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CELLULAR STUDIES ON THE PATHOGENESIS OF X-LINKED LYMPHOPROLIFERATIVE (XLP) SYNDROME

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Thesis Abstract

X-linked lymphoproliferative (XLP) disease is a severe primary immunodeficiency. Immunodysregulatory phenomena are observed following EBV infection suggesting that defects exist in these effector populations. The gene defective in XLP is SAP (SLAM-associated protein), an intracellular adaptor protein that mediates signals through SLAM and other immunoglobulin superfamily receptors including 2B4. Cytotoxic T cells (CTLs) and natural killer (NK) cells play a major role in the normal immune response to Epstein-Barr virus (EBV) infection. EBV specific T cell lines (EBV-T cell lines) were generated from normal individuals and XLP patients and examined for CTL function in response to different stimuli. It has been shown that XLP patients can generate EBV-T cell lines that are phenotypically similar to those from unaffected individuals. XLP patient derived EBV-T cell lines showed a significant decrease in interferon-gamma (IFN-γ) production in response to 2B4 and autologous EBV transformed lymphoblastoid cell line (LCL) stimulation but not in response to SLAM. Furthermore, XLP EBV-T cell lines demonstrated markedly decreased cytotoxic activity against autologous LCLs. By retroviral gene transfer of the SAP gene into XLP patient derived EBV-T cell lines, reconstitution of IFN-γ production and cytotoxic activity has been shown, confirming the defects are SAP dependent. These studies demonstrate that in XLP the lack of SAP affects specific signalling pathways resulting in severe disruption of CTL function.

In addition, SLAM and 2B4 expression on immune cell lineages has been investigated, the results suggest a wider range of 2B4 expression and deserve further investigation in relation to XLP molecular and cellular pathogenesis.

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Abbreviations

AH	Acquired hypogammaglobinemia
ACV	Aciclovir
ADA-SCID	Adenosine deaminase deficient-SCID
ADCC	Antibody-dependent cell mediated cytotoxicity
ALPS	Autoimmune lymphoproliferative syndrome
APC	Allophycocyanin
APC	Antigen presenting cell
ASCs	Antibody-secreting cells
att	Terminal attachment
BL	Burkitts lymphoma
BLNK	B cell linker
bp	Base pair (s)
BSA	Bovin serum albumin
CAEBV	Chronic active EBV infection
CBF	Core-binding factor
CCR	CC chemokine receptor
CD	Cluster determinant
cDNA	Complementary DNA
CGD	Chronic granulomatous disease
CMV	Cytomegalovirus
cPPT	Central polypurine tract
CY	Phycoerythrin-cyanine 5 conjugate
C terminus	Carboxy terminus
CTL	Cytotoxic T lymphocyte
CVID	Common variable immunodeficiency
CXCR	CXC chemokine receptor
DC	Dendritic cell(s)
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleotide
EA	Epstein–Barr-virus early antigen

EAT-2	EWS-activated transcript-2
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EBV-BLCL	EBV-transformed B lymphoblastoid cell lines
EBV-HLH	EBV-associated haemophagocytic lymphohistiocytosis
ECL	Enhanced chemiluminescence
ECMV	Encephalomyocarditis virus
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
ESID	European Society for Immunodeficiencies
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FeL V	Feline leukemia virus
FIM	Fulminant infectious mononucleosis
FITC	Fluorescein isothiocyanate
FMCF	Friend mink cell focus-forming virus
GALV	Gibbon ape leukemia virus
GLPD	Granular lymphoproliferative disorder
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HIV	Human immunodeficiency virus
HIV-1 (SIN) vector	Self-inactivating HIV-1 vector
HFV	Human foamy virus
HLA	Human leukocyte antigen
HLH	Haemo-phagocytic lymphohistiocytosis
HRP	Horse radish peroxidase
HSCT	Haematopoietic stem cell transplantation
HTLV	Human T cell leukemia virus
IFN-γ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
IM	Infectious mononucleosis
IR	Inverted repeat
IRES	Internal ribosomal entry sequences
JNKs	c-Jun NH2-terminal kinase
KD	KiloDalton
LAK	Lymphokine-activated killer cell
LAT	Linker for activation of T cells
LCMV	Lymphocyte choriomeningitis virus
LDH	Lactate dehydrogenase
ΔLNGFR	Truncated low affinity nerve growth factor receptor
L-M	Lieshmania major
LMP	Latent membrane protein(s)
LPS	Lipopolysacharide
LTRs	Long terminal repeats
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MESV	Murine ES cell virus
MFI	Mean fluorescence intensity
μg	microgram
MHC	Major histocompatibility complex
ml	Millilitre
μl	Microlitre
MLR	Mixed lymphocyte reactions
MLVs	Murine leukemia viruses
MMTV	Mouse mammary tumor virus
MOI	Multiplicity of infection
Mo-MuLV	Moleny murine leukemia virus
MPMV	Mason-Pfizer monkey virus
MPSV	Myeloproliferative sarcoma virus
mRNA	messenger RNA
NC	Nucleocapside protein
NHL	Non-Hodgkin's lymphoma

NIH	National Institute of Health
NK	Natural killer cell
NKT	Natural killer T cell
NPC	Nasopharyngeal carcinoma
N terminus	Amino terminus
ORF	Open reading frame
OVA	Ovalbumin
P	Probability value
PAGE	Polyacrylamide gel electrophoresis
PAGID	Pan American Group for Immunodeficiencies
PBL	Peripheral blood lymphocyte
PBMCs	Peripheral blood mononuclear cells
PBS	Primer binding site
PCMV	PCC4-cell passaged MPSV
PCR	Polymerase chain reaction
PE	phycoerythrin
PEBP	Polyoma enhancer-binding protein
PEI	Polyethyleneimine
PER	Phycoerythrin-cyanine 5 conjugate
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PID	Primary immunodeficiency
PLC-γ	Phospholipase Cγ
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinase
PTLD	Post-transplant lymphoproliferative disorder
RANTES	Regulated upon activation normal T expressed and secreted
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase H	Ribonuclease H
rpm	Revolutions per minute
RRE	Rev-responsive element
SAP	SLAM associated protein

SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFFVp	Spleen focus-forming virus promoter
SH	Src Homology
SHP	SH2 domain- phosphtase
SHIP	SH-2 domain-inositol polyphosphate 5' phosphatase
SLAM	Signal lymphocyte activation molecule
STAT	Signal transducer and activator of transcription
TAE	Tris acetate EDTA
TCR	T-cell receptor
TE	Tris/EDTA buffer
Th	T helper cell
TNFα	Tumour necrosis factor
Tris	2-amino-2-[hydroxymethyl]-1,3 propandiol
Tween20	Polyethylene-sorbitan monolaurate
VAHS	Virus-associated haemophagocytic syndrome
VCA	Viral capsid antigen
VSV envelope (G)	Vesicular stomatitis virus G envelope
WPRE	Woodchuck hepatitis virus post-transcription regulatory element
XLP	X-linked lymphoproliferative disease

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And last not least, I would like to take this opportunity to express my appreciation to those people who have helped me in my long-term education and taking me to a dream!!! A dream of wisdom and dedication.

The cycle that encompasses our entrance and exit

Has neither a beginning nor an end in sight

No one speaks for a moment the truth about it

As to whence is our entrance and whereto our exit

Iranian Poet; Khayyam Nishapur

Chapter 1

Introduction

1.1 Definition of X-linked lymphoproliferative (XLP)

X-linked lymphoproliferative (XLP) disease is one of the X linked primary immune deficiencies. It is characterised primarily by an inappropriate response to Epstein-Barr virus (EBV) infection, which is often fatal and has excessive damaging effects on the immune system of affected individuals. Since XLP was identified as a new immune deficiency disorder in 1975, there have been many efforts to understand its molecular and cellular pathogenesis and investigate why there is extreme susceptibility to EBV. The following chapter focuses on the clinical, molecular and cellular pathogenesis of XLP and the use of gene therapy as a potential investigational and therapeutic tool in this disease.

1.2 History of XLP

The history of XLP begins with the description of EBV. Following the recognition of Burkitts lymphoma (BL) by Denis Burkitt in 1958, Epstein and Barr subsequently reported the identification of EBV, in 1964, by electron microscopy of cultured BL cells (reviewed by Epstein, 1999). Henle later established that EBV is cause of infectious mononucleosis (IM) (Henle *et al,* 1968). The earliest report of XLP in medical literature is an abstract describing two brothers who had sequentially experienced IM, acquired hypogammaglobinemia (AH) and non-Hodgkin's lymphoma (NHL) by Hambleton *et al* in 1969 but it was rejected by a referee who did not accept that EBV could cause IM, AH and NHL (Hambleton *et al,* 1969). However, at the same time, on April 10, 1969 Purtilo and his colleague Vawter, from University of Nebraska Medical Centre, performed an autopsy on an 8-year old boy, a member of the Duncan family who had died 30 days after the onset of IM with

fulminant hepatitis and bone marrow failure. This condition was excluded from a variety of ill-defined entities such as familial lymphohistiocytosis, acute lymphoblastic lymphoma and others. Later on they found that a 2 and half-year old male sibling had died of acute lymphoblastic leukemia earlier. At the same time Purtilo noticed a report on high frequency of NHL in children with inherited immunodeficiency disease by Gatti *et al* (Gatti *et al,* 1971). On further investigation of the Duncan family, Vawter found the death of a third boy and was informed by his mother that three nephews in her family had also died (Purtilo *et al,* 1991). One of AH after IM, another of NHL involving the ileocecal region without a history of IM, and a third of NHL in central nervous system in his half-brother following a protracted period of IM. In 1974 Bar *et al* also reported fatal IM in a family (Bar *et al,* 1974). In 1975, Provisor *et al* reported three males in one family who had an illness with a presentation of IM. The two surviving males had agammaglobulinemia complications (Provisor *et al,* 1975). They confirmed that acquired agammaglobulinemia is a clinical feature in three related male children after a life-threatening illness with clinical and laboratory features of IM. In a report published in the Lancet by Purtilo *et al* in 1975 this immune deficiency related to EBV was identified as a new terminology in medicine and became recognised in the medical community. Based on his observations, the inheritance pattern, the clinical and histopathological findings, Purtilo named this new disease X-linked recessive combined variable immunodeficiency or Duncan's disease (Purtilo *et al,* 1975). In their initial report the authors suggested that EBV or other viral agents may have triggered the fatal proliferation of lymphocytes and postulated that immunological shut-off mechanisms for controlling the proliferation of lymphocytes, especially B cells, were inadequate.

For the past 30 years efforts have been contributed by clinicians and scientists to address the clinical symptoms and pathogenesis of XLP. The gene for XLP has been identified offering improved diagnostic tests and a greater awareness of the clinical spectrum of the condition. Furthermore, as the molecular and cellular pathogenesis of the condition begins to unravel, the initial suggestions of Purtilo continue to hold true (Purtilo, 1976).

1.3 Clinical manifestations

Although the identification of the defective gene in XLP has provided better diagnostic tests, it has also revealed that the clinical diversity of this condition is greater than was suggested by primary reports. To track and characterise this disease Purtilo established a registry in 1980, which serves as a world wide resource for the diagnosis, treatment, and research of this condition (Purtilo *et al,* 1991). Although Purtilo died in 1992, the XLP registry continues and up until 1995, 272 cases among 80 kindreds had been identified. It has been noticed that some cases of XLP can occur without EBV infection (Seemayer *et al,* 1995). According to this database, and recent individual case reports, the phenotypes of XLP can be classified into three main subgroups and rare presentations as shown in Table 1.1 (Gaspar *et al,* 2002; Seemayer *et al,* 1995; Sumegi *et al,* 2000).

1.3.1 Fulminant infectious mononucleosis (FIM)

This forms the most common and most dramatic manifestation of XLP. The median age at onset of disease is 2.5 years. Importantly, this clinical phenotype also has the worst prognosis with over 90% mortality. The phenotype is insidious and follows a progression of fever, malaise, pharyngitis, and generalised lymphocytosis.

Table 1.1 Clinical phenotypes of X-linked lymphoproliferative syndrome

Phenotype	Prevalence (%)	Mean age of onset (years)	Survival rate (%)
Fulminant infectious mononucleosis	50	5	4
B-cell lymphoma	30	6	35
Dysgammaglobulinaemia	30	9	55
Rare presentations	3	6-8	29-50
Vasculitis, aplastic anemia, pulmonary lymphoid granulomatosis			

Based on data from: Seemayer *et al,* 1995; Sumegi *et al,* 2000; Gaspar *et al,* 2002

Multi organ failure is a general feature. Hepatic coma, respiratory distress syndrome, and renal failure accompanying bone marrow failure progress despite supportive care. The lymphoid tissues undergo biphasic changes: hyperplasia develops first and persists for 2-3 weeks and is followed by hypoplasia (depletion). The vital organ damage is caused by infiltration of atypical lymphocytes, NK cells, EB nuclear antigen positive ($EBNA^+$) B cells, macrophages, and an accompanying dysregulated cytokine release. The extensive destruction of liver and bone marrow often leads to a fulminant hepatitis concurrent with virus-associated haemophagocytic syndrome (VAHS). In early VAHS moderate pancytopenia and a myeloid hyperplastic marrow occur, follow by the appearance of numerous activated macrophages with erythrophagocytosis and nucleophagocytosis. The condition evolves with lymphoid infiltration associated with necrosis and hemorrhage can occur. The final step is adult respiratory distress syndrome and hepatic coma as VAHS persists. Whereas VAHS occurs in approximately 90% of cases with FIM, a few patients (5%) manifest

hypolasia or aplasia of the bone marrow, which should not be confused with VAHS. In nearly 30% of those with aplastic anemia, EBV genome is present in bone marrow cells (Baranski *et al,* 1988). The aplastic anemia in XLP is characterised by marked depletion of haematopoietic cells, which are replaced with fat. Infiltration in other tissues has also been described with splenic (white pulp necrosis), cerebral (perivascular mononuclear cell infiltrates), heart (mononuclear cell myocarditis) and renal (mild interstitial nephritis) involvement.

The mortality associated with FIM is high and the registry data compiled in 1995 shows a 4% survival rate. With the use of etoposide and cyclosporine to control histiocytic and T-cell proliferation and activation, the outcome for this manifestation may have improved, but the condition still remains extremely difficult to treat.

1.3.2 Dysgammaglobulinaemia

In one third of boys with XLP, EBV infection is not fatal as in FIM presentations, but is followed by abnormalities of immunoglobulin (Ig) production. This ranges from decreased levels of IgG to panhypogammaglobulinemia (Purtilo *et al,* 1982). It also has been reported that prior to EBV infection, one third have hypogammaglobulinemia (Purtilo *et al,* 1991). In more severe cases, humoral dysfunction may accompany cellular T and natural killer (NK) defects (Sullivan *et al,* 1980). The abnormalities may arise from the extensive necrosis and lymphoid depletion throughout the lymphoreticular system that follows acute EBV infection in these individuals (Purtilo *et al,* 1975). Also it has been suggested that the Ig abnormalities could be secondary to T helper cell dysfunction (Crotty *et al,* 2003; Welsh *et al,* 2003). The identification of SAP as the defective gene in XLP now

allows unambiguous diagnosis of the condition. As a result, some patients previously labelled as having common variable immunodeficiency (CVID), a primary immunodeficiency with unknown molecular basis, have been shown to have mutations in the SAP gene (Morra *et al,* 2001; Nistala *et al,* 2001). Although it has been suggested that all male CVID patients should be screened for SAP mutations, a recent study based on screening of a large cohort of men with hypogammaglobulinemia and CVID showed only 0.6% of them carried a germline SAP mutation (Eastwood *et al,* 2004). Therefore, it is unlikely that the true incidence of XLP in the CVID population is significant and there is not any benefit of SAP mutation analysis of all cases of CVID unless they present with other suggestive clinical features. The registry data suggests that this manifestation of XLP has the best prognosis, with greater than 50% survival.

1.3.3 Malignant lymphoma

The malignancies observed in XLP are mostly non-Hodgkin B cell lymphomas and have occurred in approximately one third of affected individuals with XLP. The most common lymphoproliferative types are extranodal, usually BL of the ileocecal region (Harrington *et al,* 1987). Also lymphomas commonly involve the central nervous system, hepatic and renal systems. Almost all the lymphomas were of B-cell origin but tumours with a T-cell phenotype have also been described (Gaspar *et al,* 2002). The median age of affected boys is 4–6 years of age (Sumegi *et al,* 2000). The lymphomas can be histologically classified as Burkitt's lymphomas (53% of all B-cell lymphomas), immunoblastic lymphomas (12% of all cases), small cleaved or mixed cell lymphomas (12%) and unclassifiable lymphomas (5% of all cases) (Harrington *et al,* 1987). The features of lymphomas in XLP are similar to those seen

in other primary immunodeficiencies with extra-nodal involvement, high-grade histology, and evidence of clonality and gene rearrangements. The survival figures following the development of lymphoma are poor with 35% survival (Purtilo *et al,* 1991). Remission has often been achieved with lymphoma chemotherapy protocols but there is a high rate of relapse or development of the other manifestations of XLP. There is also a risk of fatal IM many years after successful treatment.

1.3.4 Uncommon clinical presentations of XLP

In addition to the above three manifestations, which represent the main clinical features of XLP patients, the literature also describes a number of other presentations. Lymphoid vasculitis is another uncommon but well-described complication of XLP. It affects all medium and small vessels, involving retinal, cerebral, renal, pancreatic and coronary vessels. Histological evidence demonstrates arterial wall destruction with aneurysmal dilatation. T-cell pulmonary lymphomatoid granulomatosis was the initial presentation with the lymphoproliferation characterised by predominant activation of CD4+ cells in some cases (Purtilo *et al,* 1991; Dutz *et al,* 2001; Kanegane *et al,* 2005).

Aplastic anemia can also be the initial presentation rather than arising as a complication of fulminant infectious mononucleosis with haemophagocytosis. The registry reports a number of individuals who present initially with aplastic anaemia (3% of all cases) (Seemayer *et al,* 1995). As numbers are small, there is very little detail on the pathogenesis of this phenotype.

Therefore, it is likely that the phenotype is determined by a number of factors, including the genetic elements, and environmental and infectious factors on a background of disease modifier genes.

FIM presentations of XLP are often accompanied by various degrees of haemophagocytosis. However, haemophagocytic lymphohistiocytosis (HLH) as a disease entity is well recognized with established diagnostic clinical and laboratory criteria, and exists in both familial and sporadic forms. In a certain proportion of patients, HLH is caused by mutations in the gene encoding perforin (Stepp *et al,* 1999). Arico *et al* reported 16% of male patients with a HLH diagnosis carried SAP mutation and half of them had a positive family history (Arico *et al,* 2001). This suggests that XLP should be considered in all males presenting with HLH.

1.3.5 XLP without EBV infection

XLP presents many diagnostic difficulties not just as a result of the variety of clinical presentations. From the early descriptions, EBV has always been reported to be the viral trigger for the different XLP phenotypes. However, almost 10% of XLP cases have shown clinical signs of immune deficiency prior to EBV infection (Sumegi *et al,* 2000). It has been reported that some affected boys with B-cell non-Hodgkin's lymphoma were confirmed to have mutations in the SAP gene (Brandau *et al,* 1999; Strahm *et al,* 2000). Efforts to detect EBV in individuals failed despite using serological and genetic investigations (Brandau *et al,* 1999; Gilmour *et al,* 2000; Nistala *et al,* 2001). It suggests that the XLP defect is not a specific susceptibility to EBV but a more general dysregulation of cellular responses to infection. Certainly, other viruses either alone or in combination may trigger abnormal

immune responses. The reports of hypogammaglobulinemia following measles infection (Mawhinney *et al,* 1971; Purtilo *et al,* 1977) also raises the intriguing possibility that there may be specific vulnerability to this virus as well.

1.4 The genetic cause of XLP

1.4.1 Discovery of XLP gene

A major milestone in the history of XLP was identification of the affected gene. After long investigation of the partial deletion of chromosome X among XLP kindreds and using restriction fragment length polymorphism (RFLP) linkage analysis, Skare *et al* showed that the XLP locus was located between DXS42 and DXS73 (Skare *et al,* 1989). Soon Skare's laboratory established the gene locus and refined its location to a 2.5-Mb region in Xq25 (Skare *et al,* 1993). Finally in 1998, three independent groups identified the mutated gene in XLP, through positional or functional cloning (Coffey *et al,* 1998; Sayos *et al,* 1998; Nichols *et al,* 1998). This gene was designated as SAP [SLAM (signal lymphocyte activation molecule) Associated Protein] /SH2DA1 (SH2 domain protein 1A). Here we use the term SAP, indicating protein expression and *SAP* to define the gene. The *SAP* cDNA is 2,530 bp long and contains an open reading frame (ORF) of 462 bp, with an initiation codon 79 bp from the start of the ORF. It contains four exons that code for a small 128 amino acid polypeptide with a molecular weight of 15 kD size consisting of a 5-amino acid N-terminal sequence, an SH2 domain, and a 25- amino acid C-terminal tail (Fig 1.1). The promoter and regulatory regions for the control of SAP expression have not yet been defined, however, the sequences upstream of exon 1 include putative binding sites for transcription factors important for T-lymphocyte development and function,

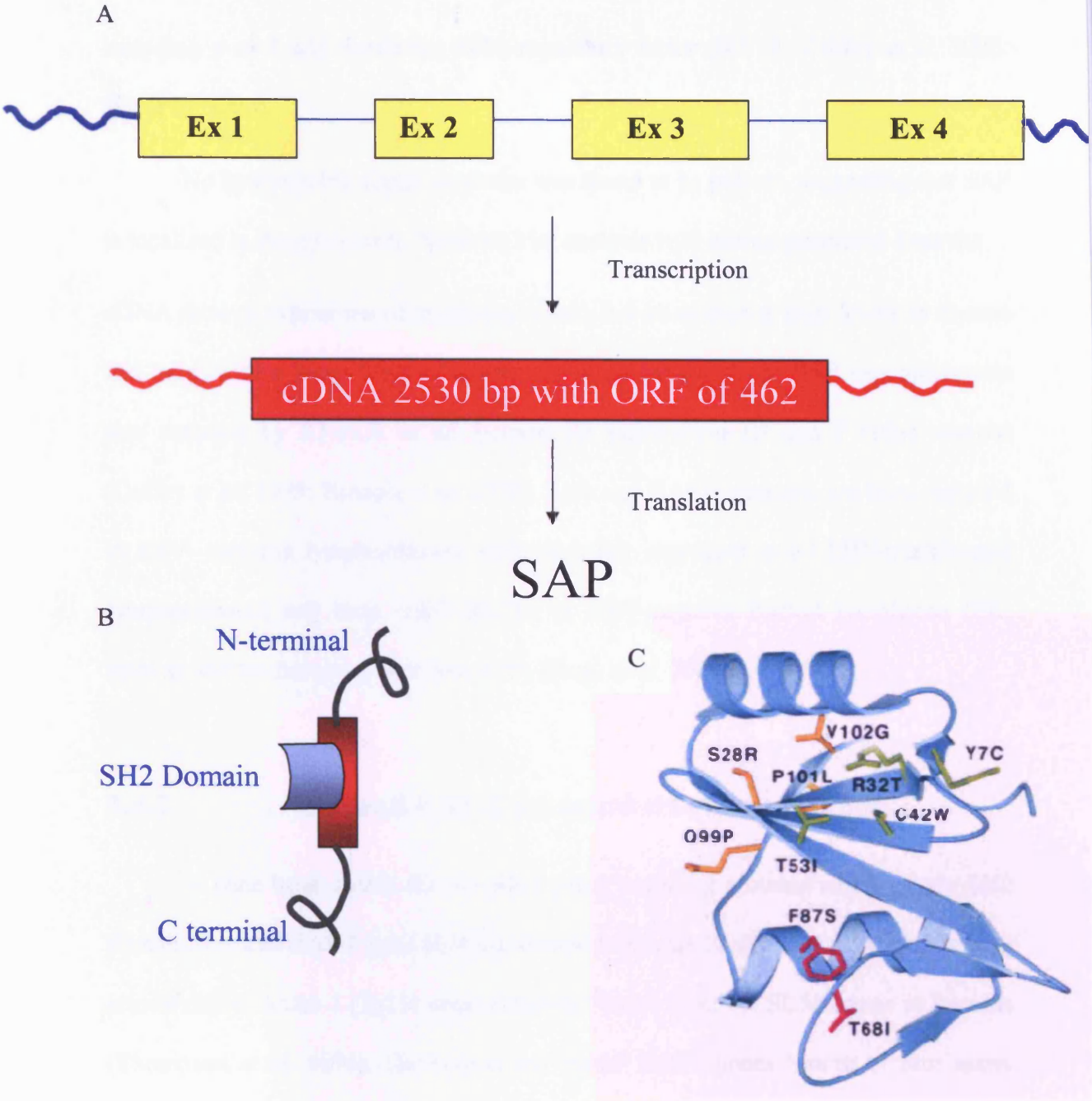


Figure 1.1 SAP gene and protein structure

A) The SAP gene consists of four exons and encodes a cytoplasmic protein of 128 amino acids. B) The protein consists of a very short N-terminal, a single SH2 domain and a small C-terminal tail. SAP protein binds to phosphorylated residues on the cytoplasmic tails of SLAM-related receptors via its SH2 domain. C) Crystal structure of SAP and location of missense mutations identified in XLP patients. SAP has the overall characteristics of an SH2 domain fold, which includes a central sheet with helices packed against either side (C is taken from Poy *et al,* 1999).

including c-est-1 and interferon (IFN) regulatory factor (IRF)-1 (Coffey *et al,* 1998; Wu *et al,* 2000).

No hydrophobic signal sequence was found to be present, suggesting that SAP is localized in the cytoplasm. Northern blot analysis with probes generated from the cDNA showed expression of an approximately 2.5-kb mRNA at high levels in thymus and lung, with a lower level of expression in spleen and liver. SAP expression was also detected by RT-PCR in all lymphocyte populations (B and T cells) assayed (Coffey *et al,* 1998; Nichols *et al,* 1998). Although SAP expression has been reported in EBV- carrying lymphoblastoid cells, it is not expressed in all EBV-transformed lymphoblastoid cell lines (EBV-BLCL) or EBV-negative Burkitt lymphoma (BL) lines as will be discussed later (see p.32) (Nagy *et al,* 2000).

1.4.2 SAP and EAT-2 are members of a gene family

A gene bank search for the other genes encoding proteins with a single SH2 domain revealed EAT-2 gene (EWS-activated transcript-2) that is located on the long arm of chromosome 1 (1q23) approximately 700 kb from the SLAM gene in humans (Thompson *et al,* 1996). The human and mouse EAT-2 genes consist of four exons and encode a 132- amino acid protein that, like SAP, consists of a single SH2 domain followed by a short C-terminal sequence. The mouse EAT-2 gene was first identified as a transcript induced in transformed mouse fibroblast by EWS/FLI1 oncogene. Using PCR and cell separation techniques, EAT-2 was found to be expressed in macrophages and B cells but not in thymus-derived lymphocytes (Morra *et al,* 2001).

As the exon-intron structures of the SAP and EAT-2 genes are identical, it appears that the SAP and EAT-2 genes represent two identified members of a new

family of genes, which are able to act as adaptor proteins and modulate cell signalling pathways.

1.4.3 SAP and EAT-2 expression in immune cell lineages

SAP expression is different in immune cell lineages as well as human and murine. SAP is expressed in T cells, including CD8, CD4 single positive T cells (Sayos *et al,* 1998; Wu *et al,* 2000). Its expression is prevalent in Th1 cells, but Th2 cells also contain the transcript (Sayos *et al,* 2000). It has been shown that SAP in human T cells is up regulated at the RNA and protein level upon specific and non-specific stimulation (Nichols *et al,* 1998; Nagy *et al,* 2000; 2002). However, its expression in T cells is downregulated upon activation in murine cells (Mora *et al,* 2001). The rapid down-regulation is the result of the presence of the AUUUA sequence in the 3' UT region of both the long and the short mRNA species (Sayos *et al,* 2000). It may suggest that SAP expression is regulated differently in human and murine systems.

The studies in human has shown that SAP is expressed at low levels in resting NK cells but its expression increases upon activation *in vivo* and *in vitro* (Nagy *et al,* 2002). Interestingly NK leukaemia lines express SAP like their normal counterparts (Nagy *et al,* 2000). However, the kinetics of SAP expression in cultured murine NK cells differed from murine T cells because of higher expression levels after activation (Sayos *et al,* 2000).

SAP is not expressed in all B cell subsets although its expression at the RNA level has been demonstrated by some groups (Yin *et al,* 2003). It has recently been

shown that SAP is expressed by murine memory B cells (Feldhahn *et al,* 2002). Significantly, the gene is not expressed in EBV transformed lymphoblastoid cell lines, and only a small number of B cell tumours express SAP (Nagy *et al,* 2000). The EBV-carrying cell panel contained type I BL lines such as Rael, Akata and Mutu I clones 148 and 216 were SAP positive. In contrast, the BL lines with type III latency phenotype (Raji, Daudi, P3HR1 and Namalwa) and EBV-negative lines (BL41, BL49, BL28, DG75, BL2, CA 46 and Ramos) were SAP negative (Nagy *et al,* 2000). Intriguingly, transformation of Mutu phenotype from type I (expressing only EBNA-1) to the type III (expressing all 6 EBNAs and 3 latent membrane proteins (LMPs)) is accompanied by the down regulation of SAP and the appearance of SLAM on the cell surface (Nagy *et al,* 2000).

Table 1.2 SAP and EAT-2 expression in different human immune cell lineages

Cell lineages	SAP expression	EAT-2 expression
CD4 T cells	+	-
CD8 T cells	+	-
NK cells	+	+
LAK	+/?	-/?
NKT	+/?	-/?
Monocytes	-	+
DCs	-/?	?
Macrophages	-	+
B cells	-/?	+

SAP expression has not been reported in other periphery immune cells including monocytes, dendritic cells (DCs), and macrophages.

In contrast to SAP, the kinetics of EAT-2 expression has not been fully studied (Table 1.2). Like SAP, EAT-2 is expressed in immune cells, including macrophages, DCs, B cells (Morra *et al,* 2001). However, it is not found in T cells. Interestingly, only in NK cells are SAP and EAT-2 co-expressed (Bouchon *et al,* 2001). This may imply a complex competitive function for these two SH2 domain adapter molecules.

1.5 SAP mutations

Over 50 different mutations in the *SAP* gene have been identified so far in XLP patients (Sumegi *et al,* 2000), although this number may be much greater (Fig 1.2). They range from large or small deletions resulting in complete loss of the gene or non-sense, splice-site and missense mutations that lead to premature arrest of protein synthesis or to a non-functional SAP (Coffey *et al,* 1998; Nichols *et al,* 1998; Yin *et al,* 1999; Sumegi *et al,* 2000). Despite the wide range of *SAP* mutations associated with XLP, similar to the experience found in other primary immunodeficiencies, there is no correlation between the type of mutation and the severity of the disease and the clinical phenotypes (Brandau *et al,* 1999; Sumegi *et al,* 2000). Indeed, within the same family, the same mutation has resulted in different clinical presentations, which implies that other factors, genetic or environmental, are important for determining the manifestations of the disease (Arkwright *et al,* 1998; Morra *et al,* 2001).

1.6 Structure of SAP and molecular association

SAP protein has a unique structure containing only a single Src homology (SH)-2 domain and a short C-terminal tail. Sayos *et al* identified SAP using a yeast two-hybrid system with the 77-amino-acid human signalling lymphocyte-activation molecule (SLAM) cytoplasmic domain as bait and a human T-cell library. It was postulated that the novel protein might be involved in protein tyrosine phosphorylation-mediated signal transduction events. Further experiments, including random peptide library screening, showed that the SAP (and EAT-2) SH2 domain preferentially recognizes the motif TipYxxV/I (where T is threonine, pY is phosphotyrosine, V is Valine, I is isoleucine and x is any other residue) (Poy *et al,* 1999). SH2 domain structure is highly conserved and forms a large antiparallel β - sheet with two flanking α-helices, which form a binding pocket for specific phosphorylated tyrosine residues (Lichtarge *et al,* 1996).

Crystallography and nuclear magnetic resonance (NMR) studies revealed that the SAP SH2 domain interacts with its ligands via an unusual mode of binding (Poy *et al,* 1999; Hwang *et al,* 2002). In addition to the classical "two pronged" interaction between an SH2 domain and its ligand peptide, a third contact point between the SH2 domain and residues located N-terminal to phosphorylated tyrosine is formed (Scott *et al,* 2000). The existence of this additional contact significantly enhances the affinity of the SAP SH2 domain for its ligand (Kd $\approx$120-150 nM; compared to Kd $\approx$500 nM for conventional SH2 domains). A similar binding mechanism exists for EAT-2 (Morra *et al,* 2001).

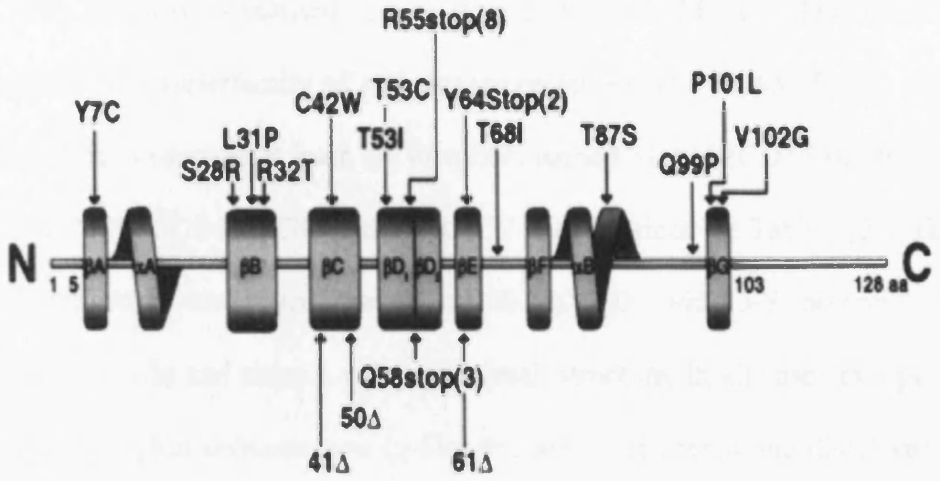


Figure 1.2 Common mutation sites in SAP gene

The mutations vary from deletions, non-sense, splice-site and missense mutations that lead to premature arrest of protein synthesis or to a non-functional SAP. Most mutations are located in the SH2 β sheets. (Taken from Sumegi *et al,* 2000).

