the exception that PBMC numbers were not adjusted to take into account the varying numbers of lymphocytes between cats before use in the susceptibility assays. In this study flow cytometry was performed to assess lymphocyte numbers in individual cats so that the number of PBMC used in the assays could be adjusted, taking the lymphocyte numbers into account. Further assays could be conducted using PBMC numbers not adjusted for lymphocyte counts to determine if the results would be more comparable to those obtained by Richardson et al. (2002). Furthermore, if lymphoid activation does indeed lead to enhancement of infection, it would be interesting to conduct further vaccine studies with varying intervals of time between the final

immunisation and challenge, in order to examine the effect of decreasing the level of immune activation with time.

CD40-CD40L interactions affect the humoral and cell-mediated immune systems in many different ways. They are involved in the activation and differentiation of B-cells, the activation of T-cells via B7-CD28 interactions, CD40-CD40L interactions enable B-cells to increase their APC function, Th cells are primed and CTL's differentiate into memory cells with the help of CD40L, furthermore, CD40L also plays a role in T-cell tolerance and has direct anti-viral activity. All these factors would seem to make CD40L the ideal co-stimulatory molecule to use as an adjuvant to boost the immune responses to FIV. However, perhaps it is this very broad range of effects that may have in fact led to CD40L DNA causing enhancement of infection, as the immune response generated was not specific enough to induce protection against FIV. Berlinski et al. (2002) have demonstrated a non-specific component of immunisation related increased susceptibility to FIV infection and have concluded that cats vaccinated with non-FIV specific antigens or even commercial feline vaccines may be more susceptible to FIV infection.

A further mode of enhancement may involve the primary receptor for FIV. As mentioned previously, the primary receptor for FIV has recently been identified as CD134 (Shimojima et al. 2004). CD134 (also known as OX40) is a T-cell activation antigen and costimulatory molecule that is expressed primarily on $CD4^+$ T-cells (Paterson et al. 1987; Mallett, Fossum, and Barclay 1990), but it has also been shown to be present on activated $CD8^+$ T-cells, B-cells and macrophages (Baum et al. 1994; Durkop et al. 1995). Therefore vaccination may induce an expansion of a population of cells expressing the viral receptor and if the immune response induced is not virus specific then vaccination may lead to enhancement of infection. Furthermore, signalling through CD134 plays an important role in the proliferation and survival of $CD4^+$ T-cells that have encountered antigen, CD134 engagement enhances expansion and migration and prevents effector T-cell apoptosis (Gramaglia et al. 1998), further increasing the FIV susceptible cell population. The eventual decline in $CD4^+$ T-cell numbers as FIV disease progresses may be explained by the shift in cell tropism of the virus to CXCR4-expressing cells (CD134-independent infection) in chronic FIV infection. It has been demonstrated previously that some primary and some cell culture-adapted strains of FIV can infect via CXCR4 alone (Lerner and Elder 2000). Further work in this field is warranted.

7.5. Conclusion

In this study, the biological activity of CD40L and the effect of CD40L as a DNA adjuvant have been demonstrated. Also the possible link between lymphoid activation and enhancement of FIV infection has been studied. Although the FIV and CD40L combined DNA vaccine did not induce protection against a virulent isolate of FIV, it did appear to increase humoral responses in the mouse against FIV and increased cell-mediated responses in the cat against FIV. The use of CD40L as an adjuvant also led to decreased viral loads following challenge and slowed the progression of disease as determined by $CD8\beta^{low}$ number counts. For lentiviral vaccination, it has been proposed that instead of sterilizing immunity being the goal of vaccination, it may be more realistic to evaluate the efficacy of vaccines based on the above determinants (Davenport et al. 2004).