SAP has another unique feature limited only to SLAM binding and not to other members of the SLAM family, which is binding to a 14 amino acid peptide in the proximity of Y281 in the cytoplasmic tail of SLAM in the absence of phosphorylation. Although the functional significance and structural basis of this feature are not known, it may further enhance the interaction between SAP and SLAM, as opposed to binding to other SLAM family members.

1.6.1 SAP interacts with SLAM family

SAP was initially identified as a ligand for SLAM, a CD2-subset of the immunoglobulin superfamily of cell surface receptors. The SLAM family of immune cell receptors comprises at least six members named SLAM (CD150), 2B4 (CD244), Ly-9 (CD229), CD84, NTBA and CRACC (summarized in Table 1.3). These cell surface receptors are glycoproteins of 60-100 kD, with 3-8 potential N-linked glycosylation sites and share a common overall structure. In all cases except Ly-9, the extracellular region contains two Ig-like domains; one membrane-distal variable (V) non-disulfide domain and a membrane-proximal truncated Ig constant-2 (C2) domain containing two intrachain disulfide bonds. This configuration of Ig domains is duplicated in Ly-9 (Tangye *et al,* 2000). The cytoplasmic domains contain three or more unique tyrosine-based motifs TxPYxxV/I (Figure 1.3). The genes encoding the human SLAM family are localized to chromosome 1 at bands 1q21-24.

In immune cells, SAP was shown to associate with SLAM, 2B4 and NTB-A (Latour *et al,* 2003). While it remains to be proven if SAP interacts with other SLAM-related receptors in immune cells, an interaction with CD84 and Ly-9 as well as binding of EAT-2 to various SLAM-related receptors was seen in transfected cells (Sayos *et al,* 2001).

Table 1.3 Members of the SLAM family

Molecule	Names	Associates with	Expression	Ligand	Function
CD84	Ly9B	SAP, EAT-2, SHP2, SHIP1	B, T cells, monocytes, platelets	CD84	Control of TCR-induced IFN-production
CD150	SLAM	SAP, EAT-2, SHP2, SHIP1, Fyn	Memory/effector T cells, activated B, monocytes & dendritic cells	CD150	Cytokine production, Th1/Th2 polarization, B&T cell proliferation
CD229	LY9	SAP, EAT-2, SHP2, AP2	B and T cells	CD229	Unknown
CD244	2B4	SAP, EAT-2, SHP1, SH2 and LAT	NK, CD8, monocytes, and Basophils	CD48	NK and CD8 cell cytotoxicity, NK co-stimulatory molecule
CS1	CRACC, Novel-LY9	SAP	NK, B and T cells and DC	CS1	SAP-independent NK-cell cytotoxicity
NTBA	SF2000, LY108	SAP, SHP1 & SHP2	NK, B and T cells	Unknown	NK-cell cytotoxicity
BLAME	----------	Unknown	Macrophages & DC	Unknown	Unknown
CD84-H1	SF2001, CD2F10	Unknown	DCs, B, T cells, macrophages	Unknown	Unknown

Modified from: Engel *et al,* 2003

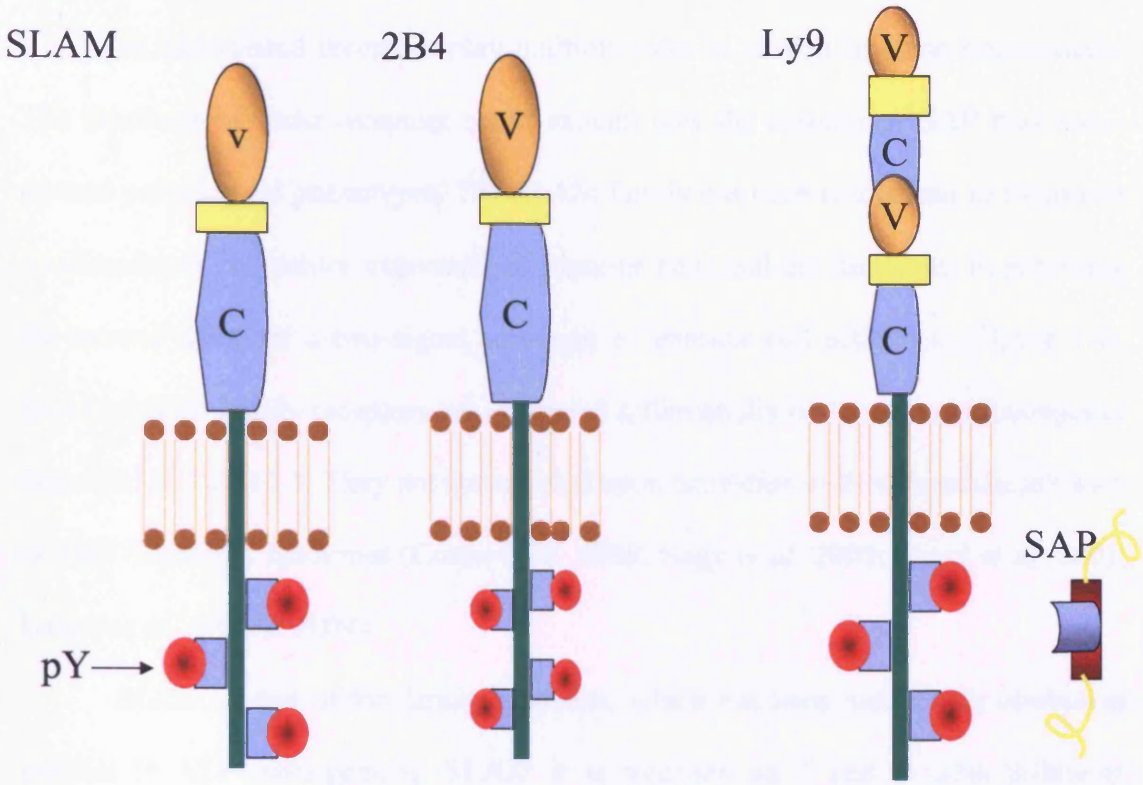


Figure 1.3 The structure of SLAM-related family receptors

The structure of SLAM, 2B4 and Ly-9 are shown. The cell surface receptors of the SLAM-related family are a subset of the CD2 family of glycoprotein (60-100 kD) cell surface molecules, which share common structures. The extracellular domains of CD2 are compromised of a N-terminal non-disulfide bonded Ig variable (V) domain and membrane-proximal truncated Ig constant-2 (C2), which in Ly-9 is duplicated. The cytoplasmic part contains two or more tyrosine-based motifs (pY). The tyrosine-based motifs interact typically with SAP SH2 domain and probably with other molecules containing SH2 domain.

1.6.2 Function of SLAM family in immune response

SLAM-related receptors play multiple roles in normal immune homeostasis. The functions of these receptors could explain how the absence of SAP may cause diverse pathological phenotypes. The SLAM family has been recognised as important co-stimulatory molecules expressed on immune cells and are important in providing the second signal of a two-signal paradigm of immune cell activation (Figure 1.4). SLAM-related family receptors are expressed differentially on lymphocyte lineages as described in Table 1.3. They are upregulated upon activation with various stimuli such as EBV infection, cytokines (Cocks *et al,* 1995; Nagy *et al,* 2000; Engel *et al,* 2003; Latour *et al,* 2003 & 2004).

SLAM is one of the family members, which has been extensively studied in relation to XLP pathogenesis. SLAM is upregulated on T and B cells following antigen receptor activation and similarly on dendritic and B cells through CD40 stimulation (Cocks *et al,* 1995; Punnonen *et al,* 1997; Castro *et al,* 1999). Its expression is also induced after activation of macrophages with LPS and IFN-γ (Howie *et al,* 2002).

Through its extracellular domain, SLAM has homotypic interaction ability to bind to SLAM on other cell lineages (Mavaddat *et al,* 2000). In the presence of SAP, these engagements conduct SLAM mediated signal transduction, which appears to modulate the cytokine response. SLAM activation on human and murine T cells using soluble anti-SLAM antibodies leads to selective production of Th1 cytokines, such as IFN-γ and tumour necrosis factor (TNFα). No effect on the production of Th2 cytokines such as IL-4 is observed (Carballido *et al,* 1997). Cytokine modulation requires pre-activation of T cells. In human preactivated T cells, SLAM activation

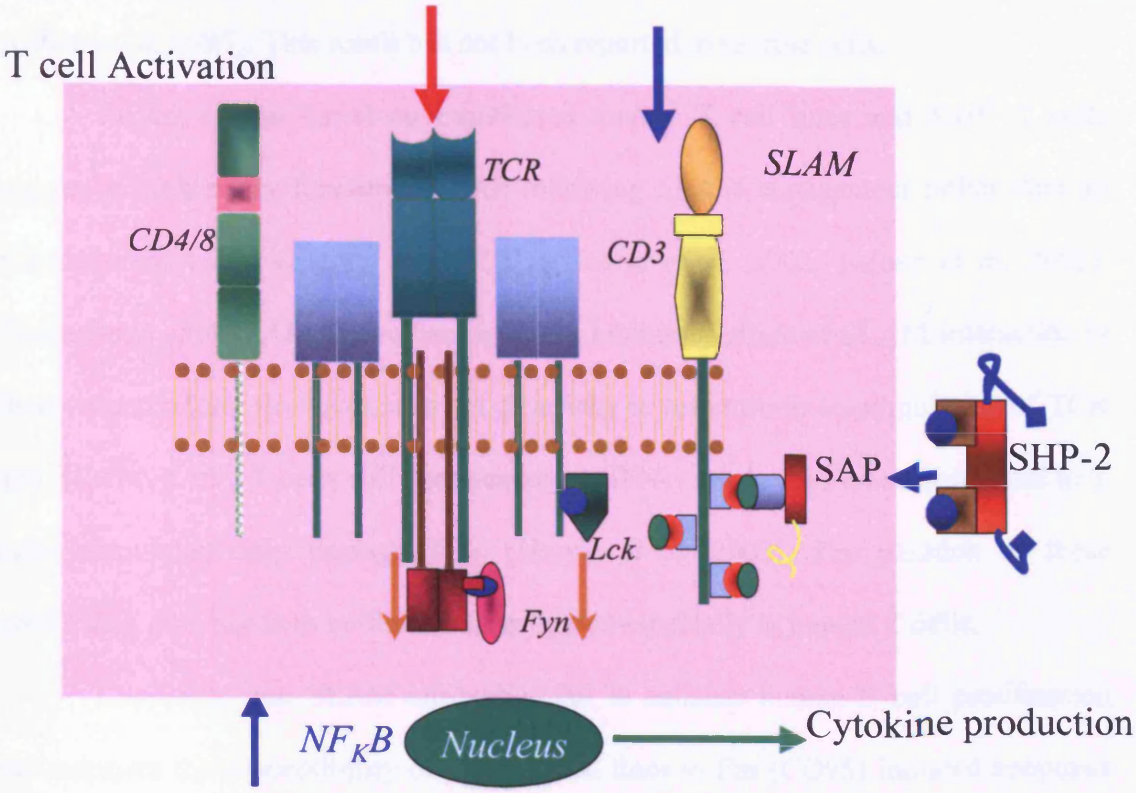


Figure 1.4 A model of SAP and SLAM function in T cell function

The binding of the SLAM-family receptors to their ligands induces the phosphorylation of their cytoplasmic tails, allowing the subsequent binding of SAP or SHP-2 through a tyrosine-containing motif located in their cytoplasmic regions. It has been suggested that SAP prevents SHP-2 binding. This prevents phosphatase activity at the SLAM cytoplasmic tail and thus prolongs phosphorylation after SLAM stimulation. SLAM activation also increases Dok1 and Dok2 phosphorylation and NFκ β levels. It leads to a Th1 upregulation in T cells.

induces proliferation in a CD28 independent manner that is resistant to Cyclosporin A (Cocks *et al,* 1995). This result has not been reported in murine cells.

Recent results based on transfected murine T cell lines and *SAP*$^{-/-}$ T cells suggest an inhibitory function of SAP following SLAM engagement rather than an enhancement effect (Latour *et al,* 2001; Howie *et al,* 2002; Latour *et al,* 2003). Conversely, anti-SLAM antibodies block the inhibitory effect of SLAM interaction in these experiments. However, it is not clear why in response to co-stimulation of TCR and SLAM, *SAP*$^{-/-}$ T cells still produce higher IFN-γ levels than when compared to T cells stimulated only through TCR (Howie *et al,* 2002). The paradox of these conflicting data needs to be further investigated especially in human T cells.

Curiously, anti-SLAM antibodies fail to enhance human B cell proliferation but augment the susceptibility of some B cell lines to Fas (CD95) induced apoptosis (Liu *et al,* 1998; Mikhalap *et al,* 1999). Some groups have reported B cell proliferation and Ig production following SLAM and anti-CD40 activation of B cell lines, but not in primary B cells (Punnonen *et al,* 1997).

Although SLAM is expressed on activated DCs and macrophages, its function has not been fully understood. It may play a major role in antigen presentation and immune response after viral infection. However, data from *SLAM* deficient macrophages has revealed that it may be involved in IL-12, TNF-α, nitric oxide (NO) and IL-6 production (Wang *et al,* 2004).

While SLAM activation seems to modulate Th1 responses, engagement of 2B4 on NK cells affects both cytokine production and degranulation–mediated cytotoxicity (Garni-Wagner *et al,* 1993; Valiante *et al,* 1993). Interaction of CD48, a member of CD2 Ig superfamily with high level expression on monocytes, DCs, NK, T and B cells, and EBV-BLCL, with 2B4 induces the secretion of IFN-γ and IL-2

(Latchman *et al,* 1998; Tangye *et al,* 2000; Chuang *et al,* 2001). 2B4, CS1 and NTBA regulate NK-cell cytotoxicity (Garni-Wagner *et al,* 1993; Bottino *et al,* 2001; Kumaresan *et al,* 2002; Falco *et al,* 2004).

In the absence of SAP, 2B4 and NTBA inhibit, rather than activate, NK-cell cytotoxicity (Parolini *et al,* 2000; Bottino *et al,* 2001). In contrast, CS1 function is SAP-independent in NK cells. CS1 might activate NK cell mediated cytotoxicity through an extracellular signal-regulated kinase (ERK)-mediated pathway. This suggests that SLAM family receptors are likely to signal through both SAP-dependent and SAP independent pathways. However, there is little known about function downstream of other SLAM family receptors, such as NTBA, Ly-9, CD84. Certainly further examination of their structure and function, is required to understand the pathogenesis of XLP and its phenotypic diversity.

1.7 Role of SAP in immune cell signalling

It has been suggested that SAP is a true adapter required for signalling through SLAM-related family receptors. In transiently transfected COS cells, SAP can prevent the SH2 domain-containing protein tyrosine phosphatase (PTP) SHP-2 binding to SLAM or 2B4 (Sayos *et al,* 1998) (Figure 1.4). By blocking SHP-2 binding or by displacement of SHP-2, SAP may modulate phosphatase activity of T cells upon SLAM activation. This is mediated by the SH2 region of SAP and the first of three tyrosines in the cytoplasmic domain of SLAM (Y^{281} in humans, Y^{288} in mice).

It has been also reported that SAP binds specifically to Dok (p62) to block its binding to SHP-2, and thereby SAP by maintaining tyrosine phosphorylation of Dok prolongs the inhibition induced by Dok to the Ras pathway (Sylla *et al,* 2000). However, the inhibitory role of SAP has not been shown in primary cells and with

other members of the SLAM family. Therefore the phosphatase blocking function for SAP may only be an observation in *in vitro* models and it may not hold true in primary cells.

The simplicity of SAP composition was an obstacle to explain its adapter functions and ability to recruit other proteins. However, this recruitment function has been recently documented in transfected T cell lines as well as in *ex vivo* normal mouse T cells. It reflects the formation of trimolecular complex involving phosphorylated SLAM, SAP and Src-related protein tyrosine kinase (PTK) FynT (Latour *et al,* 2001). The capacity of SAP to recruit and activate FynT is highly specific and does not extend to other Src-PTK family members. The crystal structure of the tertiary complex reveals that SAP binds the FynT SH3 domain through an unusual surface-surface interaction that does not involve classical SH3 or SH2 binding interactions. The interaction is mediated by a novel surface in SAP's SH2 domain that is centred around arginine 78, between the sixth β sheet and the second α helix and with SH3 domain of FynT via a nonproline-based motif (Chan *et al,* 2003). It has been suggested that the interaction of SAP with the Fyn-SH3 domain may change the auto-inhibited conformation of Fyn into a stabilised, open and active conformation (Figure 1.5). This facilitates the coupling of Fyn to SLAM (Chan *et al,* 2003). By recruiting FynT to SLAM, SAP promotes the phosphorylation of several downstream intermediates and delivers an inhibitory signal associated with the selective attenuation of IFN-γ secretion by activated T lymphocytes (Latour *et al,* 2001). In addition, following SLAM engagement, SHIP and adaptor molecules Dok1 and Dok2 are also phosphorylated (Figure 1.4). A series of experiments in Fyn-deficient mice showed lack of FynT had inhibitory effects on the tyrosine phosphorylation of SLAM. Moreover, disruption of the SAP/FynT association by

mutation of SAP Arg 78 in transfected cell lines has similar effects on SLAM tyrosine phosphorylation as did SLAM stimulation in FynT deficient mice T cells.

Although it remains unclear whether the association of SAP and FynT is true in primary human T and NK cells, co-expression of FynT and SAP in other cells expressing SLAM family receptors supports the SAP adaptor model.

In NK cells, stimulation of 2B4 triggers an activation signal that implicates phospholipase Cγ (PLC-γ), Vav, the p85 subunit of phosphatidylinositol (PI)-3 kinase and linker for activation of T cells (LAT) (Bottino *et al,* 2000). The association of LAT and 2B4 in membrane microdomains in lipid rafts, may be applicable to other members of SLAM family.

The LAT and 2B4 interaction occurs through a dicysteine motif (CXC) located at the junction of the transmembrane and cystoplasmic regions of 2B4. Consistent with the involvement of PLC-γ in this pathway, ligation of 2B4 can also induce intracellular Ca^{2+} fluxes and PI turnover (Valiante *et al,* 1993). It has been shown this cascade depends on SAP interaction with at least two of five tyrosines regions in cytoplasmic part of 2B4. Thus, in contrast to SLAM activation, SAP mediated signals downstream of 2B4 in NK cells are different, which may support its different effects in these cell populations.

In contrast, EAT-2 is unable to bind the FynT SH3 domain because of the lack of the arginine 78-based motif (Latour *et al,* 2003). This suggests an alternative function for EAT-2 or the possibility of EAT-2 binding to other Src-related kinases such as c-Fgr, Hck and Lyn.

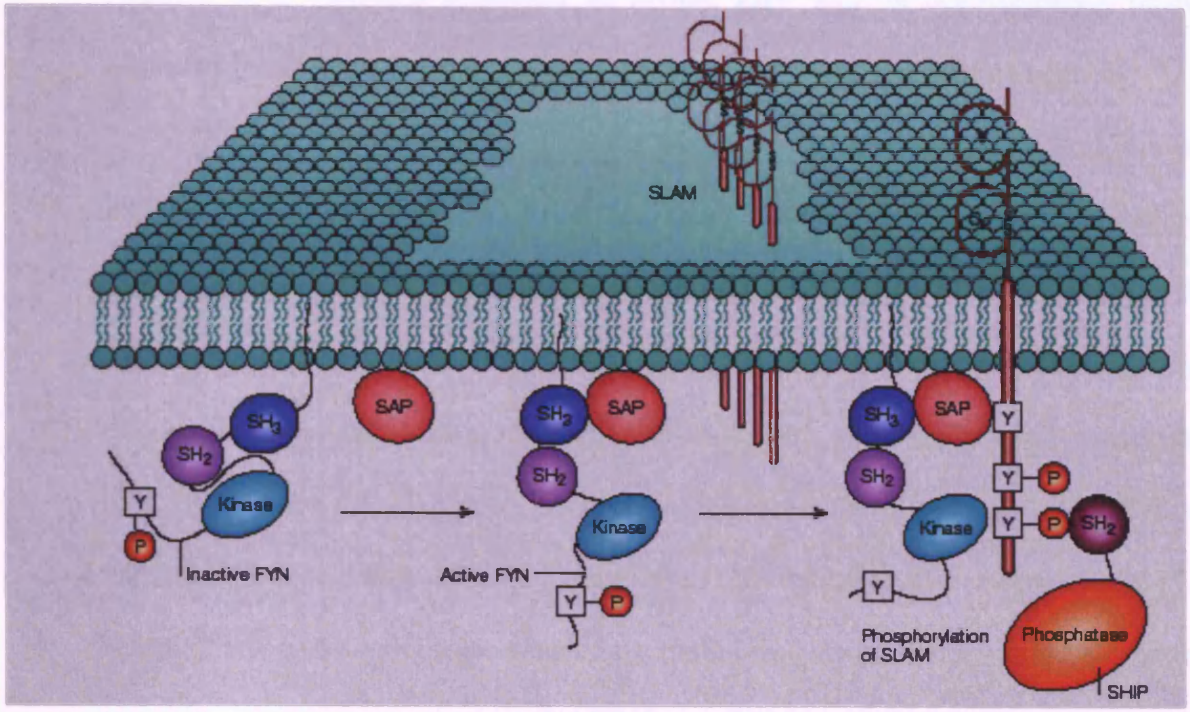


Figure 1.5 A model of Fyn activation and recruitment by SAP

The binding of SAP to the SH3 domain of the tyrosine kinase FynT via an unusual surface-surface interaction and recruitment of this complex through SAP SH2 domain by SLAM changes the inactive conformation of FynT into an active open and stable conformation. Subsequently, receptor-associated Fyn T can phosphorylate tyrosine residues in the cytoplasmic tail of the receptor or neighbouring receptor molecules. This creates docking sites for SH2 domain containing proteins such as SHP2 (SH-2 domain-phosphatase) or SHIP (SH-2 domain-inositol polyphosphate 5' phosphatase). Taken from Engel *et al,* 2003.

1.8 EBV infection and related disorders

As mentioned previously, EBV plays a trigger role in the majority of XLP patients. It is therefore important to review EBV and its heterogeneous related disorders in order to obtain a greater understanding of the pathogenesis of XLP.

EBV is one of the most successful viruses, which ubiquitously infects humans and persists for the lifetime of the individual. EBV is classified as an enveloped gamma-herpesvirus and contains double stranded linear DNA of 170-175 kb in the nucleocapsid (Bornkamm *et al,* 2001). It is well known as the causative agent of infectious mononucleosis (IM) and post-transplant lymphoproliferative disorder (PTLD) (Cohen, 2003). Also, it has been demonstrated that several types of malignancy, including Burkitt lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease and gastric cancer, are EBV-associated cancers. Recently, chronic active EBV infection (CAEBV), EBV-associated haemophagocytic lymphohistiocytosis (EBV-HLH), and mosquito allergy with granular lymphoproliferative disorder (GLPD) and CAEBV-like serological abnormality have been considered as neoplastic diseases related to EBV (Ohga *et al,* 2002). Moreover, lymphoproliferative disorders in patients with autoimmune lymphoproliferative syndrome (ALPS), which is a disorder of defective induction of apoptosis through the Fas- Fas ligand pathway of cell death, might be associated with infections by viruses such as EBV during early infancy (Dianzani *et al,* 1997; Wang *et al,* 1999). XLP disease is also well known genetic disorder for its recognition as a profound defective immune response to EBV. The EBV related immunopathological disorders are summarised in Table 1.4.

Table 1.4 Characterisation of EBV-related immunopathological disorders

	CAEBV	GLPD	XLP	ALPS
Hereditary	Unknown	Unknown	X-Linked	AD (AR)
Sex	M&F	M&F	M	M&F
Gene localization	Unknown	Unknown	Xq25	10q23
Responsible Gene	Unknown	Unknown	SAP/SH2D1A	Fas/FasL/caspase 10
Clinical Phenotype	LPD	LPD	FIM/LPD/ AGL	LPD
VAHS	+	+	++	+
Lymphoadenopathy	+/Malignant, Benign	+/Malignant/ Benign	+/Malignant	++/Benign, malignant
Immunoglobulin	High	High	Low	High
Autoimmune disease	+	–	–	++
EBV-antibodies	High	High	Low	Normal-High
EBV-genome	High	High	High	Normal-high

Adapted from Hiroshi, 2002. The abbreviations are standing for: Autoimmune Lymphoproliferative syndrome (ALPS), Chronic Active EBV infection (CAEBV), Granular Lymphoproliferative Disorders (GLPD), Virus-associated haemophagocytosis syndrome (VAHS), X-linked Lymphoproliferative Syndrome (XLP).

Although the pathogenesis of EBV and its related disorders are not fully understood, the ability of EBV to modulate the immune response may explain the pathogenesis and phenotypes of different EBV related disorders. Immune responses to EBV cover a wide range from very mild responses in asymptomatic infection to an exaggerated response in IM and potentially lethal complications in PTLD and particular susceptibility in XLP patients (Figure 1.6). Thus before discussing XLP pathogenesis in detail, a brief description of recent data on the immune response to EBV and its ability to modulate the immune response would be useful to understand XLP and diversity of its phenotypes in EBV positive patients.

In most human populations, primary EBV infection occurs during the first few years of life. EBV enters the oropharynx and adjacent structures, and preferentially infects B cells via the C3d complement receptor, CD21. Other co-receptors for virus entry have been reported although CD21 route remains the main pathway.

Primary infection during early childhood is mostly asymptomatic or mildly symptomatic, and that during adolescence causes acute IM in 30-50% of cases (Ohga *et al,* 2002). It is not clear whether the severity of age related presentation is related to virus load or to age related differences in host immunity. Acute IM patients present with fever, painful lymphoadenopathy, sore throat with pharyngotonsillitis, hepatosplenomegaly, and skin eruption. During this period the EBV-infected B cells increase to 1-20% of circulating B cells. This is followed by a vigorous immune response consisting of a brisk rise in NK cells, CD8 T cells, IFN-γ production, and antibody-dependent cell mediated cytotoxicity (ADCC) (Rickinson *et al,* 1997). The results of CD8 T cell response are an atypical lymphocytosis, which can be as many as 60% of the peripheral mononuclear cells population. CD8 T cells show the selective expansion of Vβ7 and Vβ6 in some IM patients (Smith *et al,* 1993).

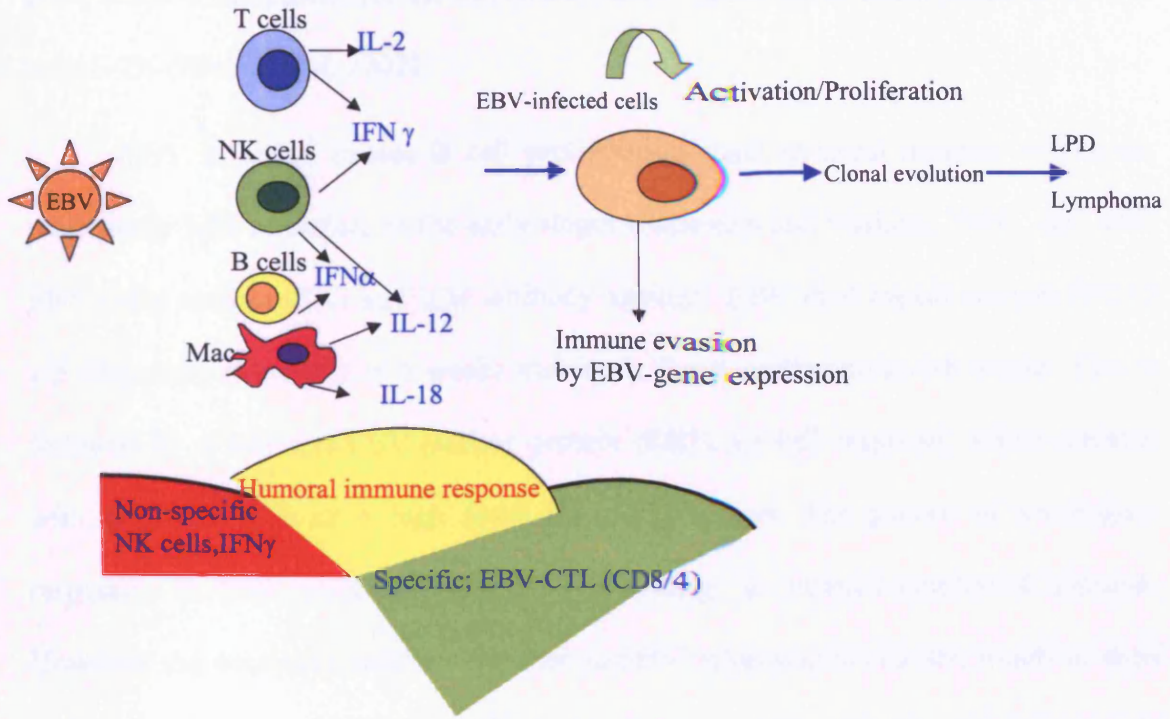


Figure 1.6 The immune response to EBV infection and disturbance of immune function leading to LPD disorders

Primary infection with EBV induces non-specific (NK, IFN-γ) (indicated as red colour and specific including humoral (indicated as yellow colour) and cellular (indicated as green colour) mainly cellular immune responses. EBV- infected cells can escape from immune surveillance with EBV genomic invasive machinery. The EBV maintains its escape with invading immune response leading to aberrant cytokine production. As a result EBV induces B cell clonal formation and finally LPD/Lymphoma disorders.

However, T cells specific for lytic epitopes account for up to 44% of the CD8 T cell pool, while CTL specific for the immunodominant proteins of latent proteins reached only 1-2% (Hislop *et al,* 2002).

EBV infection causes B cell proliferation and humoral immune responses, particularly IgM secretion, in the early stages (Papesch and Watkins, 2001). IgG anti-EBV early antigen (EA) and IgM antibody against EBV viral capsid antigen (VCA) are frequently detectable at 4 weeks and reach their maximum at 6-8 weeks. This is followed by a late anti-EBV nuclear protein (EBNA) IgG response, which remains with IgG anti-VCA at a high level for many years. The pattern of serological responses to EBV infection may be misleading in immunosuppressed patients. However, the dominant immune response in EBV infection is cellular, which in itself sometimes has a suppressive effect on Ig secretion and B cell lymphocyte proliferation.

The EBV genome encodes more than 100 proteins (Epstein, 2001). In this context EBV is capable of modulating the immune response by encoding novel homologs of cytokines. BCRF1 is one of the early lytic genes, which exhibits a human IL-10 function and negatively regulates IL-12 to inhibit IFN-γ production (Hiroshi, 2002). The overall biological effects of these proteins remain elusive.

EBV infected B cells also show the immortalisation ability of the EBV genome. Following the initial replicative (lytic) infection, the EBV genome circularises to be maintained as a multicopy plasmid in the B cell nucleus. There are 12 genes which encode different proteins involved in the latency programs of EBV. The 6 nuclear antigens EBNA1, 2, 3 (A, B), 5 (LP), 3C and 3 latent membrane proteins (LMP)1, LMP2A, LMP2B are the most important in maintenance of latency (Papesch and Watkins, 2001). Different EBV related tumours express some of these

antigens dependent on the type of latency (Type I,II,III). In persistent infection, the major latent epitopes targeted by CTLs are EBNA3A, EBNA3B, EBNA3C (Rickinson *et al,* 1997). It has been suggested that EBV- specific CD4 T cells may play a significant role in primary infection and in the maintenance of functionally competent CD8 memory (Zajac *et al,* 1998; Amyes *et al,* 2003). More recently a cytotoxic function of EBV- specific CD4 T cells has been reported particularly against EBNA1 proteins (Steigerwald-Mullen *et al,* 2000; Munz *et al,* 2000).

However, despite exciting progress in understanding the role of EBV-CTLs and Th1/Th2 polarization of the cytokine balance during EBV infection, many fundamental aspects in the pathogenesis of EBV related disorders remain unknown. Thus verification of cellular pathogenesis of XLP may shed some light not only on the EBV immune response, but also on the development of lymphoid malignancies, therapeutic and preventive strategies for the EBV-associated diseases.

1.9 Immunoserological studies in XLP

1.9.1 Immunoserological defects in XLP patients

Although most patients with XLP are healthy prior to EBV infection, it has been suspected that there may be subtle immunological abnormalities. A number of studies have been undertaken on XLP boys who had been identified as a result of previous family history and by inheritance of the abnormal X-chromosome by linkage analysis, but the data is inconclusive. A report from Purtilo *et al* showed approximately 5% of boys with XLP prior to EBV infection, had developed measles pneumonitis, with a few cases infection of Neiseria meningitis and also disseminated vaccinia following smallpox vaccination (Purtilo *et al,* 1991). Defects in immunoglobulin isotype switching (IgM to IgG) have been identified, using

vaccination with the neoantigen ϕ/X174 (Purtilo *et al,* 1989). In the largest study to date, 32 boys were identified as having XLP, all of whom were serologically negative for EBV at the time of analysis (Seemayer *et al,* 1995). Of these, only five were healthy with a normal immunological profile. Of the remaining 27, 17 had dysgammaglobulinaemia with elevated levels of IgA and IgM and /or variable defects in IgG, IgG1 or IgG3 production. However, in the majority of cases these findings did not have any significant clinical presentation.