Further studies are indeed warranted, however, it is important not to overlook the negative involvement of CD40L in diseases such as HIV (Kornbluth 2000), MAIDS (Green et al. 1996) and neoplasia (Anderson et al. 2000). Both Pinchuk et al. (1994) and Tsunetsuga-Yokota et al. (1997) demonstrated that CD40L expression was required for full $CD4^+$ T cell activation and thus, the enhanced growth of HIV-1. It would be interesting to determine if CD40L expression increased the susceptibility of $CD4^+$ T cells to FIV infection. CD40L has also been implicated in the pathogenesis of MAIDS, Green et al. (1996) demonstrated that antibody to CD40L inhibited the symptoms of MAIDS such as splenomegaly and hypergammaglobulinaemia. Furthermore, CD40L is also involved in autoimmunity and transplant rejection (reviewed in Grewal and Flavell 1998). However, this involvement may lead to therapeutic uses of anti-CD40L mAbs. Further studies are required to achieve the desirable balance between the negative and positive effects of CD40L.

In this study a DNA vaccine trial was conducted against FIV and the results raised many questions about the way in which this study and other FIV vaccine trial studies have been conducted in the past. In future, standardisation of the method of inoculation of the DNA vaccine, the method of challenge of FIV and the methods used to detect humoral and immune responses must be considered, in order for many research groups to work together in the continuing search for an effective FIV vaccine against field isolates and for the findings to be used constructively in the development of a HIV vaccine.

Currently there is a FIV vaccine available in the USA, Fel-O-Vax FIV (Fort Dodge Animal Health, P.O.Box 25945, Overland Park, KS); this vaccine is based on a dual-subtype, subtype A FIV-PET and subtype D FIV-Shizuoka (SHI), WIV vaccine (Pu et al. 2001). It was hoped that by combining two different subtypes of the FIV, the protection induced would be against a broader range of isolates. To test this hypothesis, vaccinated cats were challenged with FIV-PET, the homologous isolate and FIV-BANG, a heterologous isolate. To further test the immunity induced, two different strengths of challenge inocula were used for the heterologous isolate. Four out of five dual-subtype vaccinated cats were protected against a low-dose (10 CID_{50}) FIV-BANG challenge. However, in an additional study only 2/5 dual-subtype vaccinated cats were protected against a high-dose (100 CID_{50}) FIV-BANG challenge. Therefore, the dual-subtype vaccine did induce protection against a low-dose heterologous isolate challenge, but the vaccine was less efficacious against a high dose challenge. However, Pu and co-workers demonstrated that the dual-subtype vaccine induced stronger immunity against the homologous challenge (FIV-PET) when compared to a single-subtype vaccine. Thus, 4/4 dual-subtype vaccinated cats were protected against FIV-PET (50 CID_{50}) whereas, only 1/5 single-subtype vaccinated cats was protected against the same challenge dose. Could this be an effective strategy against virulent isolates in the UK? Recent results have indicated that Fel-O-Vax did not protect cats against challenge with the FIV-GL8 isolate (Dunham et al. 2004).

The problems involved in developing a FIV vaccine that has a broad range of protection against many isolates are similar to the problems encountered in the development of a HIV vaccine. Additional factors to consider are individual mutations and inter-subtype recombination of viruses. Therefore, it is hoped that the continued work in the field of FIV will assist the research into HIV vaccine development. Further studies are required to overcome these hurdles and hopefully the lessons learned in the FIV vaccine system will impact upon the future development of an effective HIV vaccine. SIV is another useful animal model for HIV and SIV vaccine studies also have an important role in HIV vaccine research; however FIV is an animal model in which the host is infected naturally and can suffer from immunodeficiency symptoms similar to AIDS. Therefore, the development of a FIV vaccine is an important step in the development of a vaccine against HIV infection.