1.9.2 Immunoserological defects in XLP female carriers

Carrier females in XLP are in general asymptomatic but analysis of humoral responses to EBV suggests a number of abnormalities. This includes a fourfold elevation in IgG anti-VCA titres, and persistence of IgM and IgA anti-VCA antibodies, both of which are not normally found after seroconversion (Sakamoto *et al,* 1982). IgG antibodies against the Epstein–Barr-virus early antigen (EA) were present in over half the carrier group but in only 19% of the control population. It has been suggested that these findings are indicative of continued viral replication or an attempt to compensate for a partial T-cell defect but the absence of other clinical and immunological problems in carrier females makes this unlikely. However, the recent data on murine models along with genetic epidemiological studies suggests that SAP deficiency is a recessive phenotype, therefore one normal SAP allele could keep immunological balance in carriers (Crotty *et al,* 2003; Yin *et al,* 2003).

1.10 Molecular and cellular pathogenesis of XLP

The hallmark of XLP is a dysregulated, ineffective host response against EBV and other virulent pathogens. Although the exact pathogenesis of XLP is unknown, it

has been suggested that the inability of the immune system of XLP patients to control EBV-infected B lymphocytes is likely to be due to defects of Th cells, CTLs, and NK cells. SAP is a component of the signalling mechanism used by the SLAM family receptors and represents a new paradigm in signal transduction defects associated with primary immunodeficiencies (PIDs). In contrast to other signalling molecules involved in PIDs, such as γc, JAK-3, and ZAP70 in severe combined immunodeficiency and Btk in X-linked agammaglobulinemia which, are non adaptor components in immune cell signalling, SAP exhibits a modulator role in signal duration and intensity. Lack of SAP function leads to a series of molecular dysfunctions in immune cells causing diverse phenotypes.

However, a series of studies in humans and more recently in *SAP* deficient murine models has clarified some of the molecular and cellular abnormalities in XLP. The data from human and murine models are, in some aspects, contradictory and deserve further analysis. Thus the pathogenesis of XLP is discussed in two sections as below.

1.10.1 XLP Pathogenesis based on human findings

In the first description of XLP pathogenesis reported by Purtilo, an intrinsic defect in B cells was suggested to play the main role in XLP (Purtilo, 1976). The clinical and pathological findings reported by Purtilo showed that affected boys suffered from chronic bacterial infections and low serum Ig level before and after EBV infection. Therefore, it was suggested that the dysgammaglobulinemia was caused by an arrest in B cell maturation to plasma cells. In addition, he observed a vulnerability of XLP boys to rubeola (measles) and vaccination, and concluded that T cell anergy due to the B cell defect impaired the control of B cells and was a major

part of XLP pathology. Whereas, SAP is not expressed in the majority of B cells, an intrinsic B cell deficiency is unlikely and the humoral immunodeficiency is likely due to dysregulation of T helper cell function (Lai *et al,* 1987). It is well documented that CD4 T cells play an important role in providing appropriate B cell help through costimulatory signals such as CD40L, CXCR5 expression and cytokine secretion, which may highlight SAP function in CD4 T cells in parallel with existing pathways to initiate B cells response to EBV infection (Breitfeld *et al,* 2000). It holds true when XLP males with immunoglobulin class and subclass deficiencies prior to EBV infection are considered (Grierson *et al,* 1991). However, this remains elusive in XLP pathogenesis. Also development of B-cell lymphoproliferative disease in XLP initially led to the proposal that B cells in XLP patients may be less susceptible to T cell-mediated killing (Ando *et al,* 1986). However, the lack of SAP expression in B cells particularly EBV-transformed B cells and evidence that XLP-derived B cells resemble normal LCLs *in vitro*, with respect to induction of EBV-specific cytotoxic T cells (CTL), the ability to present EBV viral antigens, and susceptibility to EBV-specific and MHC-restricted CTL-mediated killing, suggest that the cellular defect is not B-cell specific (Jager *et al,* 1998).

Large numbers of responding NK cells and CD4+ and CD8+ T lymphocytes are typically involved in the normal response to primary EBV infection. Furthermore, the large number of activated effector T cells are associated with the release of cytokines and inflammatory mediators that ordinarily lead to IM. Although XLP patients prior to EBV infection show a normal peripheral lymphocyte count, except a reverse CD4/CD8 ratio, it has been reported that B and T cells (mainly CD8) excessively proliferate and infiltrate the organs during their acute phase of EBV infection (Seeley *et al,* 1981; Lindsten *et al,* 1982; Sullivan *et al,* 1983 & 1989). This

suggests that EBV infection is the significant trigger for the phenotypic expression of XLP syndrome due to the excessive T cell activation and cytokine imbalance. Also it has been reported that EBV genomes were detected in peripheral blood lymphocytes and organs of affected males suggesting that failure of cytotoxic activity may be part of the pathology in XLP (Purtilo *et al,* 1991). As SLAM is expressed on T and B lymphocytes, it is postulated that homotypic binding, promotes T cell costimulation, proliferation, and production of Th1 cytokines. During EBV infection, SLAM-SLAM and MHC II molecule-TCR interactions at the interface between EBV-infected B cells and CD4 T cells, or CD48–2B4 interaction between EBV-infected B cells and NK cells, may promote the development of EBV-specific Th1 responses. Studies on specific T-cell immunity to EBV are contradictory. Firstly, Purtilo and his colleague suggested that T suppressor lymphocytes (CD8) might play a role in impaired immune surveillance against EBV (Purtilo, 1976). EBV-specific memory T-cell activity, as measured by inhibition of autologous EBV-LCLs outgrowth (regression assay), was defective in seven out of ten of XLP patients studied in comparison to female carriers and healthy individuals (Harada *et al,* 1982). Sullivan *et al* (1983) also demonstrated failure of T cell cytotoxic activity against a variety of targets related to EBV. In contrast Rousset *et al* (1986) reported two XLP patients with hypogammaglobulinaemia exhibited normal EBV-specific HLA-restricted cytotoxic activity, although he was not able to show the cytotoxic activity of the EBV specific T cell clone level in those patients. Later, Okano *et al* (1990) reported a 19 year-old boy with XLP and IM with defective killing activity of PBL against his LCL during the course of the disease. In another study, it was shown that the cytokines produced by T cells from ten XLP patients failed to inhibit B-LCL growth (Yasuda *et al,* 1991). For the first time, Yasuda tried to explore the underlying mechanisms of T cell

cytotoxic dysfuntion in XLP by investigation of Th1 cytokines. In Yasuda's study, IFN-γ production was normal upon allogenic LCL stimulation in XLP patients. However, T cells from XLP patients exhibit a significant decrease in IFN-γ production in response to stimulation by autologous EBV-LCLs (Yasuda *et al,* 1991). Furthermore, the production of IL-2 by T cells upon PHA or autologous B-LCL stimulation showed no difference between XLP patients and those in the normal control group. Okano *et al* reported a 20-month- old XLP boy with FIM who showed detectable IFN-γ in his serum and PBL culture supernatant, which decreased during the acute phase of EBV infection. Exogenous IFN-γ administration caused transitional improvement of his clinical condition with decreasing numbers of EBV positive cells (Okano *et al,* 1989 & 1990 a and b).

More recently, it was shown that Herpesvirus-saimiri-transformed CD4+ T cells from XLP patients exhibited severe defects in cytokine secretion (Nakamura *et al,* 2001). Stimulation with anti-SLAM caused no significant IFN-γ secretion and also activation of the TCR resulted in little or no up-regulation of IFN-γ or interleukin 2 (IL-2) in comparison with controls. Similar results were observed in mixed lymphocyte reactions (MLRs) (Nakamura *et al,* 2001). Moreover, molecular studies of XLP transformed CD4 T cells showed an enhanced level of basal and TCR-induced tyrosine phosphorylation of CD3ζ, ZAP70, and Cb1. Other key partners of TCR downstream kinase families including mitogen-activated protein kinase (MAPKs), external signal-regulated kinase (ERKs), c-Jun NH2-terminal kinase (JNKs) and also pleotropic proteins (Akt, GSK) showed an increase in phosphorylation upon TCR activation in XLP CD4 T cells, but the level of phosphorylation declined faster in comparison with phosphorylation in normal CD4 T cells. It suggests that SAP is important in TCR down stream signalling pathways and

may control cytokine production not only through SLAM family receptors but also via the TCR pathway. More recently it was shown that SAP deficient T cell lines generated by stimulation of T cells with allogeneic B-LCL demonstrated impaired TCR-CD3 signalling (Sanzone *et al,* 2003). In support of the above results, the SAP deficient lines showed an enhanced phospholipase C γ1 (PLC γ1) phosphorylation and calcium response, but phosphorylation of VAV and downstream signal transduction events such as MAPKs were diminished (Sanzone *et al,* 2003). This also leads to diminished IL-2 production upon TCR activation in XLP T cell lines. Furthermore, it is well known that LAT (linker for activation of T cells) is a central adaptor for transduction in proximal CD3 signalling associated with PLCγ, VAV and other kinase proteins (Zhang *et al,* 1998). Also LAT is associated with 2B4 via a dicysteine motif in NK cells, and the presence of SAP is important in LAT function as a central adaptor (Latour and Veillette, 2003). It is likely that SAP deficiency could provoke selective alterations in the phosphorylation of distinct LAT tyrosine residues either through SLAM family receptors or via alternative mechanisms causing imbalanced signalling in T cells from XLP patients. However, although the above information provides a better understanding of T cell dysfunction in XLP, the molecular basis of TCR mediated T cell defects, particularly in association with SLAM family receptors remains largely elusive and needs further investigation.

Studies on NK cells from XLP patients were more conclusive than T cells, and it has been suggested that the failure of cytotoxicity is 2B4-mediated, although other SLAM family receptors expressed on NK cells have not been excluded. NK cell activity was one of multiple XLP immune defects described (Sullivan *et al,* 1980). Sullivan *et al* in their report published in Science (1980) demonstrated XLP-NK cells from 12 patients aged 5-19 years old exhibit significantly lower killing activity

against K562 target cells in comparison to controls (10 out of 12) but in 2 XLP cases prior to EBV infection the NK killing activity was intact. In contrast, 10 XLP carriers, 10 patients with IM, and 33 normal individuals presented a high NK cytotoxic activity. In a second study by the same group, NK cytotoxic activity against K562 target cells in 2 XLP patients was normal but abnormal in a third XLP patient (Masucci *et al,* 1981). However, it was later shown that NK cell killing activity against K562 or Daudi (a Burkitt's lymphoma cell line) cell lines is abnormal in XLP patients (Harda *et al,* 1982; Argov *et al,* 1986; Benoit *et al,* 2000; Tangye *et al,* 2000). Moreover the effect of exogenous IFN-γ to improve NK cell cytotoxic function is controversial, and differential effects have been reported (Masucci *et al,* 1981; Argov *et al,* 1986; Okano *et al,* 1989& 1990). Argov *et al* (1986) also showed that NK cell function mediated by antibody-dependent cellular cytotoxicity (ADCC) is intact in XLP patients. Thus, when XLP NK cells were stimulated with receptors that do not bind SAP such as CD2, CD16 or NCRs, cytotoxicity was comparable to normal controls, suggesting only specific pathways are affected in these patients (Argov *et al,* 1986; Parolini *et al,* 2000; Tangye *et al,* 2000). 2B4-CD48 interaction augments non-MHC restricted cytotoxicity by NK cells (Nakajima *et al,* 2000). As K562 cell lines are CD48 and HLA negative, the recent data casts doubt over the NK cell killing failure reported previously. The more recent studies investigated cytotoxic function at a molecular level to solve this paradox. The 2B4-mediated cytotoxic activity in NK cells is Ras/Raf dependent and involves multiple MAPK signalling pathways including ERK1/2 and p38 (Chuang *et al,* 2001). 2B4 activates NK cells via a LAT-dependent signalling pathway, in which the binding of LAT and 2B4 is SAP dependent and 2B4- mediated cytotoxicity is dependent on SAP interaction with 2B4 (Tangye *et al,* 2000; Chuang *et al,* 2001). Therefore, as CD48 is highly expressed on

EBV-LCLs it was speculated that the NK cell defect in XLP is limited to 2B4/CD48-specific NK-cell killing activity. In addition, inducing expression of CD48 on a transfected K562 HLA negative leukaemia cell line enhanced killing activity of normal NK cells. XLP-NK cells showed decreased killing of CD48-expressing target cells in comparison with normal controls (Parolini *et al,* 2000). Disruption of the association between 2B4 and CD48, using a 2B4 masking antibody, restored normal cytolytic function, suggesting that this defect arises from the delivery of an inhibitory signal through the 2B4 /CD48 interaction (Parolini *et al,* 2000; Tangye *et al,* 2000).

However, it remains undefined how the lack of SAP affects cytokine production, particularly IFN-γ, and also CS1 and NTBA function in XLP patients and their specific role in pathogenesis. In this regard, a subpopulation of killer cells called lymphokine-activated killer cell (LAK) are defective against Raji cell line (a NK-resistance cell line) in XLP, and again the pathology of the defect has not been fully explored (Benoit *et al,* 2000).

1.10.2 XLP pathogenesis based on murine model findings

The first animal experiments for XLP were performed in a severe combined immunodeficiency (SCID) mouse model by Purtilo and his colleagues (Purtilo *et al,* 1991). The engraftment of PBL from 9 XLP patients showed no graft-versus-host disease (GVHD), suggesting T cell defects. In contrast, in a control group the majority of engrafted mice developed GVHD. After almost a decade, the discovery of the SAP gene made it possible to generate an XLP murine model. Three groups have generated *SAP*-deficient mice models by targeting different exons of the *SAP* gene (exons 1,2 or 3) in mice (Czar *et al,* 2001; Wu *et al,* 2001; Yin *et al,* 2003). Wu *et al* deleted exon 1 whereas Czar *et al* and Yin *et al* developed their model by targeting exon 2 and 3,

respectively. In murine *SAP* knockout models, lymphocyte development is normal as previously reported in humans (Czar *et al,* 2001; Wu *et al,* 2001; Yin *et al,* 2003). Expression of cell surface markers for lymphocyte sub-population were normal in knockout *SAP* murine models (Czar *et al,* 2001; Wu *et al,* 2001; Yin *et al,* 2003). Before exposure to any kind of infection the only immunological abnormality in the mice model was a lower level of all IgG subclasses (Yin *et al,* 2003) and low level IgE reported in two models (Czar *et al,* 2001; Yin *et al,* 2003). However, the *SAP*-deficient models showed a number of different post infection pathological immune responses. Challenging of the mice with LCMV (lymphocyte choriomeningitis virus: a virus that elicits similar responses to infection with EBV), or murine gammaherpesvirus-68 (a herpesvirus of wild rodents which genetically is related to human herpesvirus-8 and human EBV) or Toxoplasma resulted in impaired Th2 differentiation and Th1/Th2 skewing.

The *SAP* deficient mouse models present a relatively intact immune response to clear acute infection but progress into immune dysregulation with pathological presentations. However, infection with a more virulent clone of LCMV (clone 13) or a hepatotropic strain of LCMV (LCMV-WE) causes increased morbidity (Czar *et al,* 2001) or mortality (Wu *et al,* 2001) in these models. Conversely, infection with gammaherpesvirus-68 in the *SAP* deficient mice does not increase in any morbidity or mortality (Yin *et al,* 2003).

Moreover, all three models showed an increased number of activated T cells, mainly CD8 T cells, presenting with higher IFN-γ production after 1-2 weeks in comparison with the wild type mice. The CD8 T cells in response to LCMV-specific peptides produce excessive IFN-γ and TNFα production in comparison to a normal control. Also the number of antigen-specific CD8 T cells for LCMV antigens stained

with the MHC-class I Ag-specific tetramers, was significantly higher than the control group after LCMV infection (Czar *et al,* 2001). The overall proliferation of CD8 T cells in response to gammaherpesvirus-68 infection shows subsets expressing the Vβ4 T cell receptor, a characteristic feature after murine gammaherpesvirus-68 infection (Yin *et al,* 2003). These data may confirm the function of CD8 and cytotoxicity of CD8-CTLs are intact in *SAP* defective murine models. The organ lymphocyte infiltration is mainly of T cells and may be caused by over responsiveness of T cells. The higher proliferation of SAP defective murine T cells in response to TCR or SLAM stimulation is reported before infection.

CD4 T cells of *SAP* deficient mice show an enhanced IFN-γ, TNF-α and IL-2 production in response to infection but the total number of CD4 T cells was comparable between wild type and *SAP* deficient mice, except in the Wu *et al* (2001) report in which the number of CD4 T cells was slightly higher than wild type mice (Czar *et al,* 2001; Wu *et al,* 2001; Crotty *et al,* 2003; Yin *et al,* 2003). In addition, it has been reported that T helper CD4 cells have impaired production of IL-4, IL-10, IL-13 in SAP deficient mice, which would be corrected with Th2 polarization (Wu *et al,* 2001). However, the murine SAP defective T cells, before infection, upon stimulation of TCR or SLAM also show a consistently high IFN-γ and low production of IL-4, IL-10, IL-13, suggesting fundamental immune response impairment. It has been well documented that the rapid production of IL-4 by CD4 T and NKT cells in response to Lieshmania major (L-M) causes persistence of infection and susceptibility of mice (Launois *et al,* 1997). The *SAP* knockout murine model showed a Th2 defect in response to L-M infection similar to that shown in response to LCMV infection, supporting the idea that the regulation of IL-4 production is SAP dependent (Czar *et al,* 2001).

Furthermore, the *SAP*- deficient mouse model following infection with LCMV, T gondii, L.M, or challenged with ovalbumin (OVA) shows a lower level of IgE and IgG production particularly after the acute phase (Czar *et al,* 2001; Wu *et al,* 2001; Crotty *et al,* 2003; Yin *et al,* 2003). Although the presence of dysgammglobulinemia before infection reported by some groups suggests an intrinsic B cell dysfunction in addition to impaired T helper cell function, the recent results using an adoptive transfer system have emphasised the role of SAP in CD4 T cell function for late B-cell help and development of long-term humoral immunity (Crotty *et al,* 2003). The number of antibody-secreting cells (ASCs) and IgG serum level (IgG1, IgG2a, IgG2b) are increased after infection but decrease after two weeks in the *SAP* deficient murine model, confirming SAP is not important for early B cell help and class switching. In vitro studies show murine *SAP*$^-$ B cells proliferate and differentiate into ASCs normally. The normal expression of CD40L and CXCR5 on *SAP*$^-$ CD4 T cells confirm that the interaction and migration of T helper cells as an important part of B cell help is intact in XLP. The reconstitution of *SAP*$^-$ B cell function after adoptive normal CD4 T cells transfer *in vivo* underlines the crucial role of Th2 cytokine polarization in XLP (Figure 1.7).

Several groups have studied the biochemical immunopathological response in *SAP* deficient murine models. Upon SLAM stimulation the *SAP*$^-$ T cells show defective phosphorylation of SHIP, the adaptor molecules Dok2, Dok1 and Shc, and RasGTPase-activating protein RasGAP that leads Th1/Th2 imbalance (Latour *et al,* 2001; Chan *et al,* 2003). This pathway is regulated by SAP dependent coupling of Fyn to SLAM. Although, the same dysregulation has been shown in Fyn- deficient T cells, it was not addressed how the absence of SAP causes the aberrant TCR signal. To address this question, Howie *et al* suggested that the recruitment of SLAM and

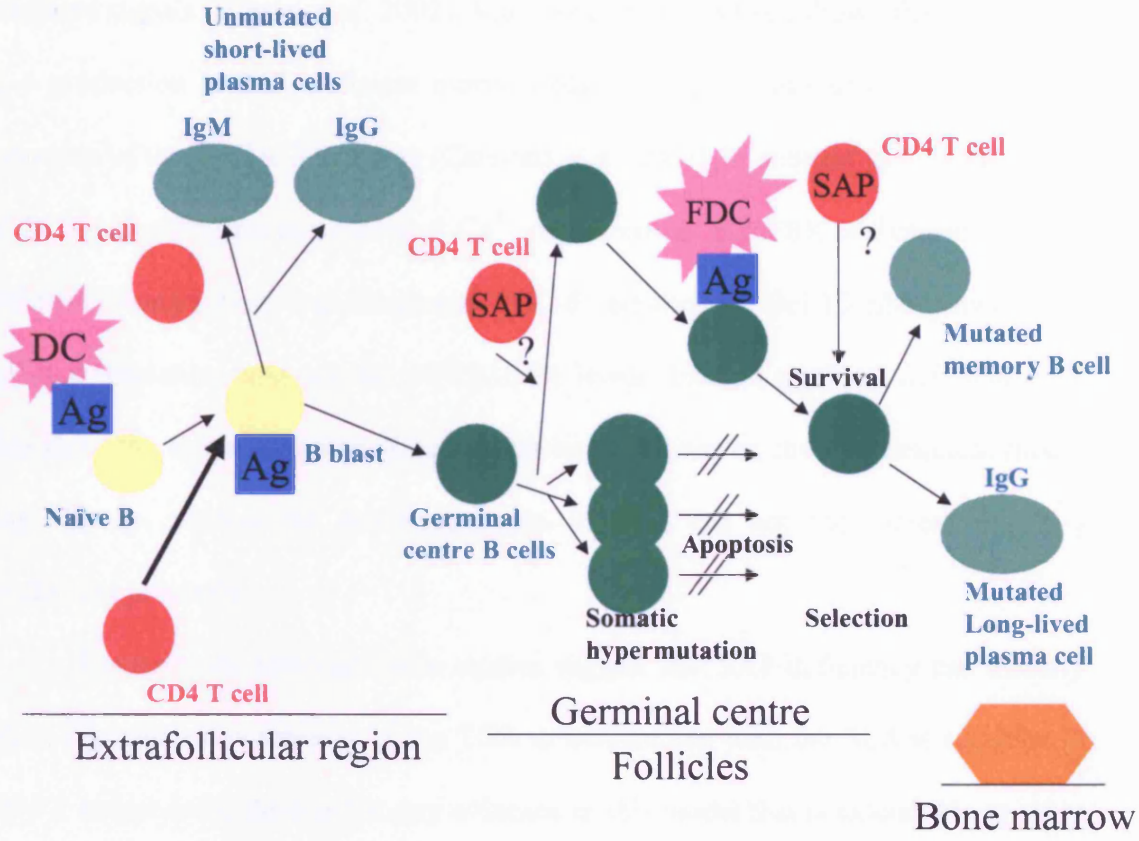


Figure 1.7 SAP function via CD4 T cells and its potential role in pathways of B cell differentiation

In germinal centres, SAP-expressing CD4+ T cells may promote the formation and/or survival of B cells which after completing affinity maturation become memory B cells and long-lived plasma cells. In a murine model it has been shown that SAP expression is necessary for the long-term humoral immune response (adapted from Welsh *et al,* 2003).

SAP to the site of TCR engagement may explain how SLAM influences TCR-mediated signals (Howie *et al,* 2002). More recently it has been shown that a defect of IL-4 production in *SAP-* deficient murine cells is caused by impaired TCR-mediated induction of the GATA-3 pathway (Cannons *et al,* 2004). In contrast to humans, study of TCR signalling revealed normal Ca^{2+} mobalization and ERK activation in *SAP-*deficient murine cells, but decreased PKC-θ recruitment, Bcl-10 phosphorylation, IκB-α degradation, and nuclear NFκB1/p50 levels. Interesting, Fyn- deficient cells also show the same TCR signalling dysregulation. However, the *Fyn* deficient model was able to address the down-regulation of IL-4 but not the increased IFN-γ production precisely.

Furthermore, although these studies suggest that SAP deficiency can directly affect signalling downstream of the TCR in conjunction with the SLAM receptor in CD4 T helper cells, there is not any evidence in this model that is extendable to other lymphocyte populations or to other SLAM family receptors affected by SAP regulatory function in the immune response.

1.11 Diagnosis and treatment of XLP

Molecular analysis of the SAP gene and protein expression are the latest methods, which are used by a number of different centres, for diagnosis of XLP. Mutation analysis with direct sequencing of the four exons using intronic primers is performed to give high sensitivity results. However, the genetic test for XLP shows a low detection rate of between 54 and 64% (Yin *et al,* 1999) even in cases with a X linked pedigree and particularly in cases with sporadic presentation. The linkage studies on XLP probands mapped the XLP gene to the Xq25 region, suggesting that another gene abnormality is unlikely. Thus mutations in SAP promoter or regulatory

regions should be sought at least in a majority of cases with a negative test of SAP mutation analysis. More recently, a SAP protein assay has shown a higher detection rate even without a positive genetic test, which again suggests the defect in some cases may lie in regulatory or promoter of SAP expression region rather than in the gene (Gilmour *et al,* 2000). However, the genetic test for SAP in affected males with an X-linked pedigree and definite XLP presentation is highly sensitive with 97% (Sumegi *et al,* 2000). The defect in cases with sporadic or atypical presentation is elusive. To improve the diagnosis of XLP cases and prevent misdiagnosis, the European Society for Immunodeficencies (ESID) and the Pan American Group for Immunodeficencies (PAGID) has recommended guidelines for XLP diagnosis (Conley *et al,* 1999). The criteria are based on SAP mutations, common and uncommon phenotypes, EBV infection, and the presence of affected maternal male relatives with a history of similar disease. According to these criteria there are three groups of cases including definite, probable, and possible criteria, where the chance of having XLP is different in each group. Still there are some cases with a negative genetic test and lack of SAP protein expression but with a similar XLP phenotype, known as XLP like disease, which still remain undefined. However, a better understanding of the underlying XLP molecular pathology will allow the design of more definite guidelines. The new technology such as microarrays or proteomics arrays would provide this along side better and more comprehensive diagnostic tools in XLP.

After diagnosis of XLP, the most striking challenge is treatment of XLP cases particularly those with a poor prognostic phenotype. At present, the only definite and preventive cure for XLP and the EBV and non-EBV complications is allogeneic hematopoietic stem cell transplantation (HSCT) either using bone marrow, peripheral

blood, or umbilical cord blood stem cell (Gross *et al,* 1996). However, it is mostly unsuccessful in affected males with an age of more than 15 years and also with pre-HSCT infective complications. Other therapeutic strategies developed for treating manifestations of XLP such as using antiviral drugs, high-dose immunoglobulin, immunosuppressive agents, and IFN-γ have been largely unsuccessful, and are palliative at best. The lack of success of conventional treatments for XLP makes the development of a gene therapy strategy for XLP an attractive proposition. There is great potential to use gene manipulation either as investigation tool or therapeutic strategy in XLP. The following section will discuss the gene therapy approach in general and specifically on advantages of retroviral gene therapy in primary immunodeficiencies.

1.12 Gene therapy and viral vectors

Somatic gene therapy is defined as the delivery of new genetic material into a patient's somatic cells for the treatment of disease and is made possible through the development of viral and non-viral gene transfer vectors. Primary immunodeficiency (PID) disorders are good candidate disorders for this type of therapy and have played a major role in the development of techniques for human gene therapy. A series of encouraging results obtained in chronic granulomatous disease (CGD) patients (Grez *et al,* 2000) and patients affected with the X-linked and ADA forms of severe combined immunodeficiency SCID (XSCID & ADA-SCID) have shown promising progress in recent years in the primary immune deficiency field (Blaese *et al,* 1995; Fischer *et al,* 2001; Aiuti *et al,* 2002; Gaspar *et al,* 2004; Muul *et al,* 2003). The technical progress will be critical for future development of gene therapy approaches for other forms of PIDs.

Since the cloning of the gene responsible for XLP, it has been confirmed as one of the monogenic X-linked primary immunodeficiency diseases, which with our developing knowledge of its molecular and cellular pathogenesis makes it an interesting disorder to develop for gene therapy. Distribution of SAP expression mainly in T and NK cells strongly suggest that gene therapy would be very possible since, both T and NK cells are readily accessible and particularly since T cells can be efficiently transduced. Gene reconstitution in XLP could be a strong tool to study the cell mediated immune response in XLP and also it might offer a new approach for treatment of this fatal genetic disorder.

To successfully achieve gene therapy for XLP, it is crucial to develop an optimal vector. An ideal vector should; (1) allow efficient and selective transduction of the target cell of interest, (2) be maintained and (3) expressed at levels necessary for achieving therapeutic effects, and, last but not least, (4) be safe in terms of avoiding unwanted side effects in the host. Viruses are considered as a good tool for gene transfer as they have evolved to deliver their genome efficiently to target cells with subsequent high level-gene expression. Vector systems for therapeutic gene transfer have been developed from different virus groups, each system having specific advantages and drawbacks. Viruses are obligate parasites and cannot replicate without the help of host cells. Thus they are, by nature, adapted to efficiently transmit genetic information to cells. Among the viruses many different strategies have evolved that allow them to enter cells, and the form of their genetic information to some extent determines the latter mechanisms used for replication and production of new virus particles or virons.

Retroviruses are particularly of interest in this respect, because although they carry an RNA genome, this genome is transcribed into a double-strand DNA in the

infected cell, which can then efficiently integrated into the host cell DNA (reviewed by Coffin *et al,* 1997). Once integrated, the virus DNA, known as a provirus, is transcribed like any other cellular gene and the virus-specific DNA is used to produce viral proteins and new genomic RNA, both of which are assembled into new virons. The integrated provirus is a stable part of the cell genome for life of the cell and is passed on to all daughter cells that arise from the original infected cell. This latter property of retroviruses makes them ideal candidates as gene delivery vehicles, or vectors, that give long-term gene expression. The ability to integrate up to 9 kb of foreign DNA into the host genome and the lack of immunogenicity has made the use of retroviral vectors very popular. So far retroviral vectors have been used in human gene therapy in different areas such as: adoptive immunotherapy, tumor vaccination, suicide gene transfer, and modified stem cell therapy (Rooney *et al,* 1995; Plavec *et al,* 1996; Qasim *et al,* 2002; Gaspar *et al,* 2003).

Retroviruses are classified based on genome structure into seven groups, which are summarized in Table 1.5. Until recently, most retroviral vector systems used for human gene therapy based on murine leukemia viruses (MLVs). These belong to the mammalian C-type retroviruses and are further classified according to the species distribution of their receptors (Coffin *et al,* 1997). To understand retroviral vector architecture the following section is dedicated to structure of simple retrovirus type C such as MLV.

Table 1.5 Retrovirus genera

Genus	Example viruses
Avian leukosis sarcoma	Rous sarcoma virus (RSV)
Mammalian C type	Murine leukaemia virus (MLV), several strains: such as Moloney, Harvey Abelson Feline Leukemia virus (FeL V) Gibbon ape leukemia virus (GALV)
D-type viruses	Mason-Pfizer monkey virus (MPMV)
B-type viruses	Mouse mammary tumor virus (MMTV)
HTLV-BLV group	Human T cell leukemia virus (HTLV)-1 and 2
Lentivirus	Human immunodeficiency virus (HIV)-1and 2
Spumavirus	Human foamy virus (HFV)

Adapted from: Coffin *et al,* 1997

1.12.1 Characterising of common structure of retroviruses

The common structure of retroviruses is based on two parts; *cis-active* and *trans- active* (Figure 1.8), which control all crucial elements for the replicative life-cycle of retroviruses (Campbell *et al,* 1997; Coffin *et al,* 1997; Pages *et al,* 2004).

The *trans-active* part of a retrovirus is the polycistronic RNA, which encodes more than one gene, and which with alternative splicing mechanisms produces two or more gene products. The *trans-active* elements of retroviruses carry at least three

major genes including: 1) *gag* encoding the internal structure protein of all retroviruses which provide the matrix protein and package the two retroviral RNA genome into a viral nucleocapsid. 2) *pol* encodes several enzymes necessary for virus replication including DNA polymerase, ribonuclease H (RNase H), integrase which is specific for each virus, and the viral protease which cleaves the *gag* and *pol* precursors into the individual proteins. 3) *env* which encodes glycosylated envelope that by its specific interaction with ligand on target cells control virus entry into host cells.

The *cis-acting* elements regulate viral gene expression and all steps necessary for viral life cycle (reviewed by: Belshe, 1991; Campbell *et al,* 1997; Coffin *et al,* 1997; Hu *et al,* 2000). The *cis-acting* elements in provirus are flanked by the long terminal repeats (LTRs), carrying the terminal attachment (att) site. The att site or inverted repeat (IR) is a short sequence (3-25bp) that forms a perfect inverted repeat at the ends of the LTR and provides recognition sites for integrase which has specific sequences in each retrovirus subtypes. Generating the linear duplication of the LTR in both 5' and 3' in retroviral DNA is necessary to establish suitable substrates for integration and to regenerate regulatory sequences (the promoter and polyadenylation site) that are lost when the provirus is transcribed into genomic RNA.

The LTR is composed of three parts the U3, R, U5, sites. The U3 region carries the viral enhancer and promoter elements. Transcription of viral genomic RNA begins at the 5' LTR and is controlled by U3 activity and in the 3' LTR, initiation of transcription is suppressed by 5' LTR interference and polyadenylation signal in R region of 3' LTR.

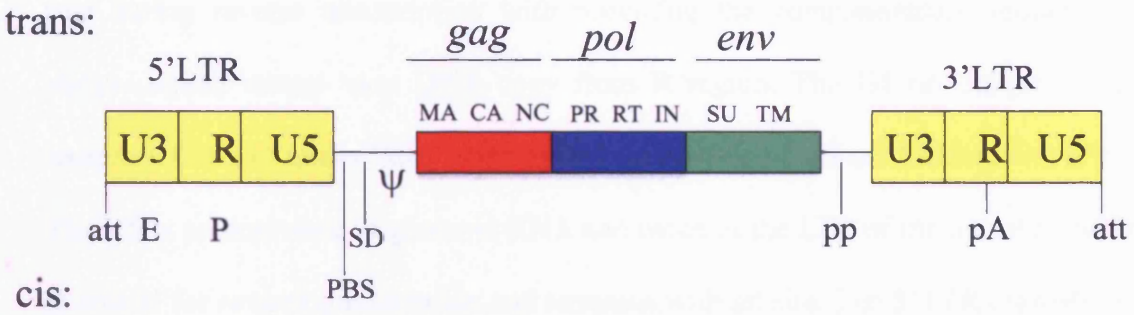


Figure 1.8 Schematic of the proviral form of a replication-competent simple C-type retrovirus

The combination of *cis* and *trans* components in a simple C-type retrovirus. (Adapted from Baum *et al,* 1999).

Components active in *trans:*

Gag : MA (matrix, p15), p12, CA(caspid, p30), NC (nucleocapsid, p10)

Pol: protease (PR), reverse transcriptase/RnaseH (RT), integrase (IN)

Env: SU (surface protein, gp70), TM (transmembrane protein, p15SE)

Components active in *cis:*

att: integration signal

ψ: dimerization and packaging signal

E P: enhancer/ promoter

PP: polypurine tract

PBS: tRNA-primer binding site

Pa: poly adenylation signal

SD, SA: splice donor, splice acceptor

The R is an identical repeated (R) redundant and short sequence (15-250 nucleotides) present at 5' and 3' ends of the RNA genome which play an important role during reverse transcription with providing the complementary sequence of minus- strand strong- stop DNA copy from R region. The U5 or Unique 5' is a sequence (70-250 nucleotides) positioned between R and primer binding site (PBS). The U5 is present once in genomic RNA and twice in the LTR of the provirus, which is crucial for reverse transcription and terminus with att site. The 5' LTR immediately is followed with untranslated leader sequence upstream of gag which is 18 nucleotides long and beginning with a 5' TGG that forms the primer binding site (PBS). The PBS is perfectly complementary to the 3' terminus of a specific host tRNA species. The PBS provides the binding site for a tRNA that acts as the primer for reverse transcriptase to initiate synthesis of the minus strand of viral DNA. Leader sequences downstream of the PBS contain the splice donor site for generating the subgenomic RNAs, as well as the packaging signal sequences. These sequences are termed "Psi" (ψ) or alternatively "E". They are required as a dimerization signal, which directs incorporation of the two viral RNA genomes into virons and thus are necessary for reverse transcription (Rein, 1994; Berkowitz *et al,* 1996). Optimal packaging can also require additional sequences, such as the first 400 nucleotides of gag in MLV-based vectors (Bender *et al,* 1987).

1.12.2 Virus life cycle

Knowledge of retrovirus structure helps us to better understand the sophisticated virus life cycle in host cells and physiological aspects of retroviral vectors function and architecture. The life-cycle of a retrovirus consists of three essential major parts: 1) virus entry, 2) reverse transcription, 3) integration

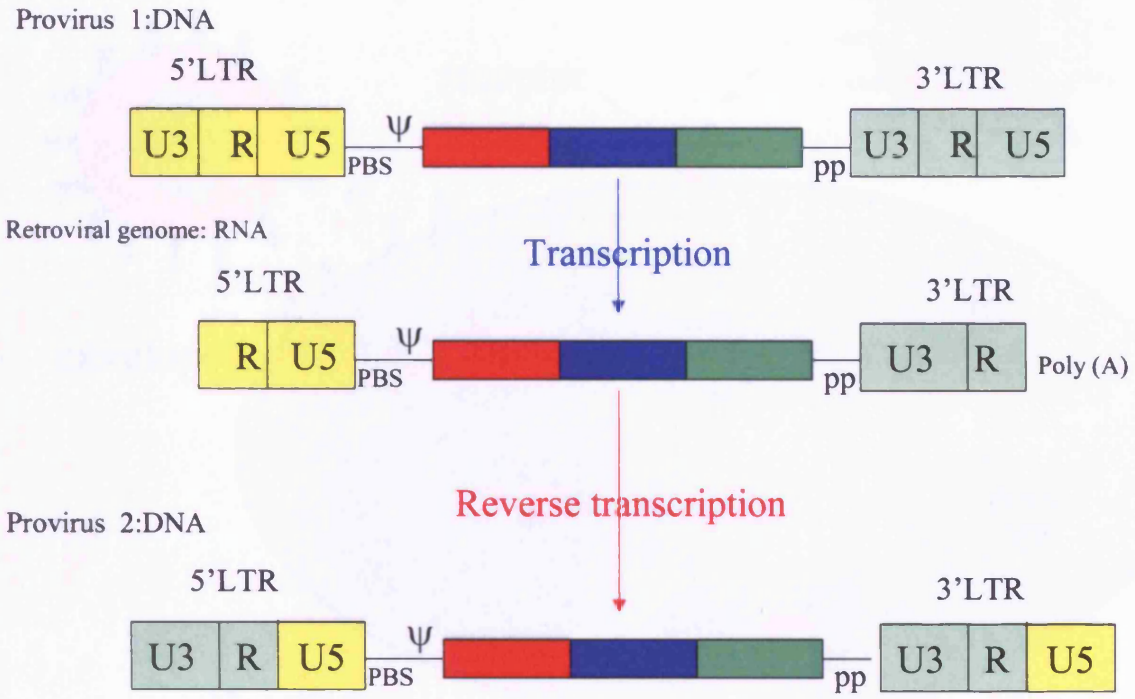


Figure 1.9 Transcription and reverse transcription steps of MLV

Provirus 1 is transcripted into the retroviral genome flanked by the R sequences. Two genomes are packaged into each virion and released from the cell. After infection of the target cell the genomic RNA is reverse transcribed into provirus 2. (Adapted from Baum *et al,* 1999).

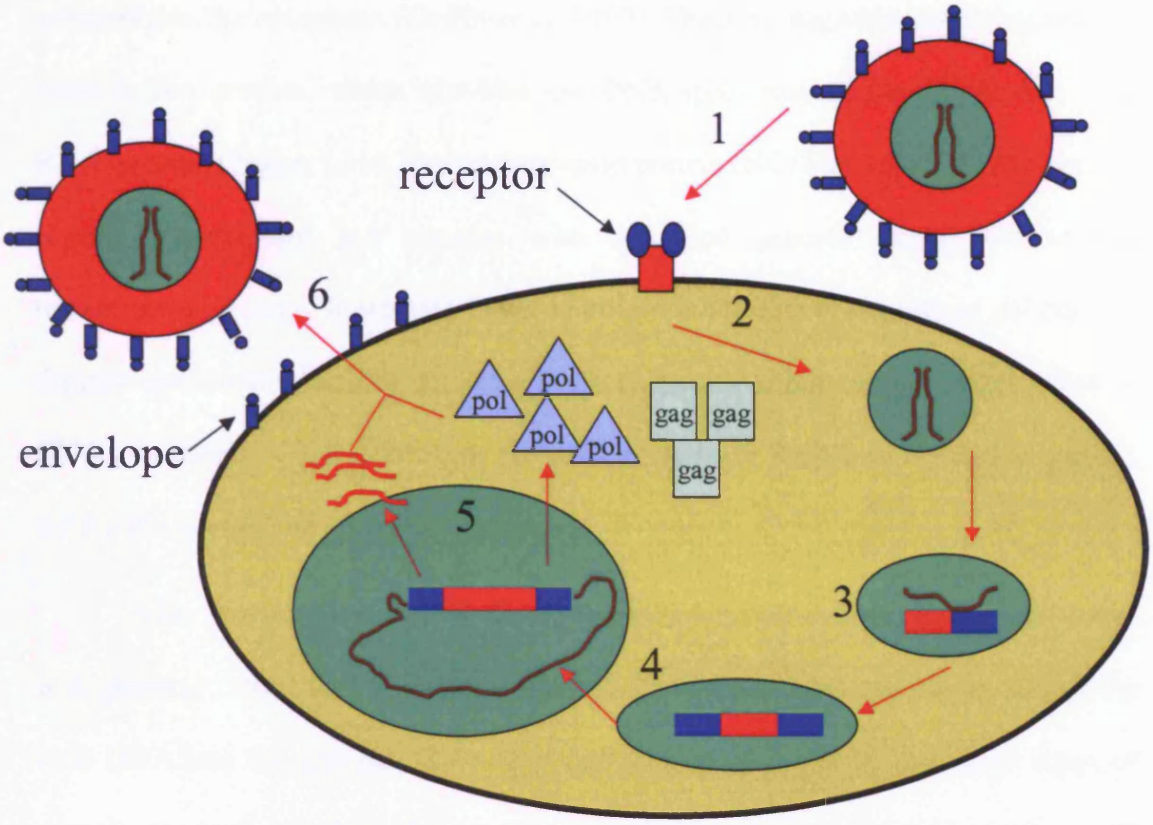


Figure 1.10 Life cycle of a replication-competent retrovirus

1) virion binding; 2) virion penetration and uncoating; 3) reverse transcription of RNA genome into proviral DNA (for detail see Figure 1.9); 4) nuclear transport of preintegration complex and integration of provirus; 5) transcription of genomic and subgenomic mRNA and translation of viral gene products; 6) nucleocapsid assembly, budding, and maturation of virion. (Adapted from Baum *et al,* 1999).

(Figure 1.9). Following fusion of viral and cellular membranes, the nucleocapsid is released into the cytoplasm (Coffin *et al,* 1997). The next stage is a multi step reverse transcription process, which provides the DNA (provirus) format of the retroviral RNA genome (Figure 1.10). The nucleocapsid protein (NC) increases the efficiency of reverse transcription and remains with the viral genome during the reverse transcription process. Retroviral DNA synthesis is absolutely dependent on the two distinct enzymatic activities of RT: a DNA polymerase that can use either RNA or DNA as a template, and a nuclease, termed ribonuclease H (RNase H), that is specific for the RNA strand of RNA: DNA duplexes.

After reverse transcription, the nucleocapsid proteins remain tightly associated with proviral DNA. This complex carries all factors necessary for integration of the viral DNA into the genome of the host cell (Coffin *et al,* 1997). For some types of retroviruses such as MLVs, this complex cannot pass through the nuclear pores, and nuclear transport requires mitosis with breakdown of the nuclear membrane. In lentiviruses, such as HIV, this step is not cell division dependent and probably the vpr protein (a protein, found in virons, that causes infected cells to arrest in G2) with virus matrix facilitates passing the complex through nucleus pores (Federico, 1999).

1.12.3 Retroviral vector architecture

Based on retroviral physiology and the flexible structure of their genome, retroviral vector systems can provide promising gene delivery systems with a large capacity and stable expression. To consider safety issues for design of a replication deficient retroviral vector, the virus production machinary is split into two parts: 1) packaging cell line (Figure 1.11) and 2) vector containing transgenic cDNA.

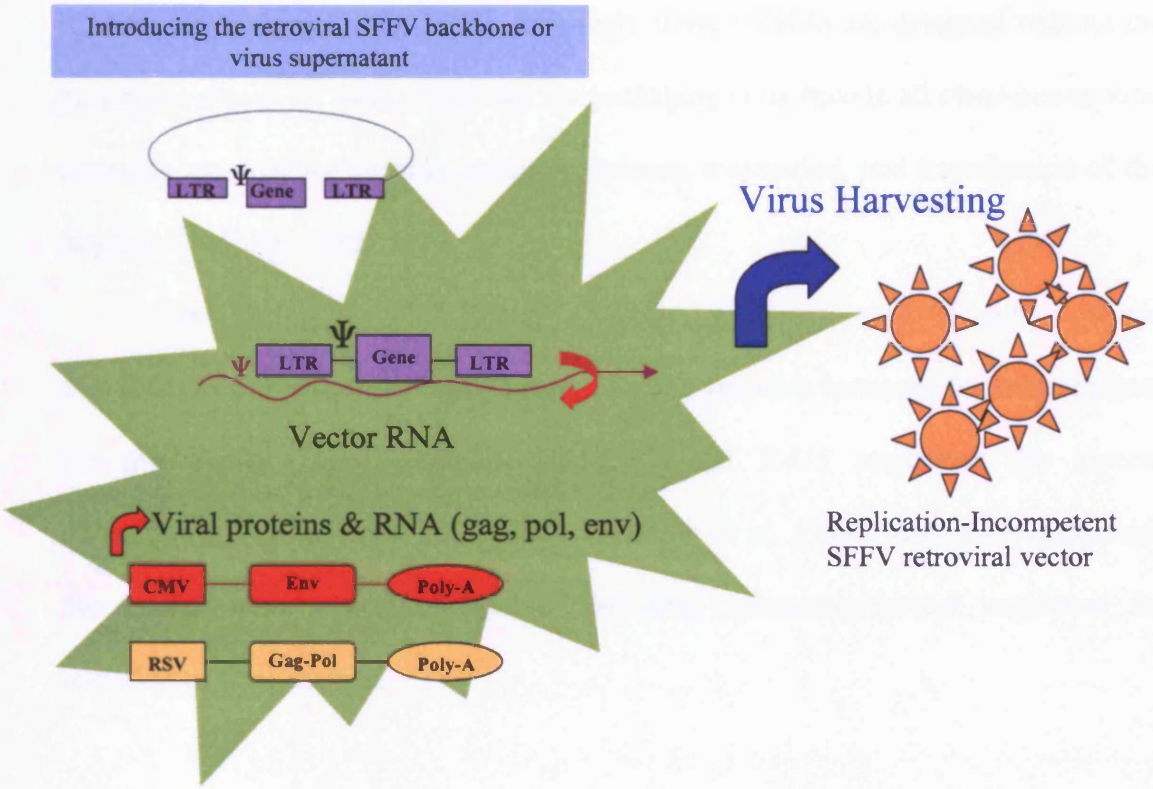


Figure 1.11 Principles of retroviral packaging cell systems

The retroviral plasmid, carrying a target gene of interest, is transfected into the packaging cells using standard chemical approaches (NX Ecotrpic). In the second step replication-incompetent retroviral particles are used to infect amphotropic PG13 packaging cell lines. The gag-pol and env components are supplied independently. Vector RNA combines with the products of gag-pol and env genes to produce infectious virus. When the virus infects a target cell, the cycle of replication is aborted once the proviral DNA integrates in the host genome as the viral structural and envelope proteins are missing. (Adapted from Lemoine and Cooper, 1996).

A modern, safety-modified packaging cell line contains at least two expression constructs for viral genes, one encoding for gag-pol and one for envelope genes (Miller, 1990; Cosset *et al,* 1995). Although, these mRNAs are designed without the packaging signal for safety purposes the packaging cells encode all *trans-acting* viral elements required for particle assembly, release, maturation, and transduction of the target cell (Miller, 1990).

The vector contains those *cis-active* elements required for a single transduction: cap site and poly (A) signal for the genomic message, packaging signal for incorporation into particles, BPS, PP and R-U5 sequences for reverse transcription, and *att* sites for integration (Baum *et al,* 1999). Moreover, it contains the transgene cassette(s) of interest including enhancer/promoter sequences for initiation of transcription.

1.12.4 SFFV hybrid retroviral vector

Moloney murine leukemia virus (Mo-MuLV) is commonly used as a retroviral vector (Gunxburg *et al,* 1996). To achieve high and constant expression by retroviral vectors, modification and hybridization of the retroviral backbone has been a key issue in vector development. The spleen focus-forming virus (SFFV) hybrid is based on Mo-MuLV vector. It has been engineered from at least four retrovirus derivatives (Baum *et al,* 1995; Hoatlin *et al,* 1998). Lastly, the polycythemic strain of SFFVp has been modified from Friend mink cell focus-forming virus (FMCF) as a mutant of replication-defective vector (Baum *et al,* 1995). The SFFVp contains the most potent retroviral enhancer configuration for gene expression in myeloerythroid progenitor cells. This currently represents the best backbone for strong transgene expression in hematopoietic cells. This hybrid has good activity in activated T cells and B cells,

which has extra advantage to high lymphotropic vector based on Mo-MuLV (Couture *et al*, 1994; Baum *et al,* 1999; Qaseem *et al,* 2002).

To get an advantage from SFFVp hybrid vector and its wide host cells expression and long- term expression, we used FMEV (FMCF-MESV; murine ES cell virus) backbone modified in 5`LTR from PCMV (PCC4 cell passaged murine sarcoma virus) a myeloproliferative sarcoma virus (MPSV) subtype (Baum *et al*, 1999). 5`LTR from PCMV provides high expression in packaging cell lines as a benefit of its active expression in embryonic carcinoma cells and further has advantage from the SFFVp 3' LTR which controls recombinant virus incompetent replication expression activity (Baum *et al,* 1995).

The vector backbone construct contains packaging signals from LX based Mo-MuLV vector (including 400bp of gag sequence with disrupting AUG) (Miler *et al,* 1989; Hawley *et al,* 1994; Baum *et al,* 1999). To enhance efficiency of this vector to transduce haemopoietic progenitors and mainly lymphocytes, a GALV pseudotype vector has been used. Pseudotype retroviral vectors are generated by co-expression of vector RNA containing the packaging signal, with retroviral Gag and Pol, and the unrelated glycoprotein (Miller *et al,* 1991; Bauer *et al,* 1995; Bunnell *et al,* 1995).

1.12.5 Third generation of lentivirus vectors

The lentiviruses are members of complex retroviruses, which have provided very efficient vectors for gene delivery. The ability of lentiviruses to integrate into non-dividing cells relies on nuclear localization signals present in the preintegration complex that allow their entry into the nucleus without the need for nuclear membrane fragmentation (Bukrinsky *et al,* 1993). Recent third-generation HIV-1 based vectors and self-inactivating (SIN) vectors represent highly efficient and more

advanced viral vehicles (Miyoshi *et al,* 1998). The HIV genome codes for three structural (gag, pol, and env) and six regulatory (Vif, Vpr, Vpu, Tat, Rev and Nef) proteins (Trono, 1995). The first generation HIV vector kept all elements and lacked packaging signal and env but in the second generation vectors, developed to be safer, more elements; Vif, Vpr, Vpu, and Nef were deleted in the packaging construct (Luciw, 1999). However, the second generation vectors kept *cis-active* elements in the transfer construct vector and rev-responsive element (RRE) and *tat* in packaging vector construct (Zufferey *et al,* 1997). It has been shown that the rev protein is strictly required for efficient viral production and with *tat* it is necessary for stable viral transcription (Emerman *et al,* 1989). However, further improvements to generate safer lentiviral vectors have been achieved by third generation based self-inactivating HIV1 virus (Miyoshi *et al,* 1998; Bai *et al,* 2003). SIN vectors are obtained by deleting all genes unnecessary for viral replication from 3' LTR of the transfer vector, corresponding to most of the U3 in 3' LTR. In other word, SIN vectors lack any LTR-driven transcription activity in target cells and no significant decreases in the viral titers were observed. In viruses generated from SIN transfer vectors, inactivation of 5' LTR prevents promoter interference with only external promoter such CMV located downstream of 5' LTR (Kim *et al,* 1998). To enhance the efficiency of SIN lentivirus vectors in our lab the SIN transfer vector has been modified by addition of a central polypurine tract (cPPT) and termination sequence upstream of external SFFV promoter and also insertion of the Woodchuck hepatitis virus post-transcription regulatory element (WPRE) in the 3' untranslated region (Demaison *et al,* 2002). The cPPT and termination sequence in this lentiviral vector facilitates reverse transcription, nuclear entry, transduction and also gene expression (Dardalhon *et al,* 2001; Manganini *et al,* 2002). It seems gene expression by this vector is boosted due

to addition of the WPRE and cPPT elements (Demaison *et al,* 2002). Efficiency of this vector particularly in non-dividing cells and haematopoietic cells, may make it a very useful tool for gene delivery into T cells.

1.13 Project plan

The project had developed in regard to study cellular pathogenesis of the XLP. The aims of this study were refined based on the study results and the data published in the literature. The main target was to use an *in vitro* model to confirm and investigate the cellular functionality of XLP cytotoxic T cells. Therefore to develop an *in vitro* model for this study we were able to achieve following aims:

1) The generation of retroviral and lentiviral vectors for the transfer of SAP gene into XLP T cells.

2) Developing transduction protocols for gene transfer into EBV-T cells using retroviral and lentiviral vectors.

3) Generating EBV-T cell lines from XLP patients and normal controls and reconstitution of cell lines with retroviral vector encoding SAP from both groups

4) Immunophenotyping of EBV-T cell lines and immune cell lineages

5) Assessment that the function of immune cells in XLP is SAP dependent for cytokine production and cytotoxicity.

Chapter 2

Materials and Methods

2.1 Reagents and buffers

All general chemicals were supplied by Sigma unless otherwise stated.

2.1.1 Reagents

Bis-tris	
Bovine Serum Albumin (BSA)	(New England Biolabs)
Dimethyl Sulfoxide (DMSO)	
Ethylenediamineteraacetic acid (EDTA)	
Ethidium bromide	
Paraformaldyde (PFA)	
Phosphate Buffered Saline (PBS)	(Gibco)
NaOH	
Sucrose	
Tryptone peptone	(Difco)
Yeast extract	(Difco)
Bacto tryptone	(Difco)
Phytohaemaglutinin (PHA)	
Phorbol 12-myristate 13-acetate (PMA)	
Ficoll-Paque Plus	(Pharmacia Biotech)
Cyclosporin (CSA)	(Sandoz pharmaceuticals, UK)
Acyclovir	(Faulding Pharmaceutical Plc, UK)
Primers	(Sigma Genosys Ltd)
HyperLadder I 1kb	

Agarose	(Gibco BRL)
Taq DNA polymerase, dNTPs buffer	(Qiagen)
Resriction enzymes	(Promega)
Shrimp alkaline phosphtase	
T4 DNA Ligase, buffer	(Promega)
Dulbecco's modified Eagle medium (DMEM)	(GibcoBRL)
RPMI 1640	(GibcoBRL)
Fetal calf serum (FCS)	(Myoclone Superplus)
Glutameax	(GibcoBRL)
Trypsin/EDTA solution	(Gibco BRL)
L-glutamine	(Gibco BRL)
Penicillin-Streptomycin	(Gibco BRL)
X-Vivo10	(Bipwhittaker)
Recombinant Retronectin	(Takara Biomedicals, Shiga, Japan)
Recombinant IL-2	(Proleukin, Chiron, Emeryville, CA)
ElectroMAX DH10B Cells	(Gibco BRL)

2.1.2 Buffers

4% PFA/3% (w/v) Glucose: 4g of PFA was weighted out in a fume cupboard and added to PBS warmed to 60°C. Concentrated NaOH was added to aid dissolution. 3g sucrose was added and the solution was made to pH 7.4 using concentrated HCl and to 100 ml with PBS. Stocks were divided into 5 ml aliquots and frozen for use.

1% (w/v) PFA: 4% PFA was made as above and diluted with PBS to make 1% PFA.

1% (w/v) BSA: 1g bovine serum albumin (BSA) dissolved in 100 ml PBS.

1M Tris-HCl pH 8.0: 60.7 g Tris and 400 ml dH_2O, stirred to dissolve. Once dissolved concentrated HCl was to achieve a pH of 8.0 and the solution was made up to 500ml with dH_2O.

TAE (x50): 0.2M Tris, 1M glacial acetic acid (BDH), EDTA, pH 8.0

L-Broth: 10g Tryptone peptone (Difco), 5g yeast extract (Difco), 10g NaCl in 500ml water.

CSA: For stock: 10 mg powder added to 10 ml absolute alcohol and filtered through 0.2 μm filter. To make working solution: (100μg/ml), 1ml of stock CSA added to 9ml RPMI and filtered through 0.2 μm filter.

Acyclovir: Making 100μM of Acyclovir in RPMI (The molarity of Acyclovir is 1.1 M and molecular weight is 225g).

Western Blotting buffers:

PBS-Tween (PBS-T): 500ml PBS, 500 μL Tween-20.

2.5% milk-PBS-T (w/v): 1.25 g milk (Marvel), 50ml PBS-T

Stacking gel buffer: 30.35 g Tris was dissolved in 400 ml dH_2O and the pH was corrected to 6.8 with HCl. 2.5 ml 20% sodium dodecyl sulphate (SDS) was added and the solution was made up to 500ml with dH_2O.

Main gel buffer: 91.06g Tris was dissolved in 400ml dH_2O and the pH 8.0 with HCL. 2.5ml 20% SDS was added and the solution was made up to 500ml in dH_2O.

Running buffer: 3g Tris and 14.4 glycine were dissolved in 997.5 ml dH_2O. 2.5ml 20% SDS was added and mixed.

Transfer buffer: A10x stock was made by dissolving 29.1g Tris and 14.65g glycine in 450ml dH_2O. The pH was corrected to 9.2, the solution was made up to 500ml with dH_2O, and filter sterilized for storage. 10ml of the 10x stock were added to 70ml dH_2O and 20ml methanol immediately before use.

NP40 lysis buffer: 1% (v/v) NP40 (Calbiochem), 20mM Tris-HCL pH 8.0, 130mM NaCL, 10mM NaF, 1mM diothiotheitrol (DTT), 20μM leupeptin, 1% aprotinin, 100μM Na_3VO_4, 1mM phenylmethylsulfonylfluoride (PMSF). The solution was filter sterilized before use.

2x SDS buffer: 75g Tris, 0.5mg bromphenol blue, 10ml glycerol, 20ml dH_2O and 5ml 20% SDS were mixed. The solution was corrected to pH 6.8 with HCl, made up to 50ml with dH_2O and filter sterilized before use.

SDS sample buffer: 3ml 2x SDS buffer, 60 μl β-mercaptoethanol.

2.1.3 Kits

Plasmid miniprep/maxiprep/megaprep kit	(Qiagen)
ELISPOT Kit	(Biosource)
CytoTox 96 (Non-Radioactive Cytotoxicity assay)	(Promega)

2.1.4 Equipment

0.4 cm glass cuvette	(Biorad)
Gene Pulser Electroporator	(Biorad)
Qiaex gel extraction kit	(Qiagen)

Table 2.1 List of Antibodies that were used in this study

Labelled Antibodies

Antibody	Label	Species	Isotype	Company	Use
Anti-human CD3	PE, FITC, PER	Mouse	IgG1	Pharminogen	F.C
Anti-human CD4	PE, FITC, APC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD8	PE, FITC, APC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD11C	PE	Mouse	IgG2b	Pharminogen	F.C
Anti-human CD14	PE	Mouse	IgG2a	Becton Dickinson	F.C
Anti-human CD16	FITC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD19	FITC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD25	PE	Mouse	IgG1	Pharminogen	F.C
Anti-human CD27	PE	Mouse	IgG2a	Becton Dickinson	F.C
Anti-human CD28	PE, FITC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD45RA	PE, FITC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD45RO	PE, FITC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD56	PE, APC	Mouse	IgG1	Pharminogen	F.C
Anti-human CD69	PE	Mouse	IgG2	Pharminogen	F.C
Anti-human CD95 (Fas)	PE	Mouse	IgG1	Pharminogen	F.C
Anti-human CD150 (SLAM)	PE	Mouse	IgG1	Pharminogen	F.C
Anti-human CD224 (2B4)	FITC, CY	Mouse	IgG_1	Pharminogen	F.C
Anti-human CCR5	PE	Mouse	IgG2b	R&D Systems	F.C
Anti-human CCL5	FITC	Mouse	IgG2	Pharminogen	F.C
Anti-human CCR7	FITC	Mouse	IgG1	R&D Systems	F.C
Anti-human αβTCR	FITC	Mouse	IgG1	Dako	F.C
Anti-human γδTCR	PE	Mouse	IgG1	Dako	F.C
LNGFR	PE	Mouse	IgG1	Dako	F.C
Anti-Rabbit	HRP	Goat	Poly	Sigma	Western Blot

Abbreviations: F.C; Flow Cytometry

Cont. Table 2.1 List of Antibodies that were used in this study

Purified Antibodies

Antibody	Purified	Species	Isotype	Company	Use
Anti-human CD3	Purified			Janssen-Cilag	Cell Activation
Anti-human CD28	Purified	Mouse	IgG1	Becton Dickinson	Cell Activation
Anti-human CD150	Purified	Mouse	IgG1	BD PharMinogen	Cell Activation
Anti-human CD224	Purified	Mouse	IgG_2	Immunotech-Coulter	Cell Activation
Anti- SAP	Purified	Rabbit			Cell Activation
Anti-human IFN-γ	Purified	Mouse	IgG1	Mabtech AB	ELISPOT
Antihuman IL-5	Purified	Mouse	IgG1	Mabtech AB	ELISPOT
Antihuman IL-10	Purified	Mouse	IgG1	Biosource	ELISPOT

2.2 Cloning

2.2.1 Plasmids

pGEM-T containing human SAP cDNA: Human SAP (SH2D1A) cDNA (OMIM no: AL023657) cloned into pGEM-T (Promega, Madison, USA) was a gift from Dr. Alison Coffey (The Sanger Center, Cambridge, UK).

pBS (plasmid Bluescript): contains the internal ribosomal entry sequences (IRES) from Encephalomycarditis virus and red shifted variant of the green fluorescent protein (eGFP) or LNGFR.

SFFVp retroviral backbone vector: A murine replication deficient oncoretroviral vector containing the U3 modified 3` LTR from SFFV, 5`LTR from PCMV (PCC4 cell passaged murine sarcoma virus) and PBS (Primer binding site) (a gift from Dr K Parsley, ICH).

SIN HIV1 backbone vector: A HIV1 self inactivated (SIN) based vector was used which takes advantage of the U3 part of the spleen focus forming virus (SFFV) strain P long terminal repeat sequence (SFFV-U3LTR), upstream of the central polypurine tract *cis-active* sequence (cPPT) and central termination sequence (CTS) and also the non-translated Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence down stream of 3'LTR of vector backbone.

pMD.G. plasmid: encoding VSV-G envelope protein

pCMVR8.91 packaging plasmid: providing (gag-pol, cPPT, Rev, and tat elements.

2.2.2 Conventional gel electrophresis

DNA fragments were resolved by electrophoresis through 1% agarose gels. Agarose was dissolved in 1x TAE buffer by boiling it in a microwave. The gel was allowed to cool to 60°C before ethidium bromide was added at 0.5μg/ml and the gel poured into a gel former with a comb fitted. The gel was then immersed in 1xTAE buffer and liquid samples of DNA mixed with loading buffer (bromophenol blue in 0.1 M EDTA, 20% Ficoll) were loaded into the wells. DNA standard size markers were also run to enable accurate size determination of DNA fragments. The gel was then subjected to a voltage of 50-100V (up to 150mA) and the DNA was visualised by exposing it to ultra violet light.

2.2.3 Restriction endonuclease digestion of DNA

DNA (generally 0.2-2 μg) was cut in a 20 μl final volume of 1 x buffer (10 x supplied by manufacturer and diluted in sterile water). The amount of enzyme used varied depending of the concentration of the enzyme stock and the amount of DNA,

but never exceeded 10% (v/v) of the total reaction volume. The endonuclease reaction was carried out at the manufacturer's recommended temperature for 1-2 hours the reaction parameters were altered accordingly. If a double digest was required, in which the two enzymes were active in incompatible buffers, the DNA was first digested with one enzyme, and then the cut DNA cleaned by PCR DNA Kit and resuspended in the buffer compatible with the second enzyme.

2.2.4 Dephosphorylation of vector ends

In cases in which a digested vector had compatible termini, the ends of the linear molecule were dephosphorylated to prevent vector self-re-ligation. Shrimp alkaline phosphatase (SAPh) is a phosphomonoesterase purified from shrimp intestinal mucosa that hydrolises 5'-phosphate groups in DNA and was used in all reactions. The enzymes was supplied as a 26 $U\mu l^{-1}$ stock and was diluted in 1x SAPh dilution buffer such that after dilution it contributed no more than $1/10^{th}$ of the final restriction volume. The enzyme was generally added to restriction enzyme digests that had been inactivated, by heating or a phenol/chloroform extraction, prior to gel purification and therefore did not require additional dephosphorylation buffer.

Dephosphorylation of 1 pmol of DNA 5'-protruding ends requires 0.01 U of SAPh at 37 °C for 30 minutes, whilst 1 pmol of DNA 5'-recessed or blunt ends required 1 U at 50 °C for 1 hour. If the reaction is allowed to proceed for too long, the SAPh damage the strand termini so the enzyme was inactivated by heating to 70 °C for 10 minutes, followed by a gel extraction.

2.2.5 Ligation

The amount of DNA added to ligation reactions varied depending upon the nature of the reaction. In general however, the ratio of vector: insert ranged from 1:1 to 1:3. Ligations were carried out in a total volume of 10 μl. A 1:10 dilution of the T4 DNA ligase enzyme was prepared in 1x ligase buffer and the DNA, dH_2O, ligase buffer and 1 μl (1u) of the diluted enzyme were mixed together. Ligations were carried out 16 °C for 16 hours or 37 °C for 2 hours. The ligation were used to transform competent bacteria immediately, or were stored at –20 °C for later use.

2.2.6 Ethanol precipitation

This procedure was used to remove salts from DNA solutions or to concentrate DNA by precipitating it from solution. The volume of DNA solution was estimated and one-tenth the volume of 3 M sodium acetate (pH5.2) and two times the volume of 100% ethanol were added. The solution was mixed by inversion and placed at –80°C for 15 minutes to precipitate the DNA that was then pelleted by centrifugation at 17,000 xg for 30 min at 4°C. The supernatant was then discarded and the pellet washed in 70% ethanol, with centrifugation for 10 min under the previous conditions. The ethanol was removed and the pellet was briefly air dried and resuspended in an appropriate volume of 10 mM Tris-HCl pH 8.0. Plasmid DNA was stored at –20°C.

2.2.7 Gel purification of DNA

Purification of DNA fragments from agarose using silica particles. Following electrophoresis of the DNA in an agarose gel the required band was excised using a clean scalpel blade under low intensity long-wave ultraviolet (UV) light in order to

minimise DNA degradation. Then DNA was extracted from the agarose using Qiaex II or QIAquick kits (Qiagen). Wash steps were performed under high salt conditions to eliminate the agarose and subsequently in an ethanol-based solution to eliminate residual salt and dyes. The DNA was eluted from the silica gel particles/column under low salt conditions with TE or Tris buffer. The procedure was carried out according to the manufacturer's instructions and the DNA eluted in a total volume of 30 µl TE pH 8.0 or 10 mM Tris-HCI pH 8.0.

2.2.8 Polymerase chain reaction (PCR)

PCR DNA amplification was generally performed in a total volume of 10 µl containing 100ng of DNA, each primer at 0.5 µM, dNTPs each at 200 µM, 2.5 U *Taq* polymerase and the appropriate buffer (Qiagen). The appropriate buffer for each primer pair was defined by titration of the concentration of $MgCl_2$ in the supplied buffer. The cycle conditions were optimised.