Appendices

Appendix A. Haematology results for cats A721 and A722 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A721								A722							
Test	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**
RBC $x10^9$/l (5-10)	7.86	7.14	7.47	7.67	9.34	8.77	9.44	7.23	7.78	6.68	7.25	7.71	6.54	7.91	9.5	6.68
Hb g/dl (10-15)	12.3	11.3	11.6	11.9	14	12.7	13.8	10.7	12.3	10.5	11.5	11.7	10.3	11.9	14.2	10.3
HCT % (30-45)	32.9	30	30.9	30.6	36.2	33.6	36.1	27.2	32.8	27.1	29.3	30.9	26.1	31.1	36.8	25.9
MCV fl (39-55)	41.9	42.1	41.3	40	38.8	38.3	38.2	37.7	42.1	40.5	40.4	40.1	39.9	39.3	38.7	38.8
MCH pg (12.5-17.5)	15.6	15.9	15.5	15.5	15	14.5	14.6	14.8	15.8	15.7	15.8	15.2	15.7	15.1	15	15.4
MCHC g/dl (30-36)	37.3	37.8	37.6	38.7	38.6	37.9	38.1	39.3	37.5	38.9	39.2	37.9	39.3	38.4	38.7	39.8
WBC $x10^9$/l (5.5-15.5)	11.9	13.6	16.2	11.1	8.68	6.74	8.95	7.38	14.9	10.6	21.9	12.7	5.23	7.55	8.94	9.3
Band neutrophils $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0.157	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	6.307	7.072	8.91	4.44	1.562	0.674	2.506	4.502	3.725	4.77	9.855	6.35	0.209	0.453	1.788	nd
Lymphocytes $x10^9$/l (1.5-7)	4.403	4.488	6.318	5.328	6.423	5.999	6.265	2.362	9.834	4.134	10.074	5.461	4.079	7.021	6.705	7.905
Monocytes $x10^9$/l (0-0.85)	0.476	0.544	0.162	0.555	0.434	0.067	0.089	0.517	0.298	0.212	0.657	0.381	0.523	0.075	0.179	0.465
Eosinophils $x10^9$/l (0-1.5)	0.714	1.36	0.81	0.777	0.26	0	0	0	1.043	1.272	1.314	0.508	0.262	0	0.268	0
Basophils $x10^9$/l	0	0.136	0	0	0	0	0	0	0	0.212	0	0	0	0	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A. (contd) Haematology results for cats A723 and A724 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A723								A724							
Test	-9w	-6w	0w	3w	6w	10w	13w	15w	-9w	-6w	0w	3w	6w	10w	13w	15w
RBC $x10^9/l$ (5-10)	7.32	7.76	7.87	7.72	8.44	8.71	9.16	7.86	7.4	7.19	7.42	7.52	8.2	8.31	7.74	7.34
Hb g/dl (10-15)	11.8	12.2	12.1	11.7	12.8	13	13.5	11.8	11.2	10.7	10.8	11	12.2	12.2	11.4	11
HCT % (30-45)	31.5	31.5	31.4	30.9	32.8	33.3	34.7	29.9	29.8	27.8	28.6	29.2	31.3	31	29	30
MCV fl (39-55)	43	40.6	39.9	40	38.8	38.3	37.9	38	40.3	38.7	38.6	38.8	38.2	37.3	37.4	40.9
MCH pg (12.5-17.5)	16.1	15.8	15.4	15.2	15.2	14.9	14.7	15	15.1	14.9	14.6	14.7	14.8	14.6	14.8	15
MCHC g/dl (30-36)	37.4	38.9	38.7	37.9	39.2	39.1	38.8	39.4	37.4	38.5	37.8	37.8	38.9	39.2	39.5	36.7
WBC $x10^9/l$ (5.5-15.5)	17.2	11	22.2	14.7	11.4	7.14	7.5	6.96	21.6	16.1	21.3	16.6	14.9	15.2	13.4	18.3
Band neutrophils $x10^9/l$	0.344	0	0	0	0	0	0	0	0.216	0	0	0	0	0	0	0.183
Neutrophils $x10^9/l$ (2.5-12.5)	13.072	6.49	17.982	8.526	5.586	2.356	3.525	2.854	12.96	9.177	12.141	8.964	7.599	8.512	5.628	12.627
Lymphocytes $x10^9/l$ (1.5-7)	2.752	3.96	3.552	5.145	5.472	4.213	3.675	3.55	6.696	5.152	7.029	6.972	6.556	6.08	6.968	4.575
Monocytes $x10^9/l$ (0-0.85)	0.172	0.11	0	0.441	0	0.143	0.15	0.139	0.648	0.322	0.639	0.166	0.447	0.304	0.402	0.732
Eosinophils $x10^9/l$ (0-1.5)	0.86	0.44	0.666	0.588	0.342	0.428	0.15	0.348	1.08	1.127	1.491	0.498	0.298	0.304	0.402	0.183
Basophils $x10^9/l$	0	0	0	0	0	0	0	0.07	0	0.322	0	0	0	0	0	0
Normoblasts $x10^9/l$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A. (contd) Haematology results for cats A725 and A726 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A725								A726							
Test	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**
RBC $x10^9$/l (5-10)	5.45	6.62	6.6	7.43	8.02	8.12	9.31	7.18	6.82	7.01	7.3	7.46	8.23	7.96	9.2	6.49
Hb g/dl (10-15)	8.8	10.2	10.4	11.2	11.9	12.3	14.2	11.1	10.2	10.2	10.7	10.6	11.5	11.3	12.9	9.88
HCT % (30-45)	23.4	27	26.8	30	31.8	32.1	37.7	28.8	27	26.7	27.7	27.8	30.4	28.6	33.1	25
MCV fl (39-55)	43	40.8	40.6	40.3	39.7	39.6	40.5	40.1	39.5	38.1	37.9	37.2	36.9	36	36	38.5
MCH pg (12.5-17.5)	16.2	15.4	15.7	15.1	14.9	15.2	15.2	15.4	14.9	14.6	14.6	14.3	14	14.2	14	15.2
MCHC g/dl (30-36)	37.6	37.8	38.7	37.4	37.5	38.3	37.6	38.5	37.7	38.2	38.6	38.3	37.9	39.4	38.9	39.5
WBC $x10^9$/l (5.5-15.5)	13.1	6.6	11.9	13.4	14.1	10.5	10.2	10.4	15.2	13.2	12.2	7.7	4.69	3	5.42	4.67
Band neutrophils $x10^9$/l	0.131	0	0	0	0.141	0	0	0	0	0.132	0	0	0.047	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	7.86	2.6	6.307	6.7	8.46	3.57	3.57	2.704	8.056	8.448	6.1	3.311	1.407	0.69	2.602	2.709
Lymphocytes $x10^9$/l (1.5-7)	3.799	2.665	4.522	5.628	4.371	6.195	6.222	6.864	5.928	3.564	5.368	3.619	2.861	2.13	2.71	1.681
Monocytes $x10^9$/l (0-0.85)	0.393	0.13	0.119	0.134	0	0.105	0	0.104	0.456	0.264	0.244	0.077	0.094	0.12	0.108	0.28
Eosinophils $x10^9$/l (0-1.5)	0.917	1.04	0.952	0.804	0.987	0.525	0.408	0.624	0.76	0.66	0.488	0.693	0.188	0.06	0	0
Basophils $x10^9$/l	0	0.065	0	0.134	0.141	0.105	0	0.104	0	0.132	0	0	0.047	0	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A. (contd) Haematology results for cats A727 and A728 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A727								A728							
Test	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**
RBC $x10^9$/l (5-10)	6.68	7.1	8.09	8.37	9.21	9.28	8.18	7.29	6.67	6.94	6.95	7.14	6.7	8.51	6.47	5.78
Hb g/dl (10-15)	10.7	11.2	12.7	12.8	14.1	14.2	12.4	11.2	10.6	10.8	10.9	10.9	10.5	13	10.3	9.03
HCT % (30-45)	27.3	29.2	32.5	33.2	36.1	35.9	32.2	28.9	27.5	27.6	28.2	28.6	26.5	33	25.3	24
MCV fl (39-55)	40.9	41.1	40.2	39.7	39.2	38.7	39.3	39.7	41.1	39.7	40.6	40.1	39.6	38.7	39.1	41.5
MCH pg (12.5-17.5)	16	15.8	15.7	15.3	15.3	15.3	15.2	15.4	16	15.5	15.7	15.2	15.6	15.2	15.9	15.6
MCHC g/dl (30-36)	39	38.5	39.1	38.5	39.1	39.6	38.6	38.8	38.8	39.1	38.7	37.9	39.5	39.3	40.6	37.6
WBC $x10^9$/l (5.5-15.5)	16.7	15.6	14.5	14.3	11.8	8.87	10.8	9.46	16.2	13.7	13.5	17.2	12.7	10.7	8.42	7.55
Band neutrophils $x10^9$/l	0	0.156	0	0	0	0	0	0	0.162	0	0	0	0	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	12.024	10.296	7.25	6.721	7.08	3.459	4.536	3.216	7.938	8.357	5.13	8.944	3.683	3.424	2.526	3.624
Lymphocytes $x10^9$/l (1.5-7)	2.338	2.808	5.655	6.149	3.894	4.612	5.508	5.487	7.29	4.521	7.965	7.568	8.128	6.42	5.389	3.7
Monocytes $x10^9$/l (0-0.85)	0.501	0.624	0.29	0	0.236	0.266	0.324	0.095	0.162	0	0.135	0.344	0.381	0.321	0.253	0.075
Eosinophils $x10^9$/l (0-1.5)	1.837	1.716	1.305	1.43	0.472	0.532	0.432	0.662	0.486	0.822	0.27	0.344	0.254	0.428	0.253	0.151
Basophils $x10^9$/l	0	0	0	0	0.118	0	0	0	0.162	0	0	0	0.254	0.107	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A. (contd) Haematology results for cats A729 and A730 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A729								A730							
Test	-9w	-6w	0w	3w	6w	10w	13w	15w	-9w	-6w	0w	3w	6w	10w	13w	15w
RBC $x10^9$/l (5-10)	6.65	6.93	7.7	7.99	8.14	7.45	7.3	6.39	7.11	7.11	8.28	8.11	8.54	9.26	9.11	6.37
Hb g/dl (10-15)	10.4	10.6	11.9	11.8	12.2	10.7	10.7	9.12	10.6	10.5	12.4	12	13.2	14	13.8	10.2
HCT % (30-45)	27.2	27.8	30.4	31.2	31.1	28.4	27.7	24	28.8	28.1	33	31.8	33.6	36.3	35.6	25.2
MCV fl (39-55)	40.9	40.1	39.4	39	38.2	38.1	38	37.5	40.5	39.5	39.9	39.2	39.3	39.2	39	39.5
MCH pg (12.5-17.5)	15.7	15.4	15.5	14.7	15	14.4	14.7	14.3	14.9	14.8	15	14.8	15.5	15.1	15.2	16
MCHC g/dl (30-36)	38.3	38.3	39.3	37.8	39.2	37.9	38.6	38.1	36.8	37.4	37.6	37.8	39.4	38.5	38.9	40.4
WBC $x10^9$/l (5.5-15.5)	10.7	10.7	11	9.5	11.8	7.79	12.7	5.67	11.6	13.4	13.9	11.4	9.82	9.72	9.45	7.04
Band neutrophils $x10^9$/l	0	0	0	0	0.236	0	0	0	0	0	0	0	0	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	7.383	5.457	4.73	6.08	7.198	5.219	9.398	3.629	5.22	7.638	7.367	5.586	4.615	4.374	3.497	2.746
Lymphocytes $x10^9$/l (1.5-7)	2.889	3.424	4.73	3.135	3.54	2.181	2.54	1.814	4.64	4.154	5.699	4.902	4.714	5.152	5.67	3.731
Monocytes $x10^9$/l (0-0.85)	0.321	0.428	0.33	0.095	0.354	0.312	0	0.17	0.696	0.536	0.139	0.798	0.393	0	0.283	0.352
Eosinophils $x10^9$/l (0-1.5)	0.107	1.284	1.21	0.19	0.472	0	0.762	0.057	0.812	0.938	0.695	0.114	0.098	0.194	0	0.211
Basophils $x10^9$/l	0	0.107	0	0	0	0.078	0	0	0.116	0.134	0	0	0	0	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0.116	0	0	0	0	0	0	0