Primers were designed with two Sal I sites (sequences shown with underline) within the forward and reverse primers (Sigma):

SAP-F: GCC CAA GAG TCG ACCAGG CCA TGG

SAP-R: GTA CAA GGT GTT TTA GTC GAC TTC ATG GGG GCT TTC

A PCR reaction was prepared in total volume of 50 µl and the PCR cycle was set up as below:

1) 94 °C for 2min/1 cycle

2) 50 °C for 1min and 72°C for 1 min and 94°C for 1min/5 cycles

3) 60°C for 1 min and 72°C for 1 min and 94°C for 1 min/30 cycles

2.2.9 Transformation of plasmid DNA by electroporation

E coli DH5α electrocompetent cells were used to transform plasmid DNA. Cells were stored in 10% glycerol in 50μl aliquots at 70°C. Ligation mix or plasmid preparation was dialysed on 10mm filter paper floating on dH_2O for 20-25 minutes. 10μl of the preparation was added to an aliquot of DH5α on ice in a 0.4 cm glass cuvette. The sample was immediately electroporated (Voltage 2.5kV, Resistance 200Ω; Capacitance 25μF) using a Gene Pulser. The cells were then resuspended in 940μl of LB medium and incubated in a shaking incubator at 37°C for 60 minutes, after which they were plated onto LB-Ampicillin agar plates. Plates were incubated overnight at 37°C and colonies picked using sterile pipette tips, and used to inoculate 3-5ml LB-Ampicillin cultures.

2.3 Tissue Culture

2.3.1 Peripheral blood mononuclear cells prep

Whole blood anticoagulated with EDTA was diluted 1:1 in Dulbecco's PBS, layered over lymphoprep (Nycomed) and spun for 25 minutes at 2400 rpm (750g), no brake. Peripheral blood mononuclear (PBMCs) were collected from the interface and washed in HBSS at 1200 rpm (250g) x 10 minutes. A further 2 washes were carried out at 1100 rpm (150g) for 5 minutes to reduce platelet contamination. PBMCs were used for further experiments where used to generate different cell lines or were frozen according to instruction to be used in future work.

2.3.2 Cell counting

Cells were counted using a Naubauer haemocytometer by Trypan blue exclusion and phase contrast microscopy.

2.3.3 Cell cryopreservation and thawing

A cryopreservation solution of 90% FCS and 10% DMSO was used for cells other than primary T cells, for which human AB serum was used. Cells were re-suspended in DMEM or RPMI, cooled on ice and equal volume of cryopreservation solution added dropwise. Aliquots were then frozen at -70°C using a cooling chamber, before transfer to liquid nitrogen.

Table 2.2 List of cell lines and suppliers

Cell Line	Origin	Supplier	Medium
B95-8	EBV carrying marmoset	ECACC, Salisbury, UK	RPMI
HeLa	Human cervical carcinoma	ECACC, Salisbury, UK	DMEM
PG13	NIH 3T3 mouse fibroblast	Gift from Pro. Collins, UCL. London, UK	DMEM
Phoenix	293T human embryonic kidney	Nolan Laboratories, Stanford, USA	DMEM
293T	293T human embryonic kidney	ECACC, Salisbury, UK	DMEM

ECACC: European collection of cell culture, NIH: National Institute of Health, UCL: University College London

Thawing of frozen cells was carried out by immediately placing cryovials in a 37°C water bath. The cells were transferred slowly to 10 ml of appropriate pre-warmed growth medium and pelleted by centrifugation. They were then resuspended in 1 ml of growth media and transferred to 4 ml of pre-warmed medium in a $25cm^2$ tissue culture flask.

2.3.4 DC preparation

PBMCs were resuspended in complete medium and plated at $3\text{-}6x10^6$ cells per ml in 6 well tissue culture plates (Falcon). After 2 hours incubation at 37°C /5%Co_2, non-adherent cells were gently washed off. The adherent fraction was cultured in complete medium with 100ng/ml, recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leucomax, Sandoz/Schering Plough) and 25ng/ml recombinant interleukin-4 (IL-4) (gift from Dr S.Burns, ICH). Cytokines were refreshed on day 3 or 4 of culture.

2.3.4.1 Harvest of DC

On day 6-7 non-adherent, immature DC were removed, layered over Lymphoprep and spun at 1600rpm (400g) for 25 minutes, brake off. Cells from the interface were washed twice in HBSS AT 1200rpm (250g) for 10 and 5 minutes, respectively. DC were purified to approximately 90% by immunodepletion using CD3 and CD19 Dynabeads (Dynal) to extract T and B lymphocytes. DC and beads were incubated on a rotator at 4°C for 20-30 minutes before magnetic removal of beads.

Mature DCs were harvested on day 7 or 8 after 24hr incubation with LPS by the same method. Mature cells from large loosely adherent clumps were washed into suspension in the well for collection.

2.3.4.2 Maturation of DC

Immature DC were matured by addition of 50ng/ml LPS (from E. coli 026.B6, Sigma) on day 6 of culture for a total of 24 hours.

2.3.5 Generation and culture LAK cell lines

Non-adherent PBMCs were plated at 2 x10^6/ml in six-well tissue culture plates in complete RPMI 16 medium with 1000 IU/ml recombinant human (rIL-2) incubated at 37°C/5% CO_2. The supernatant (non-adherent cells and media) was removed on day 3 and replaced with 2 ml of media and 500 IU/ml of rIL-2. Every 3 days, for 12 days, media replaced accordingly. On day 14, adherent cells were harvested for use in the designed assays.

2.3.6 Preparation of concentrated EBV from B95-8 cell line culture

The B95-8 cell line was cultured in complete RPMI medium. The volume was expanded to 1000ml during two weeks according to the cells growth. The vented cap of flasks containing cell lines were sealed with parafilm for 7-10 days to let viruses go into lytic phase. The cell culture was centrifuged at 2000rpm for 10 minutes. The supernatant was filtered into 0.8 μ filter. The suspension was transferred into sterile Beckman tubes and centrifuged at 16,000rpm for 2 hours at 4^0C.The supernatants were removed gently and pellet resuspended in RPMI medium and aliquoted 100 μl per cryovial and stored at-70^0C

2.3.7 Generating EBV-BLCLs from XLP patients and normal individuals

EBV-BLCL lines were produced by mixing $2x10^6$ PBMCs with 100-200 µl concentrated B95-8 virus and 10µl Cyclosporin A (CSA) in 1ml complete RPMI 20% medium. The PBMCs were incubated at 37^0C for 1 hour then were diluted (final concentration $1x10^6$ cells/ml) and dispensed in 48 well plates. They were fed weekly with completed RPMI and after two weeks were treated with acyclovir 100 µM twice per week. After 4weeks, the generated EBV-BLCL lines were transfered to 25ml flask and kept in RPMI 10% FCS ($1x10^6$ cells per ml) at 37^0C incubator for further use.

2.3.8 Generation and culture of EBV-T cell lines

EBV transformed LCLs were generated from normal donors and EBV seropositive XLP patients (P1 and P2) standard techniques. EBV-T cell lines were generated by stimulating 1×10^6/mL PBMCs cells with 2.5×10^4/mL autologous LCLs (40/1 cell ratio) using standard culture conditions. Cells were restimulated and re-cultured at a concentration of 1×10^6/mL as described in Chapter 4. Cells were either prepared for gene transfer or kept in culture with re-stimulations weekly at an effector:target ratio of 4:1. A total of 20 IU of IL-2 was added to the cultures for the first time at day 10 and twice weekly thereafter. All cell lines were cultured for up to 8-10 weeks. EBV-T cell lines with mainly a CD4+ phenotype were produced by stimulation with PHA (5 ng/ml) and IL-2 (20 IU/ml) as described in Chapter 4 continued in the same conditions as described above.

2.4 Generation of viral supernatant

Retroviral vector supernatants were generated using Phoenix ecotropic and PG13 amphotropic packaging cell lines. Single cell sorting by FACS (Beckman Coulter EPICS) into 96 well plates was used to select clones with high virus production. In the case of lentiviral vector supernatants, the virus was generated by a co-transfection system (using 293T cell line). The viral titre was measured by measuring the efficiency of transduction of HeLa cells by single exposure to a range of virus dilutions for 48 hours.

2.5 Gene transfer

Stimulated EBV T cells with different protocols (Protocol No 1-3, see Table 4.1) were washed with Xvivo 10 and counted by staining and $1x10^6$ cells in Xvivo10 cultured for two hours at 37 C, 5% CO_2. In cases using retronectin, non tissue culture 24 well plates were coated with 15 μl retronectin (1mg/ml) in 185 μl PBS per well (total volume 200 μl) overnight at 4^0C or at least for two hours at room temperature. Then plate was blocked with 1% human serum albumin for 1 hour at room temperature and washed with PBS. Coated plates were preloaded with at least 100-200 μl per well retrovirus supernatant or in case of lentivirus with 10-20 μl diluted with Xvivo 10 per well (to reach 200 μl total volume) and centrifuged at 2000 rpm (550g) for 20 mins. Cultured cells were replated into coated plates in Xvivo 10 medium at present rIL-2 and virus in Xvivo 10 used at different MOI ratio to transduce cells. When using polybrene, cells were cultured in Xvivo 10 in the presence of polybrene at 4 μg/ml and rIL-2 for 3-4 hours before transduction and virus supernatant was then added. In all experiments, cells were cultured at a final concentration of $1x10^6$ cells/ml. Then cells were centrifuged at 1500 rpm (350g) at

room temperature for 30 min and incubated at 37C, 5% CO_2 overnight. The next day half the medium was replaced with fresh Xvivo 10 and additional rIL-2. After 48 hours of the first round of transduction, the second round infection was carried out with the addition of viral supernatant. On the fourth day, cells were stained and analyzed for CD3, CD4, CD8, eGFP expression with flow cytometry or cultured further to generate EBV specific CTL clones.

2.6 LDH release cytotoxic assay

The cytoxicity of EBV-T cell lines was measured using LDH release assays (Promega). Briefly, autologous LCLs were used as target cells with EBV-T cell lines as effector cells at different effector:target cell ratios. All targets were plated in triplicate. After 4h incubation, supernatants were harvested and the specific cytotoxicity determined using a microplate ELISA reader (Dynatech). The percentage of specific lysis was calculated as 100% × (experimental release [-] spontaneous release)/(maximum release [-] spontaneous release). Maximum release was obtained by adding 100 µl of 5% Triton X-100 to the 100 µL medium containing target cells. Spontaneous release was consistently below 15% of maximum release in all assays. Ideally a specificity control should be have done using autologous PHA blast to measure probability of cytotoxic activity of EBV- T cell lines against self-antigens. This has not been done completely due the shortage of patient blood's samples (data on some lines not shown in this thesis).

2.7 Antibody stimulation

For antibody stimulation of PBMCs and EBV-T cell lines, all antibodies (CD3 or OKT3, CD28, α-SLAM, 2B4) used at concentrations of 5µg/ml (as soluble) or 1

μg/ml (pre-coated non-tissue culture plates). The stimulated cells were used for ELISPOT assay or for SLAM-2B4 expression studies.

2.8 MHC class I blocking of target cells

W6/32 monoclonal antibody (a gift from Dr Bin Gao, ICH) was used to block MHC class I antigen presentation on LCLs. Cells were washed twice with PBS and then incubated with W6/32 mAb (1μg/ml) on ice for 1 h. The cells were then washed and used in cytotoxicity assays.

2.9 ELISPOT assay

PBMCs and EBV-T cell lines were plated at 1×10^4/well for IFN-γ detection assay and $1x\ 10^5$ /well for IL-5 and IL-10 detection assays. Responder cell populations were seeded across a range of concentrations to achieve 10 to 100 spots/well so as to facilitate accurate and reproducible counting. For LCL stimulators with PBMC responders, the concentration used was 5×10^3 LCLs to 2×10^4 PBMC/well; stimulators with EBV-T cell line responders, this was 5×10^3 LCLs to 2×10^4 EBV-T cell lines/well. For antibody stimulation of PBMCs and EBV-T cell lines, all antibody concentrations used in the ELISPOT assay were optimized at 5μg/ml (CD3 or OKT3, CD28, α-SLAM, 2B4 and as a positive control PBMC or EBV-T cell lines stimulated with 2.5 μg/ml PMA, or Ags (gift from Dr L. Weddelouin, ICH), or LPS and 1 μg/ml ionomycin (Sigma). All cells were cultured in RPMI-FCS 10% supplemented to a final volume of 200 μL/well. After undisturbed incubation for 24 to 48 h at 37°C, with 5% CO_2, plates were washed 4 times with PBS containing 0.05% Tween 20 (PBS/0.05% Tw). Wells were incubated with biotinylated 'detection' antibody against IFN-γ, IL-5 or IL-10. The plates were

washed 6 x with PBS/0.05% Tw. 100 µl of Avidin-Peroxidase-Complex (AEC; prepared according to manufacturer's instructions; Vector Laboratories) was added per well for 1 h at room temperature. The plates were washed 3 x with PBS/0.05% Tw, followed by 3 washes with PBS. AEC substrate (Sigma, St. Louis, MO) was prepared according to manufacturer's instructions and filtered through a 0.45 µM filter prior to use. 100 µl was added per well. After 4 minutes the reaction was stopped with deionized water and the plates dried overnight prior membrane removal. The spot number was determined in an independent blinded fashion (Bioreader 3000; Bio-Sys). In each experiment, the result was expressed as spots/1000 cells (for IFN-γ) or spots/10000 (for IL-5 and IL-10) and the results of three experiments were used to calculate means and standard deviations.

2.10 SAP immunoblotting

Cell lines from patients and healthy donors used to produce cell lysates according to previous protocols. Briefly cells were pelleted by centrifugation x 5 minutes at 2000 rpm (550g) in Eppendorf tubes, washed with PBS to remove medium and repelleted. Approximately 100µl NP40 lysis buffer was added, on ice for every 1 x 10^6 cells. After 10 minutes debris was pelleted by centrifugation for 5 minutes at 14,000 rpm, 4°C and supernatant was transferred to a fresh tube. To 100µl of lysate, 100µl SDS sample buffer was added and the sample was then stored at –20°C.

2.10.1 Gel preparation

Glass gel plates were cleaned with 70% ethanol, dried and assembled. In a uinversal tube, 6ml 30% (w/v) acrylamide (Protogel from National Diagnostics), 5ml main gel buffer, 8.8ml water, 20µl tetramethylenediamine (TEMED) and 200µl 10%

(w/v) ammonium persulphate (APS) were added together to make a 10% gel. The gel was poured into the assembled gel plates and overlaid with water. Once polymerized, the water was poured off. A 4% stacking gel was made by adding together 1.3 ml 30% acrylamide, 2.5ml stacking gel buffer, 6.1ml water, 10µl TEMED and 100µl 10% APS and was poured on top of the main gel. Combs were inserted and the gel was left to polymerase. Once polymerized, the gel assembly was placed into a gel tank with approximately 500ml of running buffer in the top chamber and 500ml of running buffer into the bottom chamber. Cell lysates were heated to 100°C for 3 minutes, spun briefly at 13,000 rpm in a microcentrifuge and loaded to the gel. Recombinant protein molecular weight markers (Amersham) were also loaded to the gel. The gel was run at between 100-200V.

2.10.2 Gel transfer

A sheet of nitrocellulose (MSI) and six pieces of Whatmann 3MM paper of a similar size to the gel being transferred were soaked in transfer buffer. A transfer stack was assembled as follows: 3 pieces Whatmann, gel, nitrocellulose, 3 pieces Whatmann. Air bubbles between layers were removed by rolling a pipette carefully on the stack. The gel was transferred using a semi-dry blotter (Biorad) at 12V for 20-30 minutes before the stack was dismantled and the membrane was either placed in 2.5% milk-PBS-T for immunostaining or stored at 4°C wrapped in Saran Wrap.

2.10.3 Immunoblotting

The membrane was blocked in 1% milk-PBS-T for 1 hour at room temperature and then incubated for 2 hours at room temperature with anti-SAP antibody generated against the C-terminal end of SAP protein at a final concentration

of 1:1000. The membrane was washed with PBS-T and incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (Sigma) for 30-45 minutes. After a further 5 washes in PBS-T an enhanced chemiluminescence (ECL; Amersham Biosciences) was applied for 1 minute for detection. In the dark room film was placed in the cassette and exposed to the blot for 30 seconds. A longer or shorter exposure was occasionally required.

2.11 Flow cytometric analysis and FACS

To prepare samples for analysis, a minimum of $2x10^4$ cells/tube in a small volume (<100µl) and appropriate amounts of antibody (see Table 2.1) were added to FACS tubes on ice and incubated in the dark for 15-30 minutes. Cells were washed with PBS, before cenrifugation at 1500 rpm (300g) for 5 minutes at 4°C. Supernatants were flicked off and pellets resuspended in 200µl 1% PFA. Samples were stored at 4°C in the dark till flow cytometric analysis. Flow cytometry of EBV-T cell lines was performed using an EPICS XL flow cytometer (Beckman Coulter, High Wycombe, UK)

Gene-modified EBV-T cell lines were purified using an EPICS Altra fluorescence activated cell sorter (FACS) (Beckman Coulter, High Wycombe, UK). Cells were stained with phycoerythrin (PE) conjugated CD3 (BD Pharmingen, San Diego, CA) and analyzed for PE-CD3 and eGFP expression. Dual-color positive cells (positive fraction) and single-color CD3 cells (negative fraction) were sorted to high purity (more than 99%). One unsorted fraction was retained. The three cell fractions were subsequently used separately for cytotoxicity assays.

2.12 Statistical analysis

The T test was used for statistical analysis, unless otherwise stated.

2.13 Ethical approved, consenting and materials of normal individuals and XLP patients

The Institutional Research Ethics Committee approved this study and informed written consent was obtained from all subjects before taking blood. Anti-coagulated whole blood was derived from three healthy donors (C) who were EBV seropositive and three XLP patients (P1, P2 and P3) for this study. The three patients were all from different kindreds and each had a different mutation in the SAP gene, which led to the absence of SAP expression (see Chapter 5, Table5.1).

Chapter 3

Generation of SAP Retroviral and Lentiviral Vectors

3.1 Introduction

Gene therapy was initially developed as a replacement strategy for treatment of monogenic diseases. It is a new area in biology that has taken feasible advantages from genomic techniques, particularly in developing sophisticated vectors to introduce a transgene into the new host. Vector construction involves the generation of a plasmid that contains all of the elements necessary for the expression of interested genes. Viral vectors are the most common vectors in the gene therapy field. They are genetically engineered to incorporate the transgene with the aim of selective expression in the target cell following transfection.

To study the feasibility of gene therapy for XLP, retroviral and lentiviral backbone vectors (discussed in Chapter 1) encoding SAP and a reporter gene or only a reporter gene were generated in this study (summarized in Figure 3.1). The Encephalomyocarditis Virus derived internal ribosomal entry sequence (IRES) was used to design bicistronic vectors. An enhanced variant of the green fluorescent protein (eGFP) from the jellyfish Aequorea victoria was used as a reliable bioluminescent reporter for retroviral mediated transduction (Zhang *et al,* 1996). Expression of the molecule is not thought to be toxic to lymphocytes or have any known influence on function. The truncated form low affinity nerve growth factor receptor (ΔLNGFR) was also chosen as the alternative cell surface marker in this study to investigate the effect of reporter protein on the expression of two transgenes from our bicistronic vectors.

Replication-incompetent retroviral particles were produced using packaging cell lines. The Phoenix-Ecotropic (Φ_e) cell line has been used to produce SFFVp retrovirus encoding SAP and a reporter protein. The Phoenix cell packaging line is a

bipartite packaging system, which minimises the potential for recombination overlap and for the creation of wild-type virus. The Phoenix ecotropic packaging cell line has been generated by introducing the gag-pol and env genes under non-Moloney promoters to reduce recombination potential in the two separate constructs, firstly the gag-pol with a hygromycin resistant gene selection and also a second vector encoding the env gene (ecotropic envelope) with diphtheria resistance as a co-selectable marker. These packaging lines have been engineered to allow monitoring of gag-pol production through linkage to a CD8α surface marker downstream of the reading frame of the gag-pol construct. Phoenix packaging lines are capable of carrying episomes for long-term stable production of retrovirus. High efficiency transfection was achieved using polyethyleneimine (PEI) and the retroviral virions produced were harvested from the spernatant of subconfluent cultures. The filtered supernatant from phoenix ecotropic cells was used to stably transduce the PG13 line. PG13 cell line was derived from TK-NIH/3T3 mice fibroblast cells (Miller *et al,* 1991). This packaging cell line is based on gibbon ape Leukaemia Virus (GALV) and provides retroviral vectors with GALV envelope. The GALV envelope has been reported to be more efficient for retroviral delivery to haematopoietic cells in humans (Von Kalle *et al,* 1994; Bunnell *et al,* 1995). The GALV receptor (GALVR-1) is expressed on lymphocytes at much greater levels than receptors for amphotropic viruses, and the PG13 packaged vectors have been shown to transduce T cells at a higher efficiency than the previous generation of vectors (Lam *et al,* 1996).

The lentiviral virions (VSV-G pseudotype vector) were produced by a transient co-transfection system using 293T cells line as discussed in section 3.8.2. A reliable virus titre was achieved using this system.

Then the SAP expression in transduced cells was assessed by immune blotting technique.

3.2 Preparation of SAP cDNA

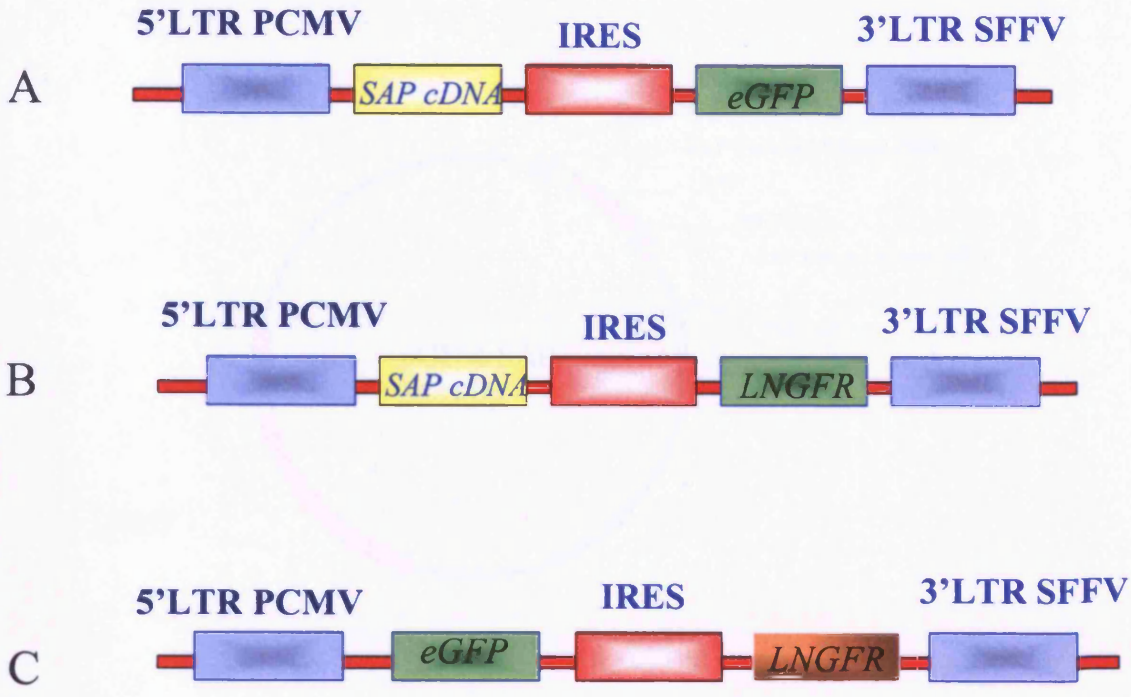
The SH2D1A cDNA is 2,530 bp long and contains an open reading frame (ORF) of 462bp, with an initiation codon 79bp from the start of the ORF (Coffey *et al,* 1998) (SAP (SH2D1A) cDNA OMIM: AL023657). The SAP cDNA (670bp) was cloned into pGEM-T (Promega) vector, which was kindly provided by Dr. A Coffey (Sanger Center-UK) (Figure 3.2 A). To avoid the poly A signal downstream of the SAP ORF and to prepare a reliable site for the next step of cloning, a PCR based mutagenesis site technique was used. By PCR, two Sal mutation sites upstream of the ORF and downstream before the poly A signal were designed.

The PCR product was run on a 1% agarose gel and the SAP band was identified around 400 bp as expected (Figure 3.2 B).

3.3 Cloning of SAP in pBluescript (pBS) vector

pBluescript (pBS) (Stratagene) is a vector with multiple cloning site (MCS). It confers ampicillin resistance on the host, allowing for selection of bacteria transformed with plasmid. We used a pBS vector which already encoded the internal ribosomal entry sequences (IRES) from Encephalomyocarditis virus and enhanced green fluorescent protein (eGFP) downstream of the multiple cloning site (MCS). The Sall site of MCS of pBS was used in conjunction with the Sal 1 site of SAP.

Retroviral vector constructs



Lentiviral vector constructs

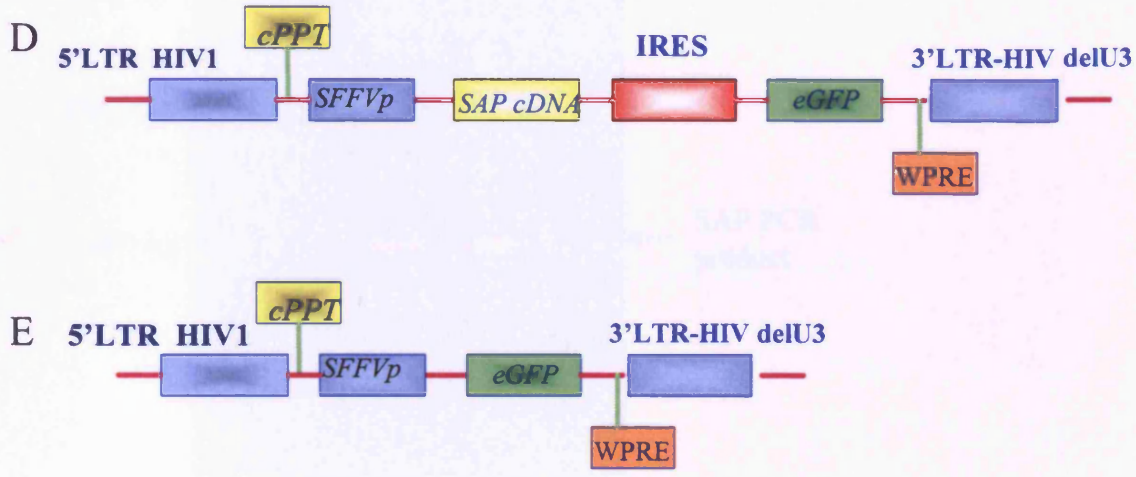


Figure 3.1 Summary of vectors used in this study

The SFFVp retroviral vectors are shown which encoding human SAP cDNA and reporter genes eGFP or LNGFR (A-C). The two lentiviral vectors encoding human SAP cDNA and reporter genes eGFP or only eGFP are presented (D, E). For the abbreviations refer to Chapter 1.

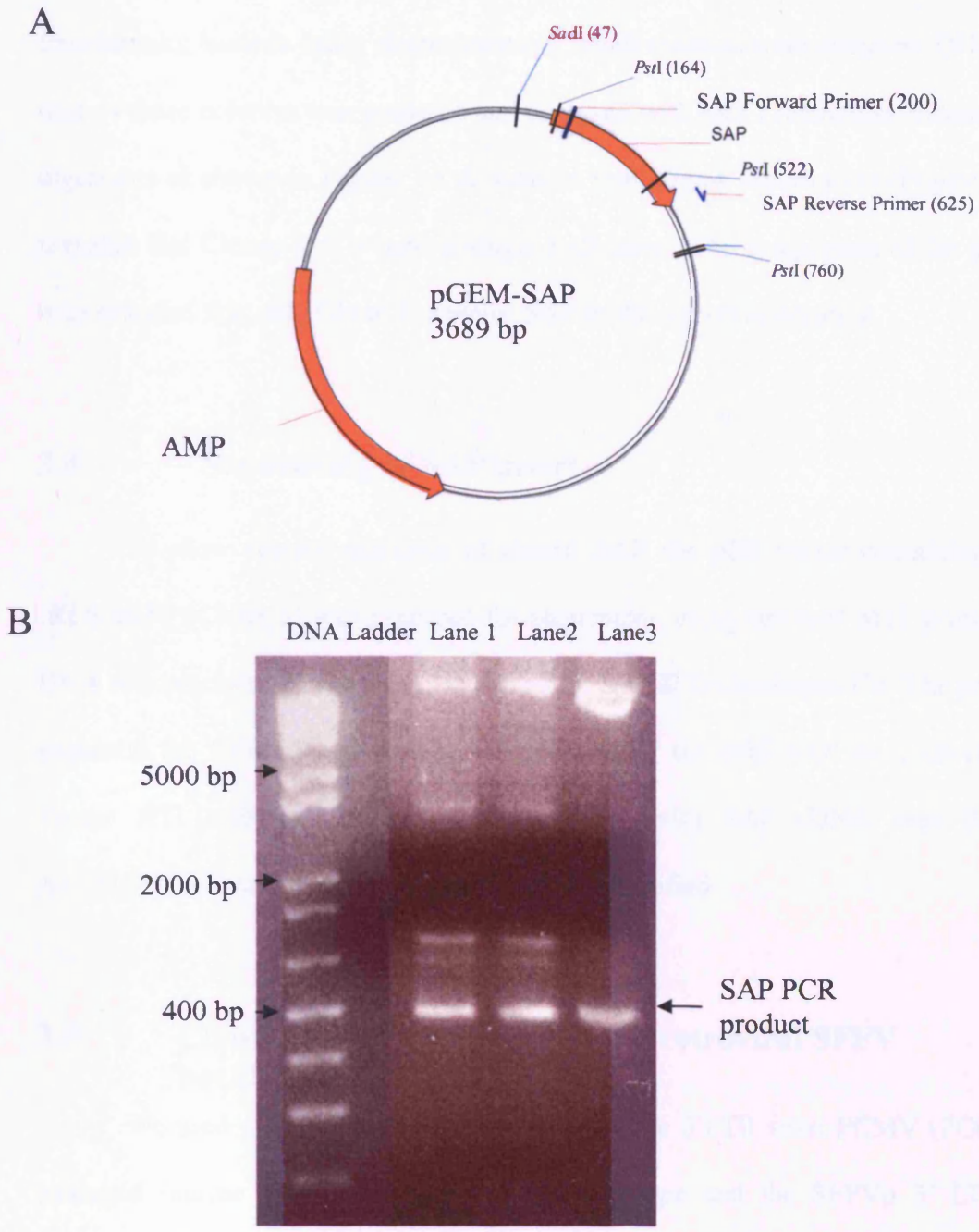


Figure 3.2 Plasmid map of human SAP cDNA and PCR product

A) The pGEM-T (Promega) vector including the SAP cDNA (670bp). B) The SAP PCR products were run on an agarose gel. Different SAP DNA concentrations were used for PCR reaction (0.1 µg, 0.2µg and 0.5µg for PCR reaction 1, 2, 3 respectively). The PCR products were loaded to a 1% agarose gel (Lines 1, 2, 3 represent the PCR product from PCR reaction one, two and three, respectively). The PCR product band was identified as about 400 bp as SAP band in all lanes. The PCR reaction 3 was selected for the next cloning step.

The SAP PCR product was cloned into the pBS vector at the Sal I site. After transforming bacteria using electroporation, 20-40 colonies were obtained. DNA from nine of these colonies was prepared and analysed with Nco I restriction endonuclease digestions as shown in Figure 3.3 A. Results from digest experiments (Figure 3.3 B) revealed that Clones 3, 6, 9 have a single SAP insert. The comparison of the plasmid map revealed that only Clone 3 contains SAP in the correct orientation.

3.4 Sequencing of SAP insert

To show correct sequence of cloned SAP, the pBS vector containing SAP-IRES-eGFP (Clone 3) was prepared for sequencing using standard M13 primer. The DNA was precipitated and then sequenced by LARK Technologies Co. The provided sequence for forward and reverse complement of the pBS SAP were checked by Vector NTI software (InforMax) to compare with SAP cDNA map (OMIM: AL023657) and no difference or mutation was identified.

3.5 Cloning SAP-IRES-eGFP in the retroviral SFFV backbone

We used a FMEV backbone modified in the 5`LTR from PCMV (PCC4 cell passaged murine sarcoma virus), a MPSV subtype and the SFFVp 3' LTR and containing a packaging signal from LX based Mo-MuLV vector (including 400bp of gag sequence with disrupting AUG) (see Chapter 1). The backbone vector included a MCS for insertion of a transgene of approximately 5.8 Kb in size.