Appendix A. (contd) Haematology results for cats A731 and A732 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A731								A732							
Test	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**
RBC $x10^9$/l (5-10)	6.7	6.09	7.14	7.12	7.23	8.38	6.84	Nd	6.9	6.77	8.06	8.86	7.69	Nd	6.88	Nd
Hb g/dl (10-15)	10.1	9.35	10.7	10.5	10.9	12.1	10.4	Nd	9.87	9.77	11.9	12.7	11.4	Nd	10.2	Nd
HCT % (30-45)	28.4	24	28.3	28.1	28.3	32.1	26.7	Nd	27.5	26.1	31.6	34.5	29.5	Nd	26.5	Nd
MCV fl (39-55)	42.3	39.5	39.6	39.5	39.2	38.3	39	Nd	39.9	38.6	39.2	39	38.4	Nd	38.5	Nd
MCH pg (12.5-17.5)	15.1	15.4	15	14.7	15	14.5	15.2	Nd	14.3	14.4	14.8	14.3	14.8	Nd	14.8	Nd
MCHC g/dl (30-36)	35.7	38.9	37.9	37.3	38.3	37.8	38.9	Nd	35.8	37.4	37.7	36.8	38.6	Nd	38.4	Nd
WBC $x10^9$/l (5.5-15.5)	13.6	8.73	13.2	8.08	7	6.86	5.35	Nd	15.7	17.3	11	9.13	6.99	11.9	9.74	Nd
Band neutrophils $x10^9$/l	0	0	0	0	0	0	0	Nd	0	0	0	0	0	0	0	Nd
Neutrophils $x10^9$/l (2.5-12.5)	8.432	5.151	8.316	4.282	2.87	1.441	1.284	Nd	11.932	12.802	6.71	5.113	3.565	7.021	4.87	Nd
Lymphocytes $x10^9$/l (1.5-7)	3.4	2.532	3.564	2.828	3.43	5.214	3.799	Nd	3.14	2.768	2.86	3.013	3.285	4.403	4.286	Nd
Monocytes $x10^9$/l (0-0.85)	0.952	0.087	0.528	0.323	0.35	0.206	0.268	Nd	0.157	0.346	0.44	0.274	0	0.119	0.195	Nd
Eosinophils $x10^9$/l (0-1.5)	0.544	0.698	0.792	0.566	0.21	0	0	Nd	0.471	1.211	0.88	0.73	0.14	0.238	0.292	Nd
Basophils $x10^9$/l	0.136	0.262	0	0.081	0.14	0	0	Nd	0	0.173	0.11	0	0	0.119	0.097	Nd
Normoblasts $x10^9$/l	0.136	0	0	0	0	0	0	Nd	0	0	0	0	0	0	0	Nd

Appendix A. (contd) Haematology results for cats A733 and A734 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A733								A734							
Test	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**
RBC $x10^9$/l (5-10)	6.2	6.08	7.58	9.2	6.87	6.26	7.25	6.04	12.3	7.05	7.85	7.48	7.93	9.84	8.75	7.04
Hb g/dl (10-15)	9.94	9.55	11.8	14.4	11.3	10	11.4	9.31	17.7	10.5	11.8	11	12.1	14.2	13.2	10.6
HCT % (30-45)	26	24.9	31.2	38.4	28.3	25.5	29.5	24.8	48	27.4	30.7	29.2	30.3	37	32.9	30
MCV fl (39-55)	42	41	41.2	41.7	41.2	40.7	40.7	41	39.1	38.9	39.1	39	38.2	37.6	37.5	42.6
MCH pg (12.5-17.5)	16	15.7	15.6	15.6	16.4	16	15.8	15.4	14.4	14.9	15	14.7	15.2	14.5	15	15
MCHC g/dl (30-36)	38.2	38.3	37.9	37.5	39.8	39.3	38.8	37.6	36.8	38.2	38.4	37.6	39.8	38.4	40	35.3
WBC $x10^9$/l (5.5-15.5)	14.4	9.44	11.4	9.36	8.77	6.2	8.19	5.54	24.6	27.8	9.25	14.3	11.4	11.5	14.3	9.01
Band neutrophils $x10^9$/l	0	0	0	0	0	0	0	0	0.492	1.39	0	0	0	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	7.776	3.87	5.358	2.434	1.579	0.496	2.293	1.662	16.728	23.908	5.55	8.151	5.7	4.715	7.722	3.784
Lymphocytes $x10^9$/l (1.5-7)	4.896	4.814	4.674	5.335	6.314	5.456	5.569	3.601	5.166	1.39	2.96	4.862	4.446	5.98	6.006	4.505
Monocytes $x10^9$/l (0-0.85)	0.576	0.189	0.114	0.468	0.351	0.186	0.164	0.111	0.738	0.834	0.185	0.143	0.456	0.115	0.286	0.18
Eosinophils $x10^9$/l (0-1.5)	1.152	0	1.14	1.03	0.526	0.062	0.164	0.166	1.23	0.278	0.463	1.001	0.684	0.69	0.286	0.541
Basophils $x10^9$/l	0	0.566	0	0.094	0	0	0	0	0.246	0	0.092	0.143	0.114	0	0	0
Normoblasts $x10^9$/l	0	0	0.114	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A. (contd) Haematology results for cat A735 at –9, -6, 0, 3, 6, 10, 13 and 15 weeks p.c.