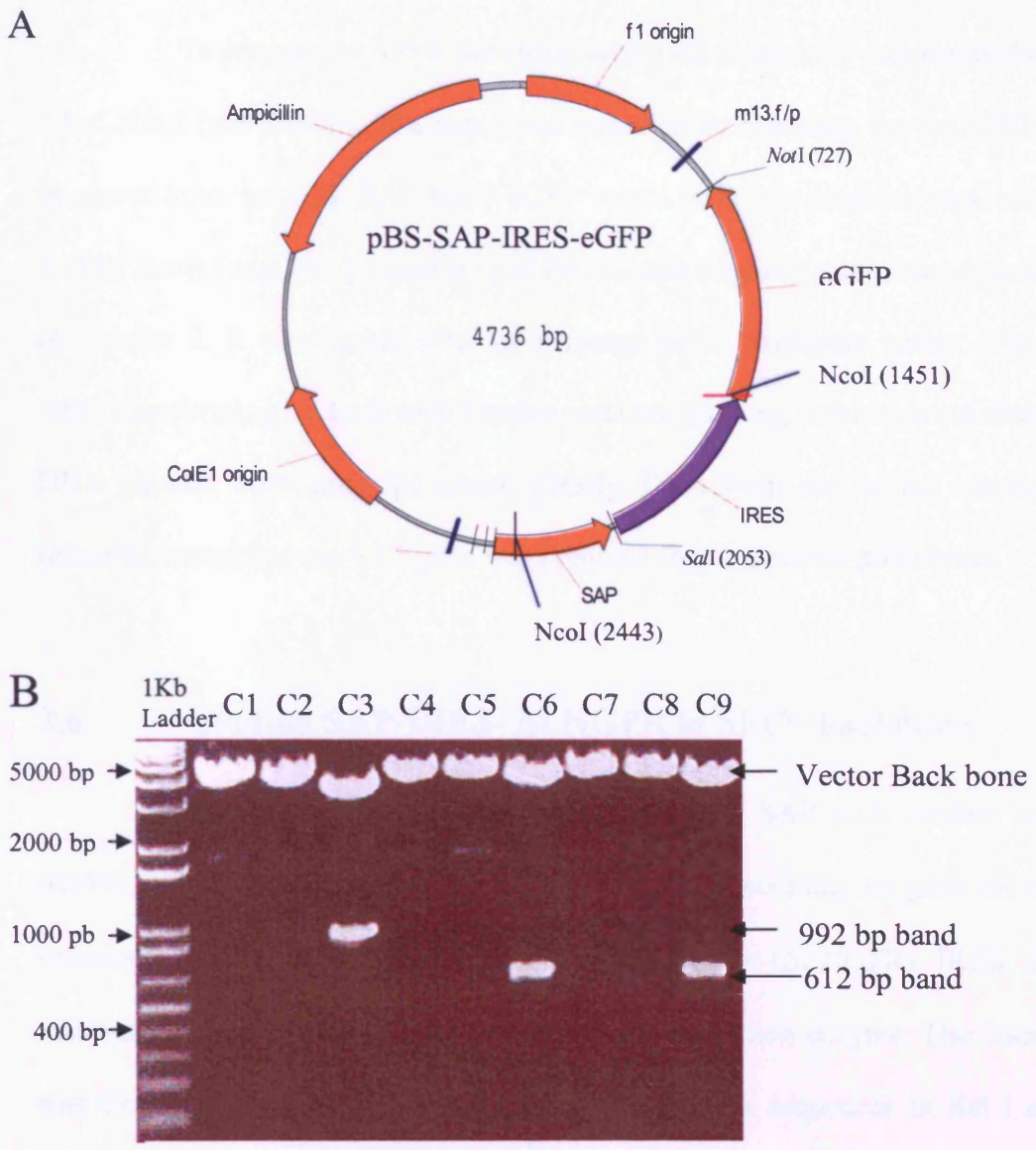


Figure 3.3 Plasmid map and restriction digestion analysis of the pBS SAP-IRES-eGFP

A) The map pBS SAP-IRES-eGFP vector containing of the *SAP* cDNA with Sal1sites. As indicated on the map, the NcoI site can be used for selecting right clone with correct SAP orientation. Digestion of the vector containing the *SAP* in the correct orientation would release a band of 992 bp. Plasmid DNA of harvested clones were digested with NcoI restriction enzyme. Digested DNA of 9 clones was run on a 1% agarose gel. According to the digest only clones 3, 6, 9 contained the *SAP* cDNA. Clone 3 contains the *SAP* insert in the correct orientation with size of 992 bp.

To prepare the SFFV backbone vector for ligation, it was digested by EcoR I and Not I (not shown). The insert was prepared by releasing the SAP-IRES-eGFP fragment from the pBS- SAP-IRES-eGFP vector (from the previous step, see section 3.3) by EcoR I and Not I digestion and the isolated fragment was cleaned as discussed in Chapter 2. It was ligated into the prepared SFFV backbone vector (Figure 3.4). After transforming bacteria with ligation reaction, growing colonies were screened for DNA plasmid containing the insert. Finally, DNA from one of the correct clones (clone 6) containing the SAP gene was prepared for viral vector production.

3.6 Cloning SAP-IRES- ΔLNGFR in SFFV backbone

To generate retrovirus SFFV vector encoding SAP with another selectable marker protein, we used the retroviral SFFV vector containing the gene encoding the truncated form low affinity nerve growth factor receptor (ΔLNGFR) -IRES. SAP was released from pBS–SAP-IRES-eGFP using Sal I restriction enzyme. The insert (SAP) was cloned in the SFFV vector down-stream of IRES sequences in Sal I site. The plasmid DNA from 30 clones was digested with Nco I to check the SAP insert had the correct orientation. The clones containing the SAP-IRES (1149 bp) band were identified according to the vector map (data not shown).

3.7 Generating retrovirus using packaging cell lines

To produce replication-incompetent SFFV retroviral vector encoding transgenic SAP and reporter proteins eGFP or ΔLNGFR, we used two steps,

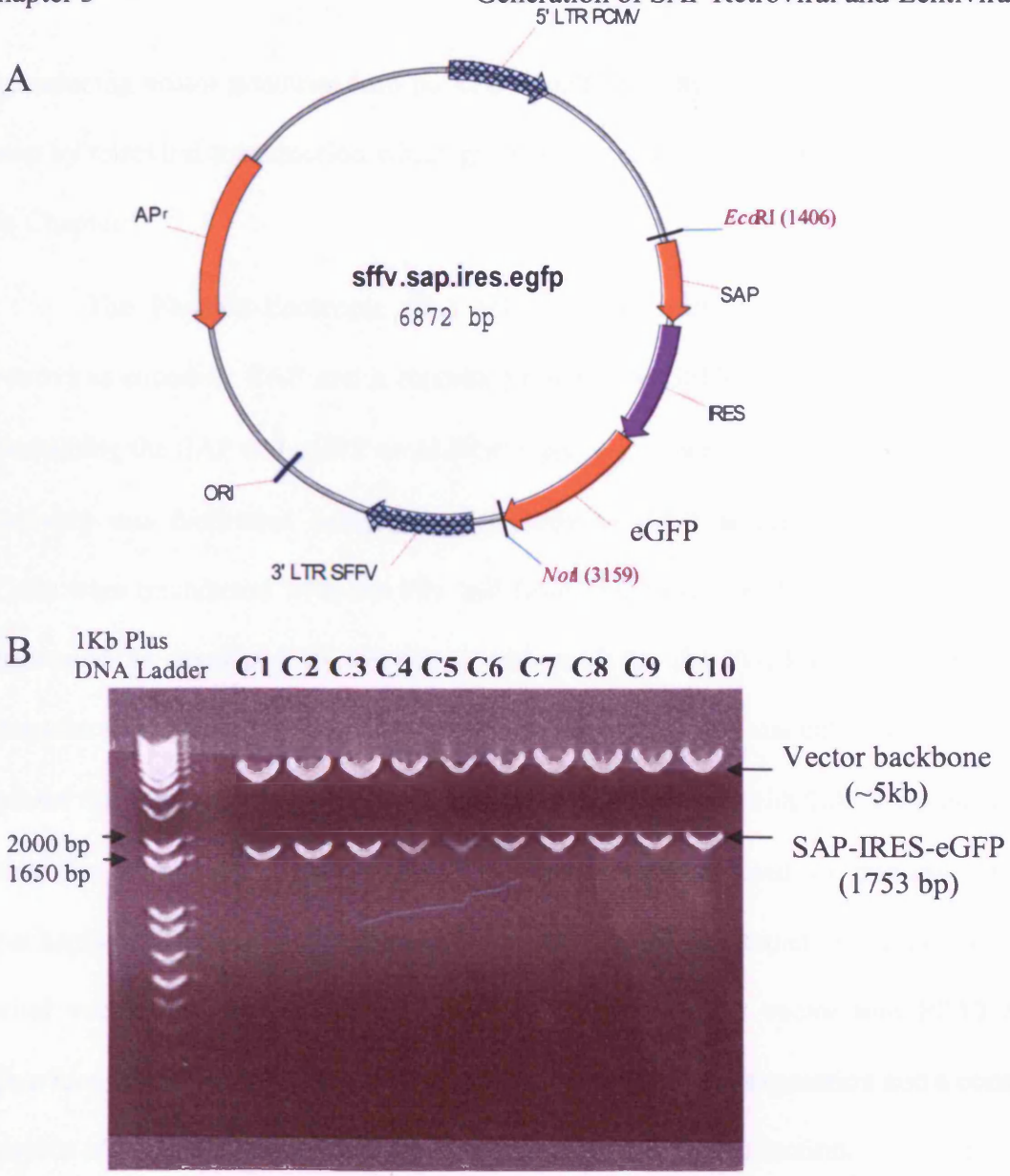


Figure 3.4 Plasmid map and restriction digestion analysis of retroviral SFFV-SAP-IRES-eGFP backbone vector

A) The map of retroviral SFFVp backbone vector encoding SAP and eGFP. B) Ten clones were investigated for Retroviral SFFV vector containing SAP-IRES.eGFP. The DNA was digested with EcoR I and Not I. The 1753 bp band of SAP-IRES-eGFP was identified in all clones. The clone 6 was selected for virus production in all experiments.

introducing vector genomes into packaging cell lines, by transfection and in the final step by retroviral transduction which gives the highest titer virus possible as described in Chapter 1.

The Phoenix-Ecotropic (Φ_e) cell line has been used to produce SFFVp retrovirus encoding SAP and a reporter protein. The SFFV vector plasmid construct containing the SAP and eGFP or ΔLNGFR genes was prepared for transfection. DNA delivery was facilitated using polyethyleneimine (PEI) as cationic DNA complex. Cells were transfected with the PEI and DNA complexes. In the next step virus was harvested as described in Chapter 2 and used for the PG13 packaging cell line transduction. We used the PG13 cell line to produce permanent SFFV retrovirus vector containing SAP-IRES-eGFP or SAP-IRES-ΔLNGFR with GALV envelopes. In this step harvested virus from the previous step was used to transduce PG13 packaging cell line. The expression was checked by flow cytometric analysis for both viral vectors (Figure 3.5). Thus introduction of retroviral vector into PG13 cells provides vector integration and permanent viral vector RNA expression and a constant source of replication- incompetent SFFV retroviral vector production.

3.7.1 Sorting PG13 packaging cell bulk culture with high expression reporter gene to isolate single clone

The bulk culture of transduced packaging cell line consists of heterogenous packaging cells with different viral production titre capability. To achieve the highest titre of virus, transduced packaging cells were sorted to single cell clones. FACS Cell sorting was used to select single cell clones of PG13 packaging cell lines transduced by SFFV-SAP retroviral vector. Transduced PG13 cell line for both viral vectors were

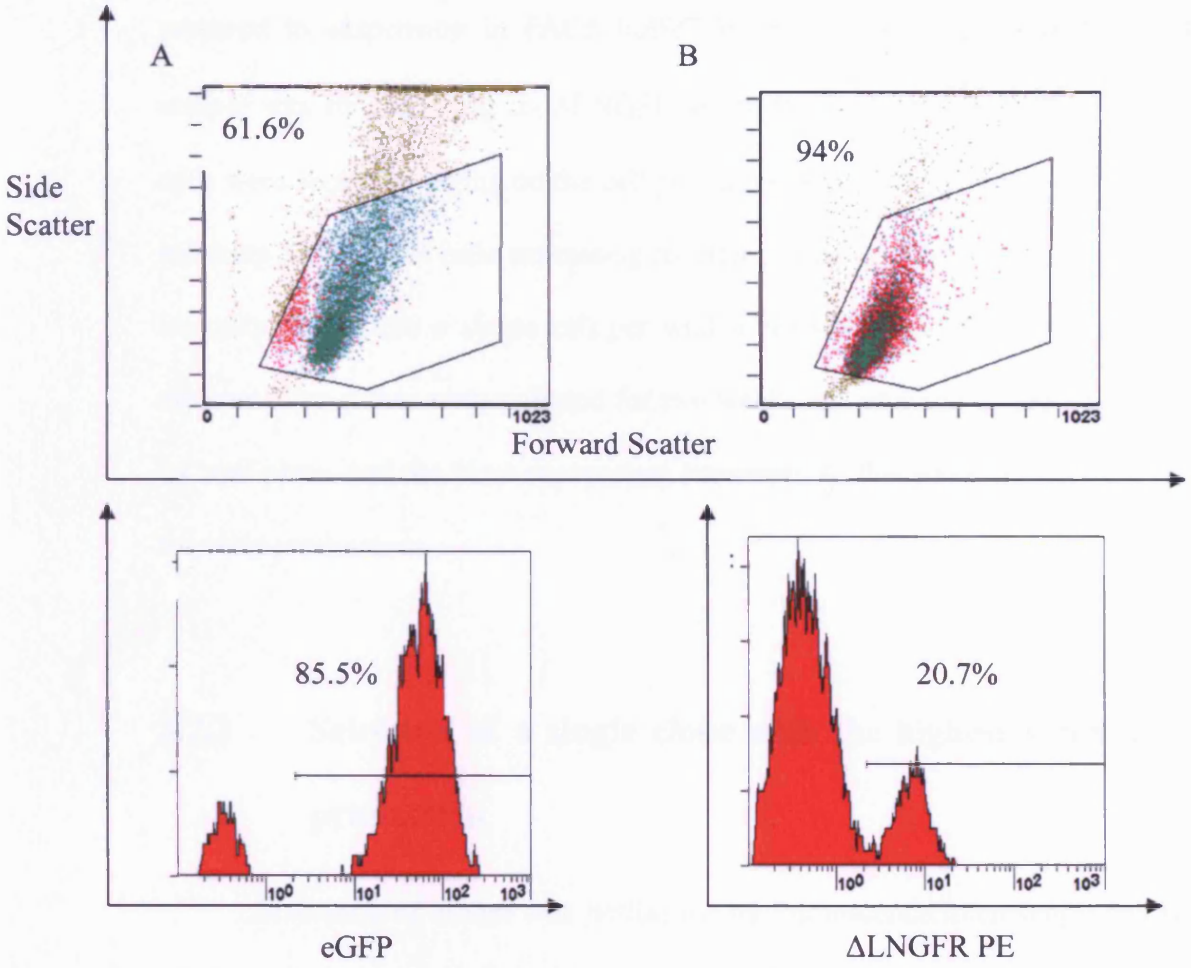


Figure 3.5 Production of PG13 packaging cell lines

Analysing transduced PG13 packaging cell lines with flow cytometry. The supernatant from Phoenix cells was used for PG13 transduction. The cells were harvested 72 hours after transduction with (A) SFFV-SAP-IRES-eGFP (B) ΔLNGFR and were analysed for eGFP or ΔLNGFR (using anti-ΔLNGFR PE antibody) expression based on fluoresence intensity. Histograms from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter and then on eGFP or ΔLNGFR positive cells.

prepared in suspension in FACS buffer. In the case of SFFV-SAP/ΔLNGFR, the sample was stained using α- ΔLNGFR antibody conjugated with PE. The prepared cells were sorted by gating on the cell population expressing reporter protein and then selecting the 1-2% of cells expressing reporter protein with highest mean fluorescence intensity (MFI) into a single cell per well in 96 wells plate with complete DMEM medium. The clones were cultured for two weeks and growing clones transferred into 24 well plates and the virus supernatant harvested. At this stage clones were screened for virus production.

3.7.2 Selection of a single clone with the highest virus titre production

Screening of clones was performed by fluorescence microscopy and titration of virus particles by measuring infection capability on the HeLa cell line. HeLa cells were infected by virus supernatant harvested from each PG13 clone at a different dilution. A day later, half the supernatant from each well was replaced with fresh medium. The infected HeLa cells were prepared for flow cytometric analysis after 48 hours (Figure 3.6). The percentage of positive infected cells with reporter protein eGFP or /ΔLNGFR was calculated using Expo analyze program.

The titre of the virus was determined according to the following formula:

$$\frac{\text{(\% of positive cells) x (dilution) x (number of infected cells)}}{100} = \text{Viral titer (Viral particles /ml)}$$

The clones with virus titres of up to 10^6 for both vectors were selected and used for reconstitution studies.

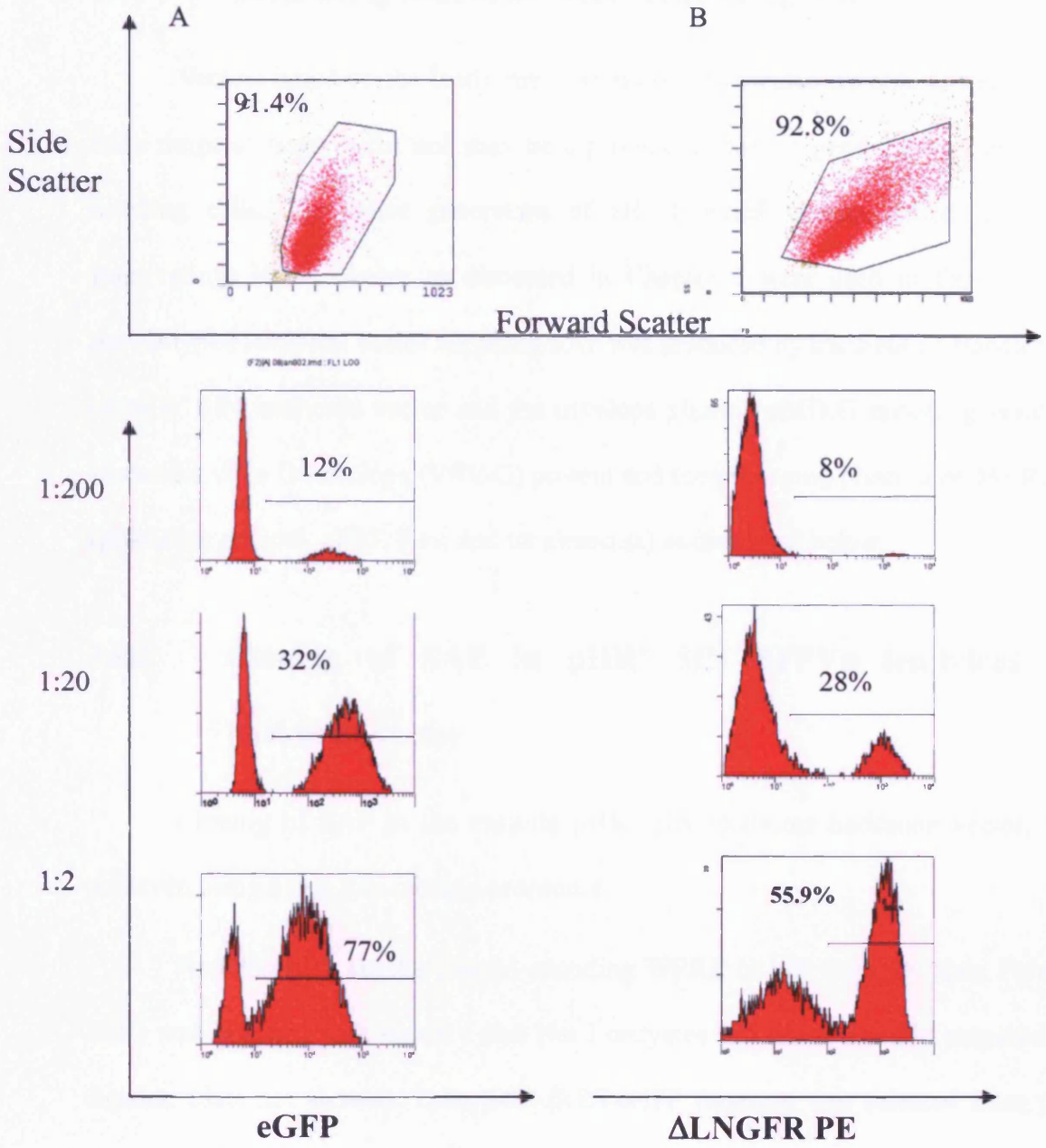


Figure 3.6 Titration of retrovirus particles produced by PG13 clones

HeLa cells were exposed to serial dilutions of virus supernatant in a final volume of 1ml (1:2, 1:20, 1:200). The infected HeLa cells were analysed by flow cytometry for reporter protein expression (eGFP/ΔLNGFR; A and B respectively). Using 5μl of supernatant (1:200) a transfer efficiency of 10-12% was observed and hence the titre of the viral preparation is estimated at 10^6/ml in this experiment.

3.8 Generating lentivirus vector containing SAP

Vectors based on the lentivirus subclass of retroviridae are able to transduce a wide range of target cells, and may be a promising tool for gene delivery into non-dividing cells. The latest generation of HIV-1 based vectors called SIN (self inactivating) HIV-1 vector as discussed in Chapter 1 were used in this study. A pseudotyped lentiviral vector encoding SAP was produced by transient co-transfection of pHR' SIN lentivirus vector and the envelope plasmid pMD.G encoding vesicular stomatitis virus G envelope (VSV-G) protein and the packaging plasmid pCMVR8.91 (providing gag-pol, cPPT, Rev, and tat elements) as discussed below.

3.8.1 Cloning of SAP in pHR' SIN SFFVp lentivirus backbone vector

Cloning of SAP in the cassette pHR' SIN lentivirus backbone vector, was achieved using a two step cloning procedure.

First the pBS shuttle plamid encoding WPRE (a gift from Dr Kate Parsley, ICH) was digested with BamH I and Not I enzymes and linear plasmid prepared for ligation (data not shown). Then SAP-IRES-eGFP fragment was selected from pBS containing SAP-IRES-eGFP (from section 3.3) by digesting with BamH I and Not I and the relevant fragments were isolated from the gel (data not shown). The vector and insert were used in a ligation reaction to transform bacteria by electroporation (Chapter 2). The DNA of transformed clones was screened for SAP-IRES-eGFP insertion with multiple restriction endonuclease digestions (BamH I and Not I) and analysis by gel electrophoresis (data not shown). The clone showing the correct restriction map was reserved for next stage in the cloning process.

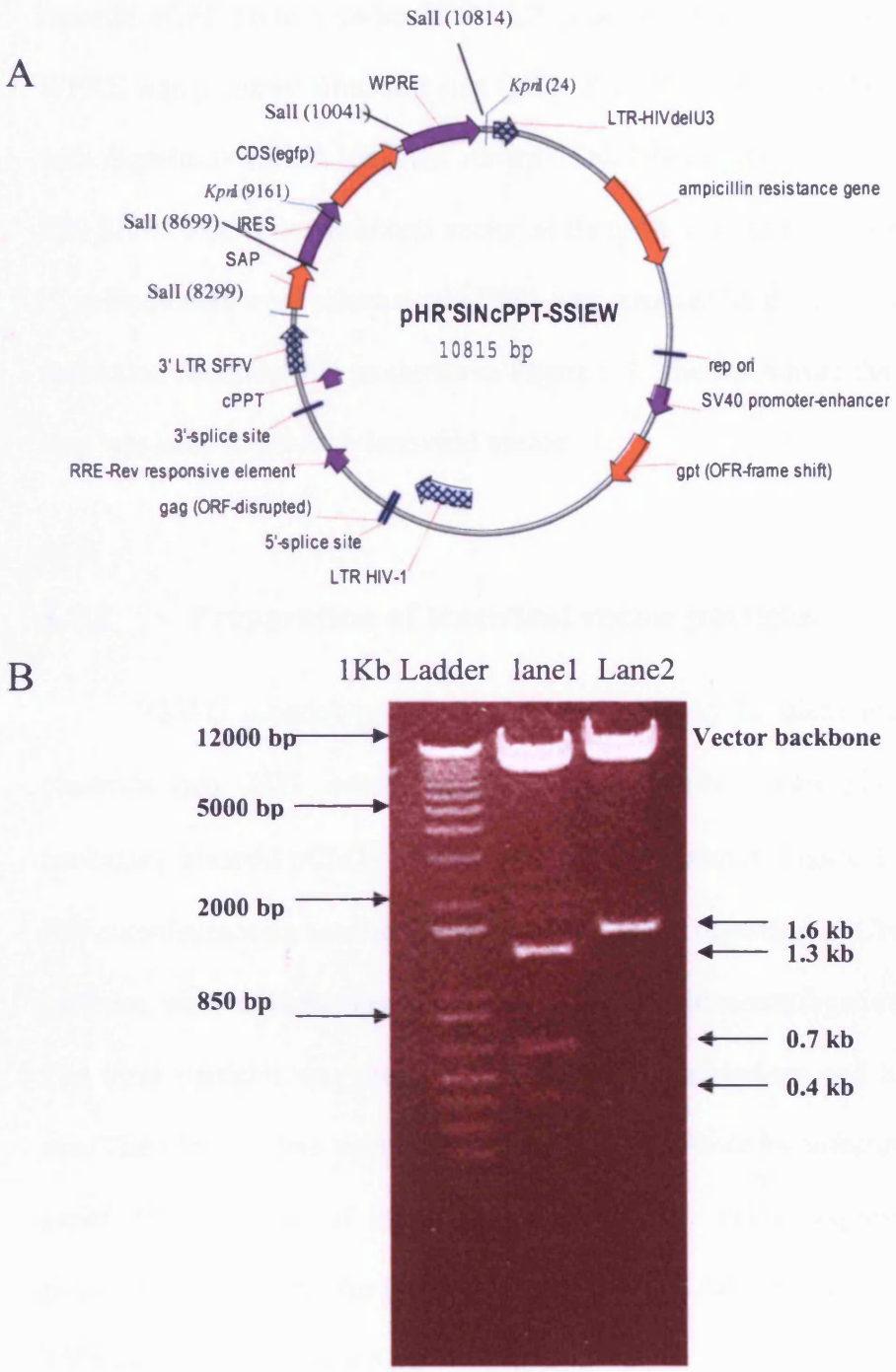


Figure 3.7 Plasmid map and restriction digestion analysis of pHR SINcPPT- SFFVp-SAP-IRES-eGFPS-WPRE (SIEW)

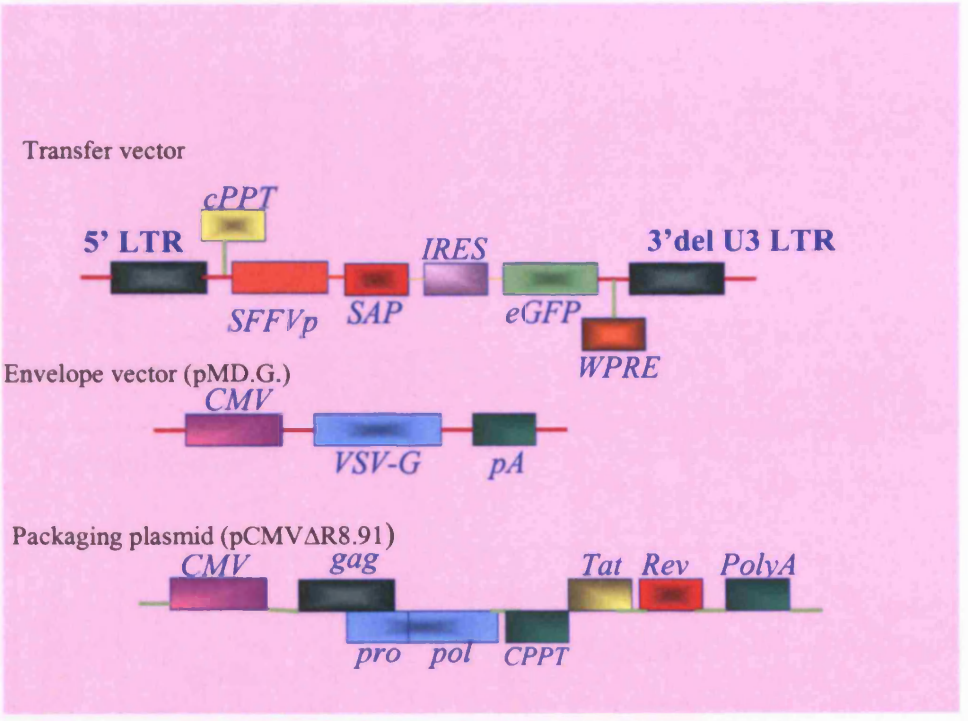
A) The map of lentiviral SIN HIV1 vector (pHR SINcPPT-SIEW) with size of 10 Kb. B; DNA from selected clones was digested with Sal I or Kpn I. Digestion with Sal I (lane 1) released four bands 8.3 kb, 1.3 kb, 0.7 kb, and 400 bp and digestion with Kpn I (lane 2) released only two bands of 9 and 1.6 kb.

To construct pHR' SIN SFFV lentivirus backbone vector encoding SAP-reporter eGFP protein under SFFV U3 promoter, the cassette of SAP-IRES-eGFP-WPRE was prepared from last step (pBS -SAP-IRES-eGFP-WPRE with ~ 6 kb size) with digestion of DNA (data not shown). The DNA fragment was cloned in the pHR' SIN SFFV lentivirus backbone vector at BamH I and EcoR I sites. The transformed E. coli colonies were selected and DNA was screened by digestion with Sal I or Kpn I restriction endonuclease as shown in Figure 3.7. The DNA from the clone with correct map was used to produce lentiviral vector.

3.8.2 Preparation of lentiviral vector particles

VSV-G pseudotype vector was produced by transient transfection of three plasmids into 293T cells: the pHR' SIN transfer vector plasmid, multideleted packaging plasmid pCMVΔR8.91, and pMD.G plasmid (Figure 3.8). The DNA plus PEI complexes were used to transfect 293T cells as described in Chapter 2. The vector particles were concentrated 20 to 100–fold by ultracentrifugation (see Chapter 2). The virus particles was resuspended in serum free medium and kept at -80^0C until use. The virus vectors were titrated as described before by infecting HeLa cells with serial 1:10 dilution of virus and analyzing for eGFP expression at 72 hours postinfection. The titre for harvested virus was calculated as explained and a final titre 10^8 was achieved (Figure 3.9).

A



B

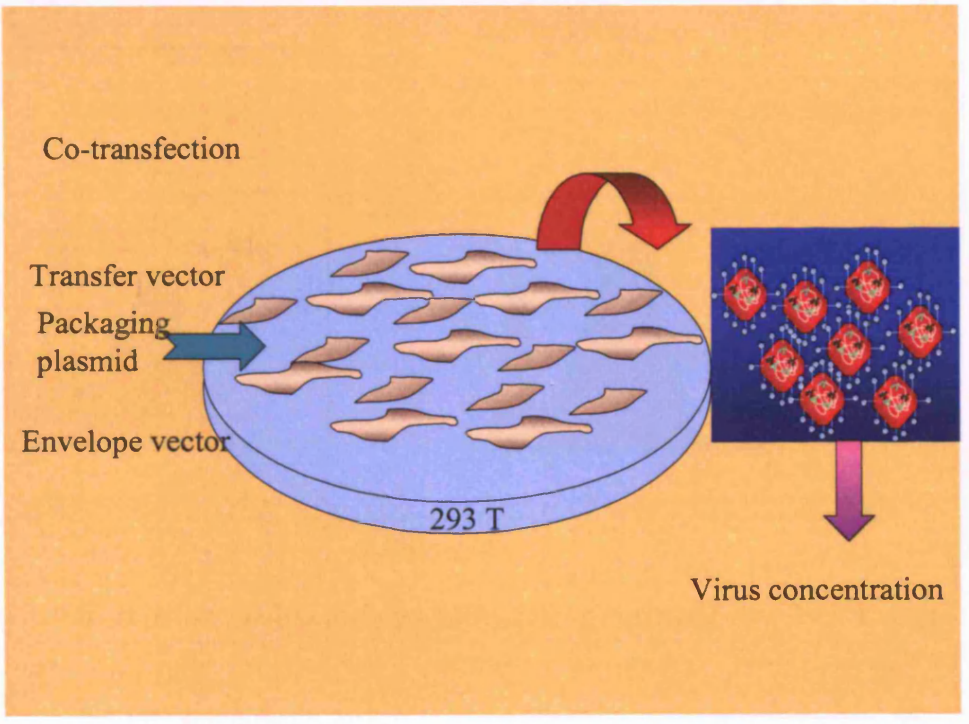


Figure 3.8 Producing lentiviral particles using a co-transfection system

A) The plasmid constructs essential for virus production

B) The co-transfection system using 293 T cell line for virus harvesting

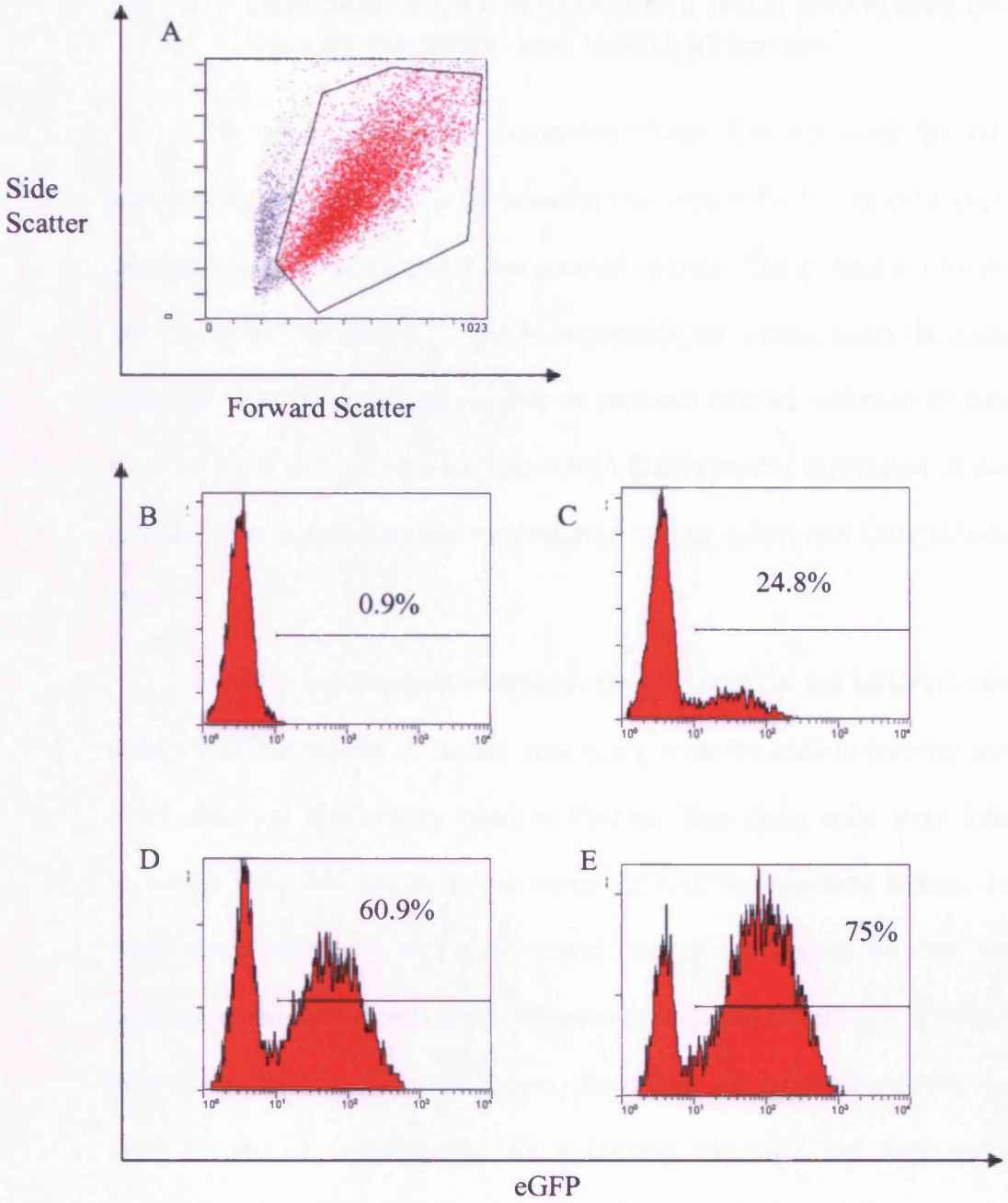


Figure 3.9 Titration of lentivirus particles produced by 293 T cell lines

HeLa cells were exposed to serial dilutions of virus supernatant in a final volume of 1ml ($1:10^6$, $1:10^5$, $1:10^4$, $1:10^3$ shown B-E respectively). The infected HeLa cells were analysed by flow cytometry for reporter protein (eGFP) expression by gating on cells with a high forward and side scatter (A) and then on eGFP positive cells (B-E). Using 1µl of supernatant ($1:10^3$) a transfer efficiency of 75% and hence the titre of the viral preparation is 10^8/ml in this experiment.

3.9 Detection of SAP expression in HeLa transduced cell lines by retroviral and lentiviral vectors

The use of bicistronic expression vectors has improved the efficiency of selection during transduction by ensuring that over 90%-95% of cells expressing the selectable marker also express the gene of interest. The retroviral vectors encoding IRES are well recognized for use in expressing two genes, under the control of one promoter. The IRES element is able to promote internal initiation of translation of RNA in a cap independent manner, which facilitates the expression of two or more proteins from a polycistronic transcription unit in eukaryotes (Martinez-Salas *et al,* 1996).

In order to investigate whether generated retroviral and lentiviral vectors could induce SAP expression in human cells along with the surface reporter protein, SAP expression was detected by western blotting. The HeLa cells were infected with harvested retroviral and lentiviral encoding SAP as described before. To measure transduction efficiency of a viral vector, the ratio of number of viral particles per millilitre to number of cells per millilitre or multiplicity of infection (MOI) is a widely used parameter in gene manipulation. Retroviral and lentiviral vectors were used at MOI 20 and 10, respectively. As a control, retroviral and lentiviral constructs encoding only eGFP protein were used to infect HeLa cells. After 48-72 hours, transduction efficiency was measured by flow cytometric analysis of eGFP expression and showed up to 50-60% transduction. The lysates from transduced and non-transduced HeLa cells were examined by western blotting, for the presence of SAP (Figure 3.10).

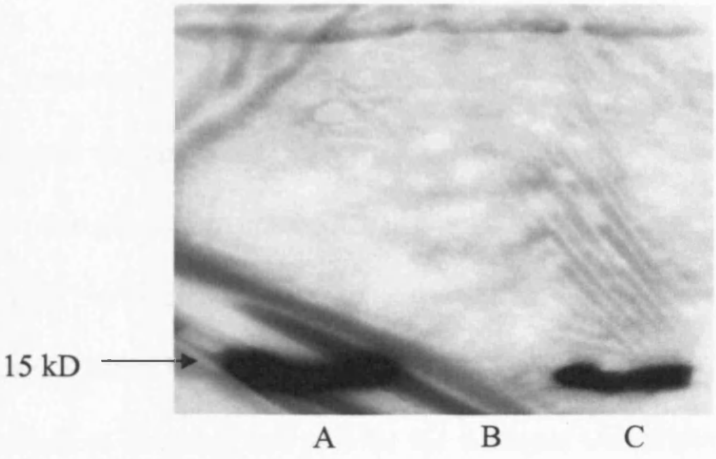


Figure 3.10 Detection of SAP in HeLa transduced cells by immunoblotting

Immunoblot analysis using an anti-SAP antibody shows a 15kD protein expressed in transduced HeLa cells with retroviral vector encoding SAP-eGFP (A) and lentiviral vector encoding SAP/eGFP (C) not in cells transduced with retroviral encoding eGFP (B).

Equivalent amounts of total protein from 10^6 cells were loaded in each lane. As shown, only HeLa cells transduced with viral vectors encoding SAP showed SAP expression.

3.10 Discussion

Retroviral vectors are the most commonly used gene delivery vehicles in clinical gene therapy trials. So far the progress of retroviral vectors have included; a) general design improvements, b) greater insight into retroviral stability and production, c) safer systems to ensure that replication-competent virus is not produced, d) vectors based on lentiviruses. The latest generation of retroviral vectors are resistant to silencing and provide the strongest transcriptional activity particularly in haematopoietic cells. Therefore, retroviral vectors are ideal for gene delivery to T cells, and hence their use in this study.

The constructs generated here are summarized in Figure 3.1. The generated retroviral vector is a SFFV hybrid based on Mo-MuLV vector with several features as described in Chapter 1, which make it an attractive vehicle for T cell gene transfer. Moreover, the lentiviral vector constructed here is a third-generation HIV-1 based vectors with self-inactivating (SIN) advantage and as described in Chapter 1, has different elements to improve its efficiency. The lentiviral vectors, unlike MLVs, are able to infect differentiated cells, and it was thought that they may give advantages to target EBV-specific T cells in our study without the need for a high number of cell division cycles. This appears to be due to the presence of redundant nuclear localisation signals both in the HIV MA protein as well as in the HIV accessory gene

product Vpr associated with MA protein (Heinzinger *et al,* 1994). The transduction efficiency on EBV- T cell lines of two vectors is studied in Chapter 4.

The cell surface markers eGFP and ΔLNGFR were designed to enable us to select transduced cells. The SAP and marker protein expression were under a single SFFV promoter using the IRES element derived from the encephalomyocarditis virus (ECMV). It has been shown there is a direct linear correlation between the genes (Zhu *et al,* 1999).

To generate retroviral SFFV vector, the PG13 packaging cell line was used and single cell high virus titre clones were selected. It has been reported that the two step approach to the production of retroviral vector from infected cell clones containing a single provirus provide higher and more stable virus production (Coffin *et al,* 1997). The clones were selected with a virus production titre higher than 10^6infectious particles/ml for this study. The titration was measured by infecting a fixed number of HeLa cells mediated with polybrene. However, because the viral envelope used here is GALV, the titration of virus particles on HeLa cells may not exactly represent the number of particles infecting T cells (further studied in Chapter 4).

The co-transfection system was used to generate lentiviral vectors because there were no packaging cell lines to produce lentiviral SIN vector at the time of our work. The transient transfection of 293 T cells with different constructs shown in Figure 3.8 produced a high number of viral particles with VSV envelope (G) known as pseudotyping. The titration of concentrated virus on HeLa cells showed a titre higher than 10^8 infectious particles/ml.

The risk of generation of the replication competent viruses for retroviral and lentiviral vectors is low in both systems. A GALV-pseudotyped retrovirus is incapable of infecting murine cells (PG13 cell lines) and so the chance of self-infection and re-combination of introduced SFFV retroviral vector with PG13 endogenous murine retroviral sequences is near impossible. Also using a SIN HIV-1 based vector and providing the trans-active elements and envelope with different plasmid constructs reduces significantly the chance of replication competent virus production.

Because SAP is a small cytoplasmic protein, the SAP expression in transduced cells with retroviral and lentiviral vectors was detected using immuno blotting. The results here shows that all steps of cloning human SAP into vectors and producing the viruses with above mentioned methods were successful and were able to express stable and detectable SAP in the human cells under the SFFV promoter. However, although the 40-50% of transduced cells were negative for eGFP expression, the SAP band was very strong showing a high level of expression. It remains to be addressed whether the constructed vectors have the same capability to express SAP in T cells, and this is further investigated in Chapters 4 and 7.

Chapter 4

Transduction of EBV-T Cells

4.1 Introduction

The first ever approved clinical trial of gene therapy began in 1990 at the NIH, and involved repeated *ex vivo* transduction of autologous peripheral blood T cells with retroviral vector expressing human ADA, followed by expansion and reinfusion into two patients (Blaese *et al,* 1995). Genetic manipulation of T lymphocytes may significantly benefit patients with various genetic or acquired haematologic disorders. Although T cells present a mature blood cell population, they can be very long lived and have the capacity for limited clonal activation and expansion. In the switch between the resting and the activated status, chromosomal organisation and transcription factor equipment is reordered. Thus, a stably integrating retroviral vector is an excellent tool for genetic manipulation of T cells, but vector expression may vary depending on the cellular activation status. At this time, the most widely used method for gene transfer into human T cells has involved Moloney murine leukemia virus (MuLV)-based vectors. MuLV based vectors allow stable and efficient gene transfer but efficiency mainly depends on cell division. In recent trials, the virus supernatant used along with co-localisation agents such as fibronectin has improved the efficiency of transduction of haematopoietic stem cells (HSC) and T cell transduction by retroviral vectors (Moritz *et al,* 1996; Stockschlader *et al,* 1999; Parsley *et al,* 2002). The fibronectin binds to the integrins VLA-4 (very late antigens) and VLA-5 (expressed on T cells) through its active CH-269 carboxy-terminal chymotryptic moiety, which is currently being used for clinical trials (Retronectin).

Furthermore, most protocols for primary T cell transduction are based on using mitogen (PHA), anti-CD3 alone or in combination with anti-CD28, and IL-2 with reliable results (Bonini *et al,* 1997; Pollok *et al,* 1999; Ferrand *et al,* 2000). In spite of this, gene transfer into antigen (Ag) specific T cells is difficult to achieve with

current techniques and methodology. New lentiviral vectors based on HIV1 may be appropriate candidates for targeting T cells without polyclonal expansion and may target Ag stimulated T cells which have limited proliferation capacity in comparison with ordinary stimuli.

While this methodology holds promise, optimism has been moderated by the difficulties encountered in achieving high levels of gene transfer into T cells without affecting their TCR repertoire distribution and function (Sauce *et al,* 2002; Qasim *et al,* 2003). For instance the stimulation of T cells with anti-CD3 caused shifting of T cells from naïve to the memory phenotype (Verhoeyen *et al,* 2003). In a recent report by Koehne *et al*, they succeeded in transducing purified EBV stimulated T cells with a dicistronic based MuLV vector which achieved 10% transduction but the efficiency was still very low (Koehne *et al,* 2000). Moreover, the third generation of lentiviral vectors based on HIV-1 as described in Chapter 1 may hold promise to improve gene delivery to Ag specific T cells without extra stimulation.

Since the well developed protocols for T cell targeting are mainly based on non-specific T cell activation and preparing patient material is a significant problem particularly in the rare diseases; developing a convenient practical system to target T cells without unwanted effects is a major aim. We have therefore tried to develop an efficient transduction protocol to minimise the ex-vivo manipulation of primary T cells and preserve the high efficiency of transduction using retroviral or lentiviral vectors. It was also a major objective to target mainly primary EBV- specific T cells with higher transduction efficiency rather than the T cell general population. Finally, we have been able to show efficient transduction of EBV- T cells from XLP patients with a defined protocol.

4.2 EBV T cells stimulation

To define conditions resulting in maximal transduction of antigen-reactive T cells, we initially examined different stimulation protocols based on autologous BLCL stimulation to achieve proper EBV specific T cell proliferation. 2 x 10^6 PBMCs from three normal EBV seropositive donors (C1, C2, C3) were stimulated with irradiated autologous BLCL at a 1/40 cell ratio (BLCL/ PBMCs) and cultured with complete RPMI medium at a concentration of $1x10^6$ cells/ml as described in Chapter 2. To prepare cells for transduction, different stimulation protocols were tested (summarised in Table 4.1) and expanded EBV T cell polyclonal lines analyzed for cell proliferation, CD4/CD8 ratio or prepared for transduction.

Table 4.1 Stimulation protocols used in this study for EBV-T cell polyclonal lines

Protocol	First Stimulation	Second Stimulation
Protocol 1	PBMCs stimulated with Autologous BLCL and rIL-2 for 3 days	On 4th day with PHA (5ug/ml) and rIL-2 (20 IU/ml) for 48 hours
Protocol 2	PBMCs stimulated with Autologous BLCL for 7 days	On 7th day with PHA (5ug/ml) and rIL-2 (20IU/ml) for 48 hours
Protocol 3	PBMCs stimulated with Autologous BLCL for 7 days	On 7-8th day restimulated with Autologous BLCL (4/1) and rIL-2

Following stimulation with each protocol, proliferative responses were analysed using Trypan blue to count live cells. There was an average 3.5 fold increase in cell number using protocol No.2 (Figure 4.1). However, using protocol No.1 and No.3, there was a 2 and 2.5 fold increase in cell number, respectively. In order to show T cell percentage and CD4/CD8 ratio upon different stimulation, stimulated cells were analysed by flow cytometry for CD3, CD4 and CD8 expression. All stimulated cells showed up to 80-90% CD3 positive phenotype with protocol No.1 showing less CD3 expression than others around 80% (Figure 4.2). In addition, the CD4/CD8 ratio presented considerable diversity using the different stimulation methods. Cells stimulated with protocol No.1 and No.3 exhibited 2.5:1 to 1.7:1 CD4/CD8 ratio whereas cells stimulated with protocol No.2 shifted to 1:1 CD4:CD8 ratio. Thus although cells stimulated with protocol No.2 showed a greater increase in cell number, it had an unwanted effect on the CD4:8 ratio.

4.3 Optimising EBV T cells transduction with viral vectors

So far optimal ex vivo conditions for T cell manipulations, transduction protocols, and vector constructs that maintain adequate transgene expression, have been under refinement. We next investigated the optimal transduction of EBV T cell clones with retroviral and lentiviral vectors using different stimulation and transduction protocols (Figure 4.3). Firstly, the transduction protocol was justified to prepare prestimulated T cells using fibronectin or polybrene to enhance gene delivery efficiency. In this study, retroviral SFFV vectors encoding SAP-eGFP or eGFP alone (a gift from Dr.W. Qasim, ICH) and SIN lentiviral (pHR'cPPT SFFV) vectors

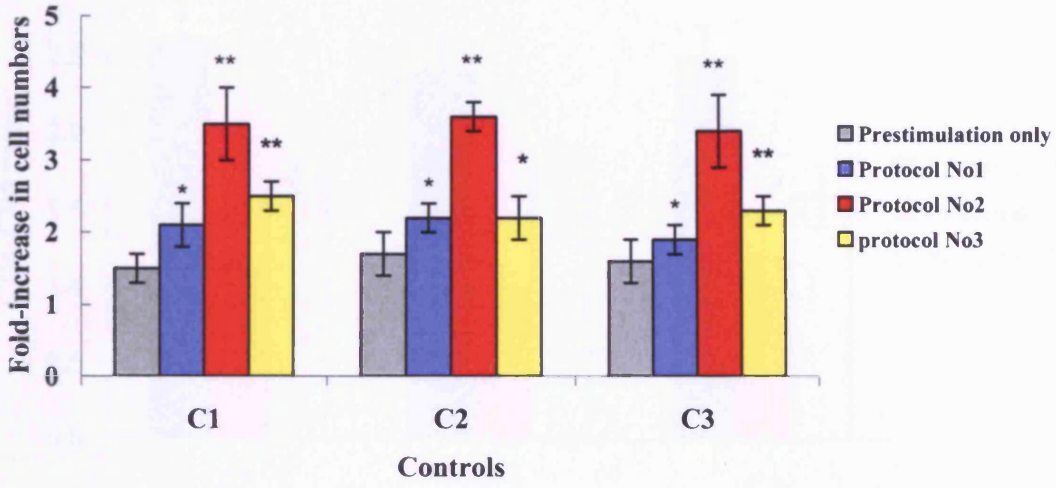


Figure 4.1 T cell proliferation rate in response to stimulation protocols No 1-3

Prestimulation of PBMCs (C1-3) with stimulation protocols No1-3 (see Table 4.1) induced T cell proliferation. The number of cells in culture were counted at the end of pre-activation (48 hours) using Trypan blue dye exclusion. Prestimulated cells (C1-3) without additional stimulation were used as the control for analysis. Data from three experiments on three volunteer donors is presented. The mean value (± SD) for each condition was used for statistical analysis. Significant differences are indicated by asterisks (no asterisk, no statistical significance; *, $0.05 \geq p \geq 0.001$; **, $0.001 > p$).

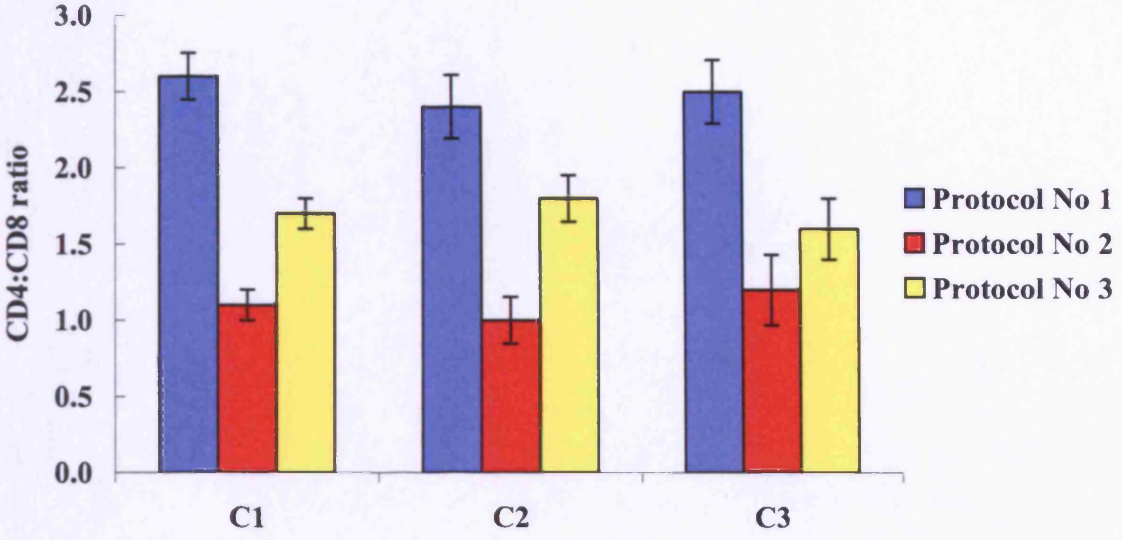


Figure 4.2 The effect of stimulation with protocols No1-3 on CD4/8 ratio

CD4/CD8 T cells ratio were studied in response to different types of stimuli combination (protocol stimulation number 1-3) in three normal individuals. The CD4/CD8 ratio were measured 48 hours after final step of stimulation with flow cytometric analysis respectively. The data is based on three experiments. The mean (± SD) for each condition was used for statistical analysis.

encoding SAP-eGFP or eGFP were used (Figure 3.1). The viral vectors were prepared as explained previously.

4.4 EBV-T cells transduction with viral vectors

Stimulated EBV T cells, as described in Chapter 2, were prepared for transduction. The titre of virus, established in Chapter 3, was 10^6 particles/ml for retroviral vectors and 10^8 particles/ml for lentiviral vectors. Two commonly used facilitation agents, polybrene and retronectin, were used to increase transduction efficiency. EBV-T cells were exposed to two rounds of infection with virus. The introduction of an additional step, whereby EBV-T cells and viral supernatant were centrifuged for 20 minutes, before the addition of fresh supernatant and rIL2. On the fourth day, cells were stained and analysed for CD3, CD4, CD8, eGFP expression with flow cytometry or cultured further to generate EBV- T cell clones as described in Chapter 2 (Figure 4.3).

4.4.1 Transduction EBV T cells with retroviral vectors

EBV T cells were infected with retroviral SFFV vector containing SAP-eGFP or eGFP alone using recombinant Retronectin. Because activated T cells express very late antigens (VLA)-4, and VLA-5, which interact with fibronectin, this may improve transduction of T cells with retroviral vectors (Stockschlader *et al,* 1999). Following stimulation with protocol No 2, the flow cytometry results showed transduction efficiencies were between 35-40%. Upon stimulation with protocol No.1,

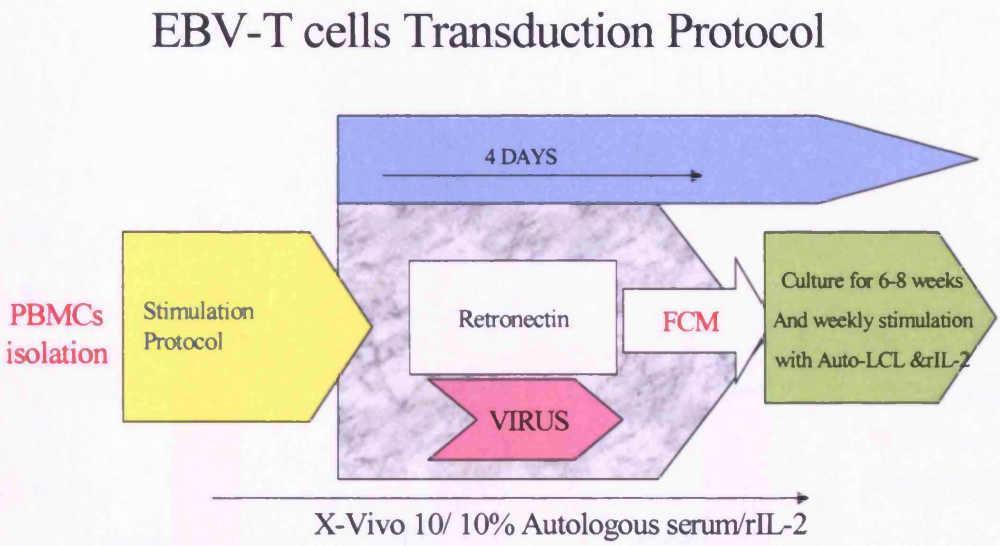


Figure 4.3 Protocol for the transduction of stimulated EBV-T cells

The following procedure was judged to achieve optimal levels of gene transfer into EBV- T cell polyclonal lines. Stimulated T cells from the stimulation protocols (1-3) were suspended in X-Vivo 10 with 10% autologous serum and 20iu/ml of rIL-2 at $0.5x10^6$/ml. The cells were transferred to fibronectin coated plates that had been pre-loaded with viral supernatant. Two rounds of 24 hours virus exposure were carried out and the cells were cultured for 48-72 hours before analysis by flow cytometry. The cells stimulated with protocols 2 and 3 were cultured for 6-8 weeks to generate EBV-T cell lines.

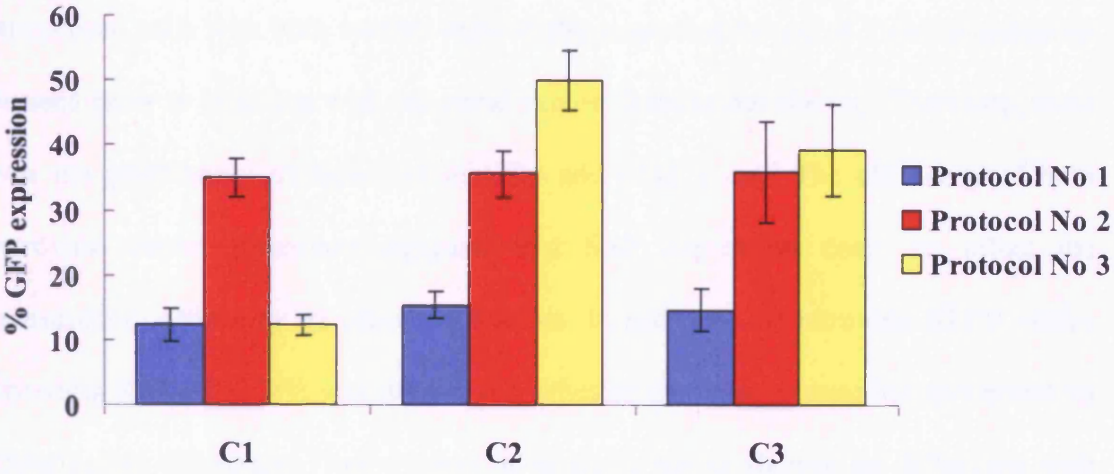


Figure 4.4 Transduction efficiency of retroviral vector in stimulated T cells with protocols No 1-3

Stimulated T cells from three normal individuals were transduced with retroviral vector encoding SAP-eGFP or eGFP following stimulation protocol 1-3. The eGFP expression was measured by flow cytometry after four days of transduction. The mean (±SD) for each condition based on three experiments was used for statistical analysis.

efficiency was very low with 10-15% (Figure 4.4). However, prestimulated T cells with protocol No.3 in which there was only stimulation with specific antigens (autologous BLCL) exhibited wide range of transduction efficiency with 10-50% efficiencies (Figure 4.4). The comparison of MFI for all experiments was constant with 200-250 mean range (data not shown). Analysis of CD4, CD8 expression on transduced cells with both vectors showed the same distribution of T cell populations as seen prior to infection with retroviral vectors (results not shown). Therefore, there was not preferential transduction of CD4 and CD8 T cell. The efficiency of both retroviral vector constructs suggests that SAP expression does not affect the transduction efficiency by retroviral vectors. In addition, the retroviral SFFV vector encoding SAP-ΔLNGFR was used to test whether the reporter gene has any effect on transduction efficiency. Flow cytometric analysis results showed no difference with above results (data not shown).

4.4.2 Transduction EBV T cells with lentiviral vectors

To investigate whether SIN lentiviral vectors based on HIV are promising vehicles for gene delivery to prestimulated or unstimulated T cells, particularly EBV T cells, transduction efficiency with lentiviral vector was examined using different stimulations and conditions. A modified lentiviral SIN backbone vector was used, which benefits from cPPT and WPRE under the SFFVp U3 promoter. Two vectors were used encoding SAP-eGFP or eGFP for these experiments. Transduction of prestimulated T cells with different quantities of viron particles demonstrated 10-20% efficiency. There was a positive correlation between MOI and transduction efficiency.

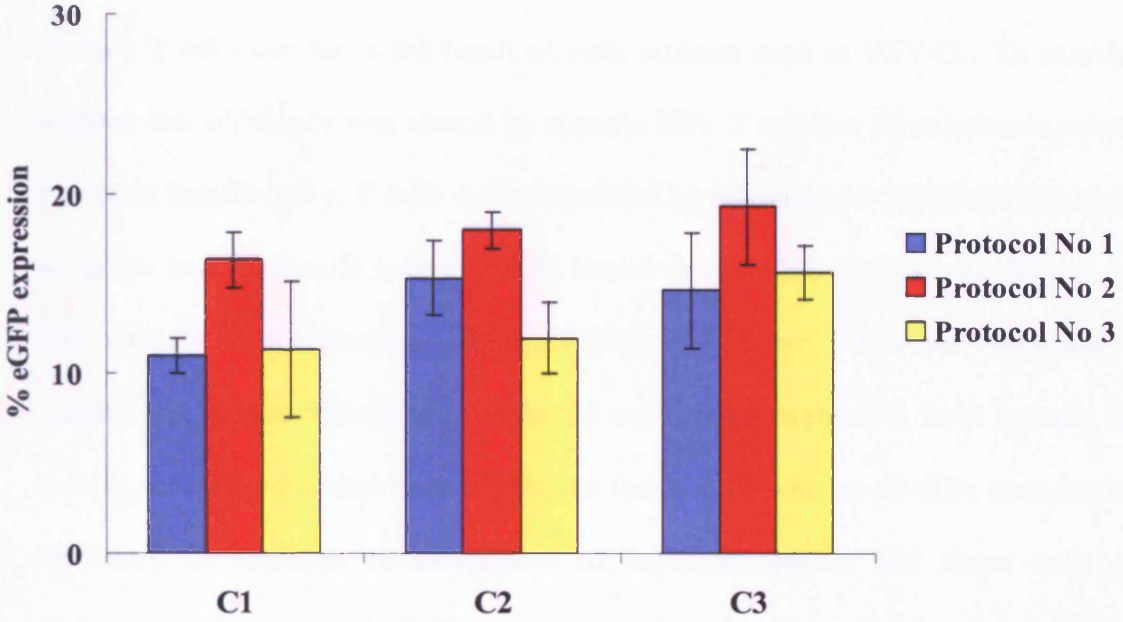


Figure 4.5 Transduction efficiency of lentiviral vector in stimulated T cells with protocols No 1-3

T cells stimulated with different protocols (stimulation protocols No 1-3, see table 4.1) were transduced with lentiviral vectors encoding SAP-eGFP or only eGFP (data presented for vector encoding only eGFP). The MOI for all experiments presented in this figure was 30. On the fourth day of transduction, they were analysed with flow cytometry for eGFP expression. The mean (±SD) for each condition based on three experiments was used for analysis.

However, this correlation was limited to MOI under 50 and the best efficiency achieved in MOI of 20-40 (units) (data not shown). Moreover, stimulation with different protocols did not increase transduction efficiency (Figure 4.5) and quantities more than 50 MOI had toxic effects on T cells. Although other studies showed a high MOI does not have toxic effect in some type of cells, the toxicity of viral particles in primary T cells can be as the result of viral proteins such as VSV-G. To examine whether low efficiency was caused by specific EBV T cell line stimulation or caused by vector insufficiency, T cells were stimulated by co-localized OKT3 and CD28 for 48 hours and then cells infected with lentivirus particles. The results showed no improvement in transduction efficiency (data not shown). However, infection of Jurkat cells or non-stimulated T cells did not change expression level (results not shown). Instead, retroviral vectors infected Jurkat cells with up 50-60% transduction efficiency. In addition co-localization of lentiviral vectors and target cells on fibronectin only increased eGFP expression by less than 5% and using polybrene did not show enhanced eGFP expression (Figure 4.6). The results from both lentiviral vectors were consistent.

4.5 Transduction of EBV T cell lines of XLP patients

In order to show feasibility of SAP reconstitution in T cells by retroviral vectors, the optimized transduction system was performed on EBV-T cell lines of XLP patients. Considering the result of transduction efficiency of retroviral and lentiviral vectors in stimulated T cells with BLCL (protocols No 1-3), the stimulation protocols No 2, and No 3 were used with retroviral vectors. The prestimulated EBV-T cells from two EBV seropositive XLP patients were prepared with protocol No 2 and No 3. Then cells were transduced either with retroviral vector encoding SAP-eGFP or

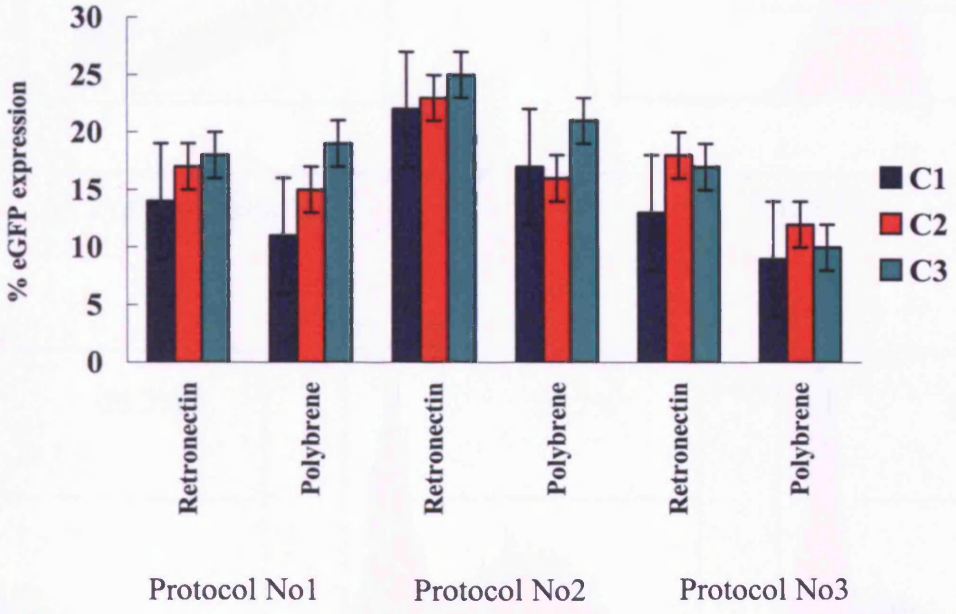


Figure 4.6 The effect of retronectin and polybrene on transduction efficiency of lentiviral vector in stimulated T cells with protocol No1-3

The effect of Retronectin and Polybrene on the transduction percentage of stimulated T cells (with protocol No1-3) with lentivirus vector was studied. PBMCs from three normal individuals (C1-3) were stimulated with protocol No1-3 and transduced with lentiviral vector (see figure 4.3) and transduction efficiency were measured with analysis of transduced cells for eGFP expression using flow cytometry. The mean (± SD) was estimated for each condition based on three experiments. The interindividual difference for each condition was not significant using stimulation protocols No 1-3.