	A735							
Test	**-9w**	**-6w**	**0w**	**3w**	**6w**	**10w**	**13w**	**15w**
RBC x10^9/l (5-10)	5.84	6.32	6.46	8.14	7.69	7	6.15	Nd
Hb g/dl (10-15)	8.68	9.39	9.57	11.8	11.4	10.5	9.25	Nd
HCT % (30-45)	23.4	24.7	25.1	31.1	29	26.2	23.2	Nd
MCV fl (39-55)	40	39.1	38.9	38.3	37.7	37.5	37.7	Nd
MCH pg (12.5-17.5)	14.8	14.9	14.8	14.5	14.8	14.9	15	Nd
MCHC g/dl (30-36)	37.1	38	38.2	38	39.3	39.9	39.9	Nd
WBC x10^9/l (5.5-15.5)	14.2	11.5	16.7	15.5	9.21	6.06	6.57	Nd
Band neutrophils x10^9/l	0.852	0	0	0	0	0	0	Nd
Neutrophils x10^9/l (2.5-12.5)	10.224	6.9	10.688	11.315	5.25	2.06	2.694	Nd
Lymphocytes x10^9/l (1.5-7)	1.704	3.335	4.843	2.48	3.408	3.636	3.811	Nd
Monocytes x10^9/l (0-0.85)	0.852	0.345	0.334	0.465	0.461	0.182	0.066	Nd
Eosinophils x10^9/l (0-1.5)	0.568	0.805	0.835	1.085	0.092	0.182	0	Nd
Basophils x10^9/l	0	0.115	0	0.155	0	0	0	Nd
Normoblasts x10^9/l	0	0	0	0	0	0	0	Nd

Nd- not determined

Appendix B. Solutions for large scale plasmid preparations

Solution I

2g lysozyme

3.6g glucose

20ml 0.2M EDTA (pH 8)

10ml 1M Tris (pH 8)

Make up to 400ml with water for 8 preparations

Solution II

25.6ml 5M NaOH

64ml 10% SDS

Make up to 640ml with water for 8 preparations

Solution III

240ml 5M KOAC

46ml acetic acid

Make up to 400ml with water for 8 preparations

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