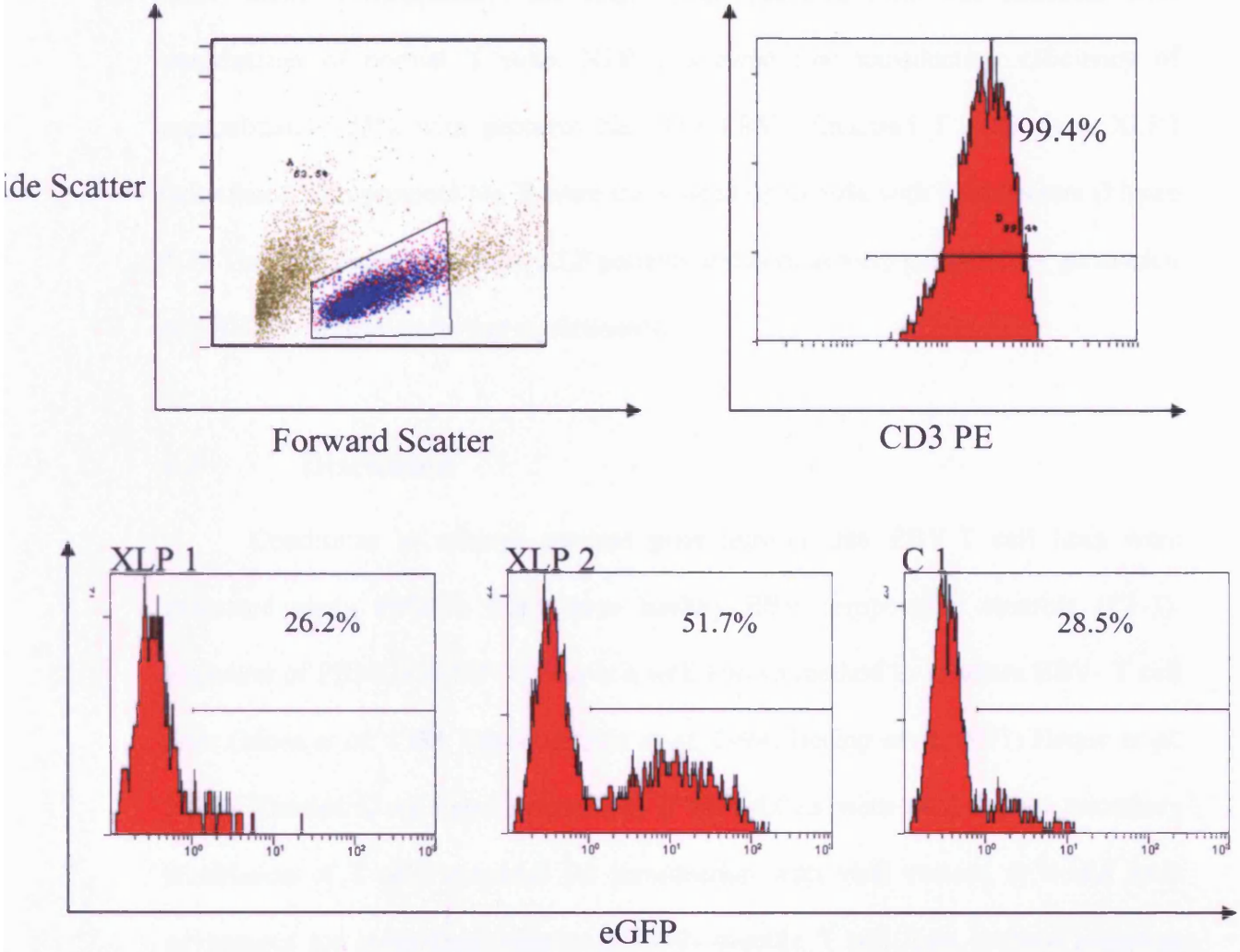


Figure 4.7 The efficiency of retroviral vector transduction of EBV-T cell stimulated with protocol No 3 in XLP patients versus controls

Transduction efficiency of retroviral SFFV vector encoding SAP and eGFP or eGFP alone on EBV-T cells. EBV-T cells from XLP 1, 2 and C 1 (C2, 3 data not shown) were stimulated with protocol No 3. Transduced cells were analysed by flow cytometry for eGFP expression on the fourth day. The flow cytometry histograms show CD3-PE expression of T cell clones and eGFP expression (XLP1, 2 and C1). The eGFP expression was significantly variable in different individuals from 25% to 50%. The data was confirmed based on three or more experiments in all three normal individuals and two XLP patients.

eGFP alone. Consequently, the result from protocol No2 was identical with transduction of normal T cells. XLP 1 showed low transduction efficiency of approximately 25% with protocol No3 but EBV stimulated T cells from XLP2 (stimulated with protocol No 3) were transduced up to 50% with both vectors (Figure 4.7). The transduced cells from XLP patients and normal were prepared for generation of EBV-T cell lines in further experiments.

4.6 Discussion

Conditions to achieve optimal gene transfer into EBV-T cell lines were evaluated using PBMCs from three healthy EBV seropositive controls (C1-3). Exposure of PBMCs to EBV-LCLs is a well-known method to generate EBV- T cell lines (Moss *et al,* 1988; Papadopoulos *et al,* 1994; Heslop *et al,* 1997; Haque *et al,* 2002). Therefore, we hypothesised that if EBV-LCLs were used for the necessary stimulation of T cells prepared for transduction with viral vectors, it would have advantages for generating transduced EBV- specific T cell lines without requiring excessive non specific T cell prestimulation. So, three types of T cell stimulation developed here are based on EBV-LCL Ags challenge. The different pre-stimulation of primed EBV-T cells were tried in protocols 1, 2 and 3 to ensure active division at the time of retrovirus or lentiviral exposure. It has been shown that although PHA and anti-CD3/28 stimulation increases T cell transduction, it causes CD4:CD8 ratio alteration (Duarte *et al,* 2002). Movassagh *et al* also reported that preferential expansion of CD8 T cells led to the CD4:CD8 ratio becoming inverted after T cell activation upon anti-CD3 and rIL-2 (high level of rIL-2) (Movassagh *et al,* 2000). Although there was considerable inter-individual variation between healthy donors, comparing the average data among individuals was consistent. In support of previous

work, the results showed that the activated cells were predominantly T cells at time of analysis with each different protocol. Moreover, the results presented here confirmed that only protocol No 2 demonstrated a reversed CD4:CD8 ratio. However, although PHA was used with protocol No1, the change in CD4:CD8 ratio was nearly similar to protocol No3 in which only Ag stimulation and a very low level of rIL2 was used. The CD4 preference seen with protocol No1 may be explained by the early analysis of the stimulated T cells whereas other studies have shown that the CD4:CD8 ratio changed after five days of *ex vivo* stimulation (Movassagh *et al,* 2000).

It has been reported recently that highly efficient primary T cell transduction with MLV based retroviral vectors can be achieved using retronectin and CD3/28 stimulation (Qasim *et al,* 2002). Also it has been shown that the additional centrifugation of target cells and virus (also referred to as spinoculation) improved infection efficiency (Qasim *et al,* 2002; Kuhlcke *et al,* 2002). The same transduction method was used here in addition to using different stimulations. However, although protocol No1 showed the highest increase in cell numbers following stimulation of EBV-T cells, the transduction efficiency results were lower (10-20%) than the two other protocols. Whereas the results of transduction with protocol No2 were more efficient and comparable with other reports, using a non-specific mitogen, such as PHA, would have an effect not only on the T cells function as reported by Duarte *et al* (2002), it also has an effect on EBV- T cell lines phenotype, as we describe later in this study (see Chapter 5). In contrast, the percentage of transduced cells stimulated with protocol No3 varied considerably amongst individuals (10-50%) but is still more efficient than previous reports (Koehne *et al,* 2000). It may be explained by the different frequencies of EBV-specific CTLp in population. The achievement of a reliable transduction efficiency with protocol No3 enables us to avoid the extra

stimulation such as PHA which has a striking effect on depletion of both EBV-specific and allospecific T cells as previously reported (Koehne *et al,* 2000). In addition, the percentage of transduced CD4 and CD8 was similar using different protocols. This is in support of previous studies (Duarte *et al,* 2002; Qasim *et al,* 2002). In all these experiments, the two retroviral constructs preserved the same transduction efficiency confirming that different transgenes do not interfere with gene transfer efficiency.

Furthermore, the lentiviral vectors are recognised for their ability to transduce the target cells without any extra stimulation. However, the reports on the efficiency of lentiviral vectors to transduce T cells showed that extra stimulation with IL-2 alone or with PHA enhance the efficiency from around 2% to 40-70% (Manganni *et al,* 2002) although others reported lower efficiency (Verhoeyen *et al,* 2003). However, these stimulations would have effects on T cell functionality and phenotype as described before. In our results using lentiviral SIN vector taking advantage of trans-active elements such as cPPT and under SFFVp as explained in Chapter 1, the efficacy of transduction was not as high as using retroviral vectors. The protocols No1, 2, and 3 showed almost the same results and applying co-localising agents did not have significant effect on outcomes. Interestingly, using anti-CD3/28 stimulation did not improve transduction efficiency. More recently, application of IL-7, a cytokine which interacts with T cells through its receptors, has been shown to improve the lentiviral transduction without any impact on T cell phenotype (Verhoeyen *et al,* 2003). It could be possible that using IL-7 may be beneficial for transducing EBV-T cells with higher efficiency without affecting EBV-specific and allospecific T cell subpopulations. However, these issues remain to be investigated in future studies.

Therefore, in these studies retroviral vectors provide the ideal tool to transduce EBV-T cells. The results presented here show that we have been able to transduced EBV-T cell from XLP patients and controls (26%-50%) with our constructs using defined stimulation protocol with transduction efficiencies appropriate for further investigations.

Chapter 5

Immunophenotyping of EBV-T Cell Lines

5.1 Introduction

During the primary response and again during secondary infection, the most important function of cytotoxic T lymphocyte (CTLs) is to eliminate noncytopathic viruses, which may otherwise cause significant immunopathology. EBV provides one of the most informative systems with which to study CTL responses in humans (see Chapter 1). EBV is well known for its growth-transforming function that is regulated by a specific subset of genes, the latent genes, which are expressed in latently infected lymphoblastoid cell lines (LCL) in a coordinated manner (Tatsumi, 1992). LCLs arise following viral infection of resting B cells *in vitro* and provide an *in vitro* model to investigate T cell responses to EBV latency Ags (Rickinson *et al,* 1997). The infected B cells have the ability to generate cytotoxic T cells by presenting the viral antigen (Ag) to TCRs. Moreover, memory CTLs in the blood of healthy virus carriers can be reactivated *in vitro* by stimulation with autologous LCL and expanded as polyclonal T cell lines or as CTL polyclones in interleukin 2 (IL2)- conditioned medium.

So far EBV-specific cytotoxic CD8 lymphocytes in normal individuals have been well characterized for memory-effector and activation immunophenotypes (Callan *et al,* 1996 & 1998; Hislop *et al,* 2001; Appay *et al,* 2002). Also their dominant reactivities are well documented to one or more latency antigens, mainly EBNA 3A, 3B, 3C (Rickinson *et al,* 1997). However, studies of specific T cell immunity to EBV in XLP are contradictory (Chapter 1; Gaspar *et al,* 2002). In order to study XLP T cell responses to EBV, an *in vitro* autologous LCL system was used in the work presented here. Although XLP patients respond to EBV infection with T cell proliferation, those who survive from FIM maintain a dominant CD8 population and a reverse CD4/CD8 ratio (see Chapter 1). However, the T cell phenotype and

responses such as HLA-restricted EBV-specific T cell memory have not been investigated in detail or compared with normal EBV carriers. More recently, cytotoxic CD4 lymphocytes have been defined and in EBV infection, particularly in response to EBNA 1, play an important role in immunity (Munz *et al,* 2000; Nikiforow *et al,* 2001 & 2003). In contrast with cytotoxic CD8 cells, these have not yet been studied comprehensively and their character with regard to effector phenotype remains undetermined. In order to compare the phenotype of EBV- T cell lines in XLP patients (with defined genetic lesions; see Table 5.1) with normal controls, two kinds EBV-T cell lines were generated following the stimulation protocols No.2 and No.3, which were explained in the previous chapter. Furthermore, they were evaluated for SAP-related receptors and chemokines, which recently have been suggested to be important in immune response to viral infection.

Table 5.1 Details of XLP patients who were investigated in this study

XLP patient	Age	Mutation	Phenotype	EBV serotype
XLP1 (P1)	4 years	G→ T (nt500)	EBV+HLH	Seropositive
XLP2 (P2)	16 years	N448-159 del	EBV+B cell lymphoma	Seropositive
XLP3 (P3)	19 years	IVS: -32—28 Del A TTTT	CVID	Seronegative

5.2 Generating EBV-T cell lines and their proliferation response to autologous EBV-LCL

Generating CTL *in vitro* has been well described for almost two decades and has been used beneficially in different clinical applications (Papadopoulos *et al,* 1994; Mackinnon *et al,* 1995; Heslop *et al,* 1997; O'Reilly *et al,* 1997; Haque *et al,* 2002). Simply, peripheral T cells can be divided into two subsets: naïve and memory cells. Memory cells have the capacity to proliferate and generate effector cells in response to specific Ags. Autologous BLCLs can be considered as effective antigen presenting cells. Although they are not as potent as dendritic cells and macrophages because of poor B7.1 and B7.2 ligand expression, they have been used in many studies to generate EBV-specific T cells in response to most latency antigens. As XLP is a rare disease and considering the difficulties of obtaining material from XLP patients, generating EBV-T cell lines using autologous BLCL as antigen presenting cells is an ideal system for this study.

To generate autologous BLCL lines from XLP patients (XLP 1, 2) and normal control donors (C1, 2, 3), 2-3 million PBMCs were infected with EBV, which was prepared from the B95-8 cell line as described in Chapter 2. Autologous BLCL from XLP3 was generated from bone marrow cells because the patient had B cell lymphopenia. The established transformed cells were analyzed using flow cytometry for CD19 (a B cell marker) and CD 3 (T cell marker). All lines were over 95% CD19 positive and negative for CD3 (data not shown). These EBV-LCL lines were used to generate EBV- specific T cell lines.

Pre-stimulated EBV- T cells were generated using protocols No2 and No3 (see Table 4.1), and used to produce EBV-T cell lines, which were either transduced (with SAP-eGFP or only eGFP) or non-transduced (as described in Chapter 4). The lines were stimulated with irradiated autologous BLCL and rIL-2 weekly and cultured in

complete RPMI as shown in Figure 4.3. EBV- specific T cell lines were successfully generated from two XLP patients and two normal controls. All lines generated using protocol No2 were mainly CD4 positive (85-95%) and lines generated with protocol No3 were mainly CD8 (80-95%) positive (see Table 5.2 and Table 5.3).

As shown in Figure 5.1, the rate of EBV- specific T cell lines expansion obtained in XLP patients was comparable to the pattern observed with lines from healthy donors, with a mean of 4-4.5- fold expansion after 4 stimulations. In addition, non-specific polyclonal stimulation of XLP T cells with lectin substances such as PHA induced a 2- fold expansion, which was consistent with results from healthy controls. To summarise, these results confirmed that T cells from XLP patients show normal proliferation capacity in response to autologous BLCL or a polyclonal stimulator demonstrating that lack of SAP expression does not affect this process.

5.3 EBV-T cell line immunophenotyping

The EBV-T cell lines generated were CD3 positive (95-99%) with more than 90% of cells positive for the αβ TCR and less than 5-10% of the lines expressing γδ TCR (Figure 5.2). Natural killer cells (defined as cells expressing CD56 and /or CD16 antigen but lacking CD3 expression) represented less than 2-5% of the population in both CD4 and CD8 lines. In addition, the EBV -T cell lines were almost negative (less than 1-2%) for lymphokine activated killer (LAK) (CD3+/CD56+). As shown in Table 5.2 and 5.3, EBV-T cell lines were examined for other markers, which are explained in detail below.

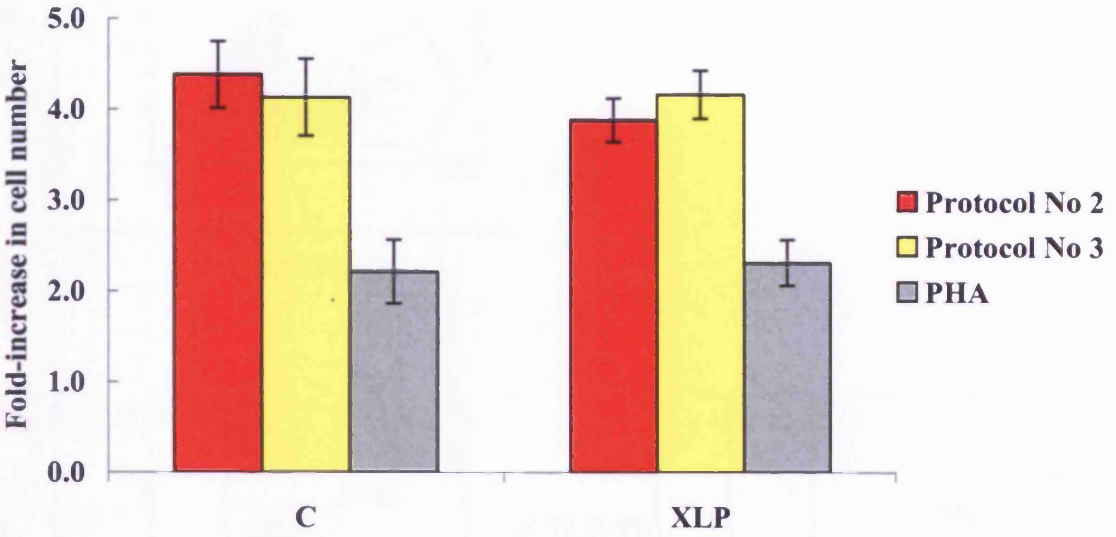


Figure 5.1 T cell proliferation in XLP patients and controls

EBV- T cell proliferation was measured by cell counting in two XLP patients and two controls under protocol stimulation number 2 and 3 after four weeks. The mean (± SD) data for each group (XLP and control groups) were calculated based on at least three occasions. As a control, PBMCs were stimulated with PHA and the increase in cell number was measured after 48 h.

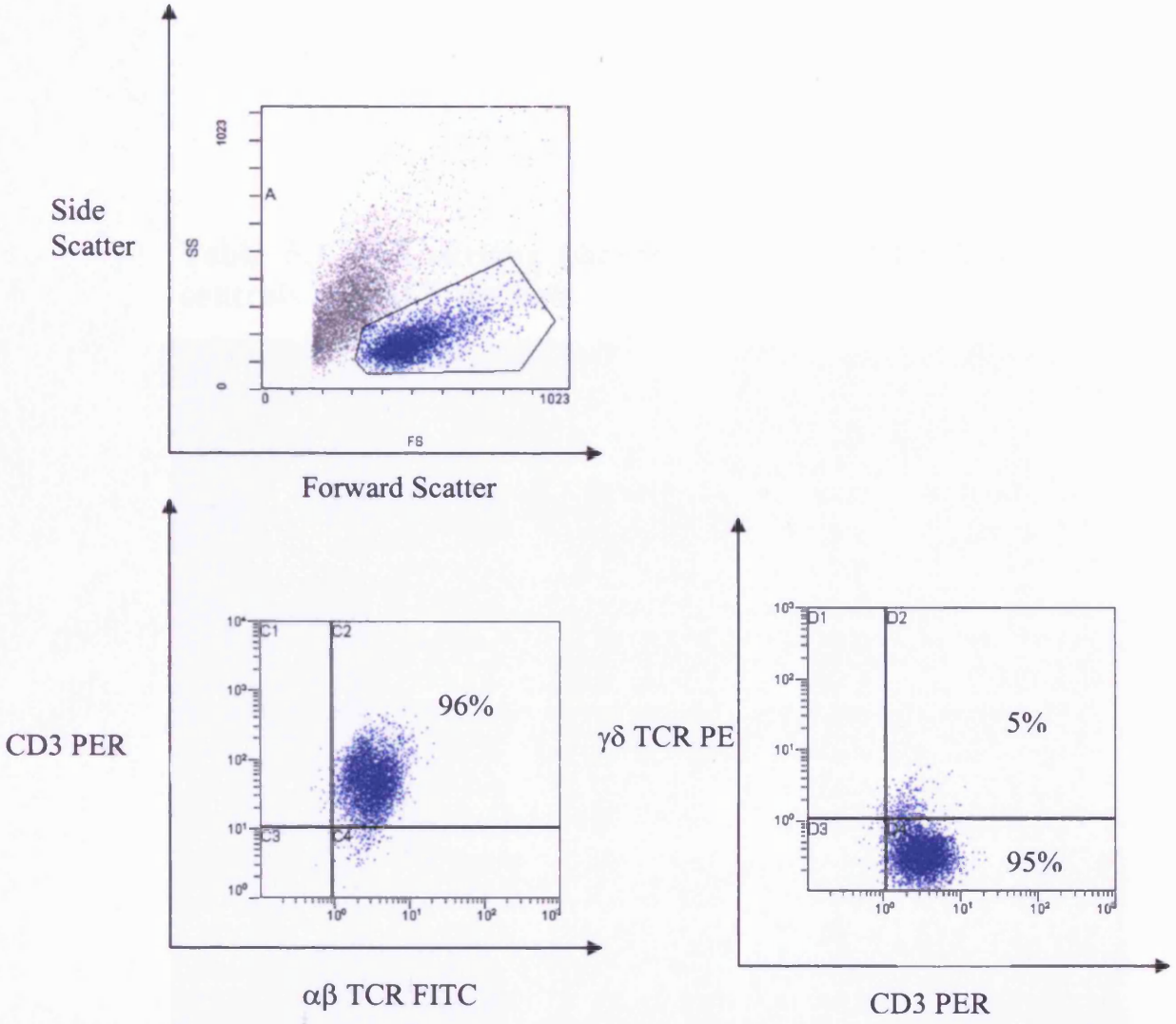


Figure 5.2 Surface expression of αβ and γδ TCR by EBV-T cell lines

The surface expression of αβ and γδ TCR EBV- T cell lines were examined by flow cytometric analysis. Plots from a representative experiment (XLP1) are displayed and were generated by gating on cells with a high forward and side scatter then on CD3 positive population. Quadrants were placed according to their isotype controls (not shown). All the lines from control and XLP patients were mainly αβ TCR positive with a small percentage γδ TCR T cell population.

Table 5.2 Cell surface phenotype of EBV-CD8 T cells from controls and XLP patients

Cell surface marker	% expression (MFI)		% expression (MFI)	
	NORMAL		PATIENT	
	C1	C2	XLP1	XLP2
CD3	95-95(136)	95-95(227)	95-98(183)	95-99(133)
CD4	5-15(19.6)	12-20(39.5)	7-8(26)	10-12(43)
CD8	80-95(283)	85-95(232)	90-95(185)	80-90(223)
CD16	3-10(1.6)	2-5(2.1)	7-13(1.3)	10-12(1.6)
CD56	5-15(3.4)	10-12(3)	8-10(2.8)	10-12(3.1)
CD45RA	2-5(3.5)	2-5(2.1)	3-6(2.5)	8-10(3.3)
CD45RO	90-95(70.6)	90-97(60.1)	94-97(40.3)	90-93(51.4)
CD28	0.5(2.5)	0.9(1.5)	1.05(2.7)	1(1.9)
CD25	1-2(12)	3-5(3.2)	2-3(11.2)	1-2(4.2)
CD27	30-50(1.2)	30-55(2.4)	25-55(2.1)	20-60(1.5)
CD69	20-70(41.9)	15-75(53)	20-65(56)	20-55(61)
CCR5	**50-55(10.4)**	**50-75(13.1)**	**3-8(6.1)**	**2-4(2.3)**
CCR7	5-12(4.2)	5-7(3.9)	10-12(3.6)	7-10(5.6)
SLAM	1-4(1.8)	2-3(2.7)	1-4(8.4)	3-5(3.2)
2B4	40-90(27)	35-95(31)	40-95(38)	30-85(41)
CD95	10-13(1.6)	6-7(1.2)	7-9(1)	7-15(1.7)

The EBV-T cell lines (from two XLP patients 1, 2 and two healthy controls 1, 2) were stimulated with protocol No 3 and were analysed for different CTL and NK markers with flow cytometry. The lines were mainly CD3/CD8 positive. The percentage expression and mean fluorescence intensity (MFI) were estimated on at least two occasions. The CCR5 expression was different in the XLP and control lines (It is highlighted with Bold font).

Table 5.3 Cell surface phenotype of EBV-CD4 T cell lines from controls and XLP patients

Cell surface marker	% expression (MFI)		% expresssion (MFI)	
	NORMAL		PATIENT	
	C1	C2	XLP1	XLP2
CD3	99-99.5(213)	98-99(234)	97-99(300)	98-99(330)
CD4	90-95(99)	85-95(102)	90-95(116)	90-97(93)
CD8	5-7(1.9)	10-15(3.4)	7-8(1.9)	5-8(1.7)
CD16	3-4(1.5)	4-5(1.2)	2-6(1.1)	5-7(1.6)
CD56	6-8(3.1)	3-4(2.8)	4-8(2.3)	5-7(2.7)
CD45RA	1-3(5.8)	2-3(2.4)	1-2(2.6)	1-5(2.8)
CD45RO	90-97(95.5)	95-98(69.3)	98-99(78.9)	94-99(70)
CD28	86-85(1.5)	70-86(1.6)	80-87(1.7)	57-75(1.5)
CD25	85-90(21)	87-92(24.6)	80-90(27.1)	85-94(21.3)
CD27	60-70(1.5)	65-73(1.7)	55-70(2.1)	47-73(1.7)
CD69	40-90(31)	35-85(28)	45-85(36)	40-80(33)
CCR5	<1	<1	<1	<1
CCR7	10-15(2.4)	8-17(3.2)	5-7(2.7)	10-12(3.3)
SLAM	1-4(2.2)	3-4(2.2)	2-5(2.6)	3-4(2.8)
2B4	85-99(22)	86-95(17)	80-95(23)	80-97(39)
CD95	2-5(1.2)	3-7(1.5)	6-7(1.3)	5-8(1.6)

The EBV T cell lines (from two XLP patients 1, 2 and two healthy controls 1, 2) were stimulated with protocol No 2 and were analysed for different CTL and NK markers with flow cytometry. The lines were mainly CD3/CD4 positive. The percentage expression and mean fluorescence intensity (MFI) were estimated on at least two occasions.

5.3.1 Memory-effector EBV-T cell lines phenotype

In order to characterize EBV-T cell lines for memory and effector phenotype, they have been analysed by flow cytometry for surface expression of CD28, CD27, CD45RA and CD45RO. CD28 and CD27 are costimulatory receptors involved in regulation of T cell activation and in the generation of antigen-primed cells respectively (Hintzen *et al,* 1994; Lenschow *et al,* 1996; Appay *et al,* 2002). CD28 interacts with CD80 (B7-1) and CD86 (B7-2), which are expressed on APCs and has a dual role as an adhesion and a signalling molecule (Mauri *et al,* 1995; Sansom *et al,* 2003). On the other hand, CD27 binds to CD70 and plays a role in TCR-induced expansion of both CD4 and CD8 T cells (Hendriks *et al,* 2000).

While CD28 is down regulated after TCR stimulation, CD27 is up regulated and the expression of CD28 and CD27 is particularly useful in distinguishing between subsets of differentiated effector/memory T cells (Hamann *et al,* 1997; Tomiyama *et al,* 2002). It has been reported that CD28- CD27+ and CD28- CD27- phenotypes define late and intermediate effector subset differentiation in humans (Tomiyama *et al,* 2002). The results of CD8 CTL lines were 25-60% positive for CD27 and almost negative for CD28 (less than 2%) in XLP patients and in normal controls. In contrast 57-87% of CD4 CTL lines were positive for CD28 at a low fluorescent intensity and were 47-73 % positive for CD27. CD27 expression was slightly higher than that seen in the CD8 population. The expression of CD27 and CD28 on CD4 CTL lines was similar between XLP patients and controls. Moreover, for both XLP and control CD4 and CD8 CTL lines very low levels of CD45RA (1-10%) were expressed but more than 90% of each population was positive for CD45RO. Interestingly, CD25 expression on CD8 lines was very low (1-5%) instead of high expression (80-94%) on CD4 lines. The results for other activation marker CD69 were dependent on the

stimulation conditions and showed similar patterns in both patients and healthy donors. Together, these results suggest that while CD4 and CD8 EBV-T cell lines demonstrate a slightly different effector phenotype, which were the low expression of CD28 and CD25 (1-10%) on CD8 lines in comparison with high expression (60-90%) on CD4 lines. However, there was no difference between XLP and normal lines for these markers.

5.3.2 SLAM-2B4 expression in EBV- specific T cell lines

To date, it has been shown that the CD2 Ig super family members SLAM and 2B4 are mainly involved in the pathogenesis of the XLP syndrome through SAP, although the role of other members of this receptor family cluster remains to be determined. It has been reported that SLAM is expressed on $CD45RO^{high}$ peripheral-blood memory T cells *in vivo* and it is rapidly induced on naïve $CD45RO^{-}$ T cells and enhanced on $CD45RO^{high}$ T cells after activation (Cocks *et al,* 1995). It has been speculated that during EBV infection, SLAM-SLAM interactions at the interface between EBV-infected B cells and T cells may promote the development of EBV-specific T responses but this has not been studied for CTLs. Moreover, 2B4 is expressed mainly in NK cells, CD8$\gamma\delta$ and almost half of CD8$\alpha\beta$ cells, but the regulation of its expression has not been fully investigated in CTLs or in T cells generally. In order to address this, EBV-T cell lines were examined for 2B4 and SLAM expression by flow cytometric analysis. Interestingly both CD4 and CD8 EBV-T cell lines displayed very low or negative (1-5%) SLAM expression but high 2B4 expression (40-95%) was seen (Figure 5.3). The level of 2B4 expression varied with different stimulation situations. The similarity of the phenotype of lines from

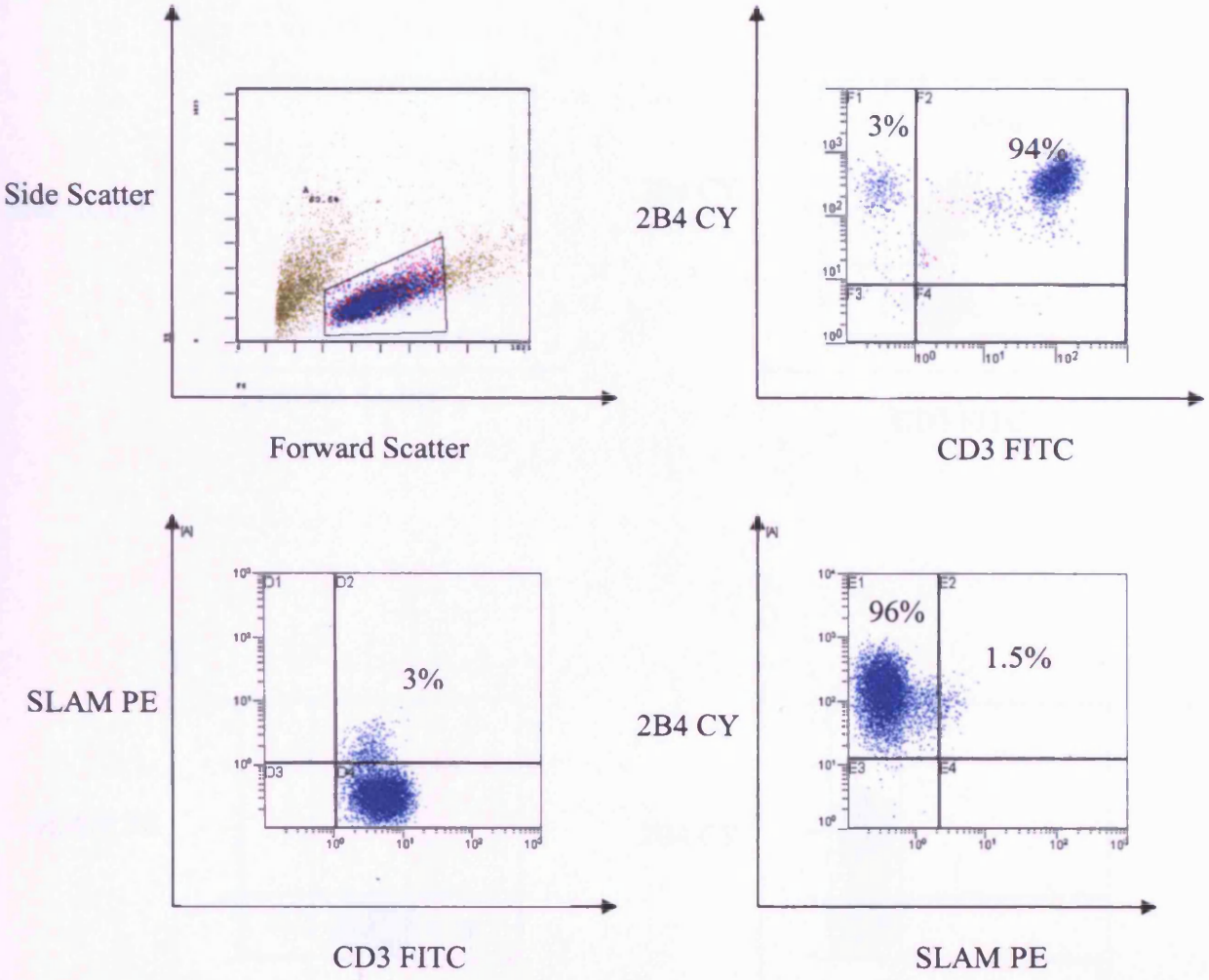


Figure 5.3 SLAM and 2B4 surface expression of EBV-CD8 T cell lines

The surface expression of SLAM and 2B4 on EBV-CD8 T cell lines from C1 was examined by flow cytometry analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on CD3 positive population. Quadrants were placed according to their isotype controls (not shown). EBV-CD8 T cell lines expressed a low level of SLAM and a high level of 2B4. Flow cytometric analysis of CD4 and CD8 lines show the same pattern for SLAM and 2B4 expression in XLP patients and healthy controls (see table 5.2-3).

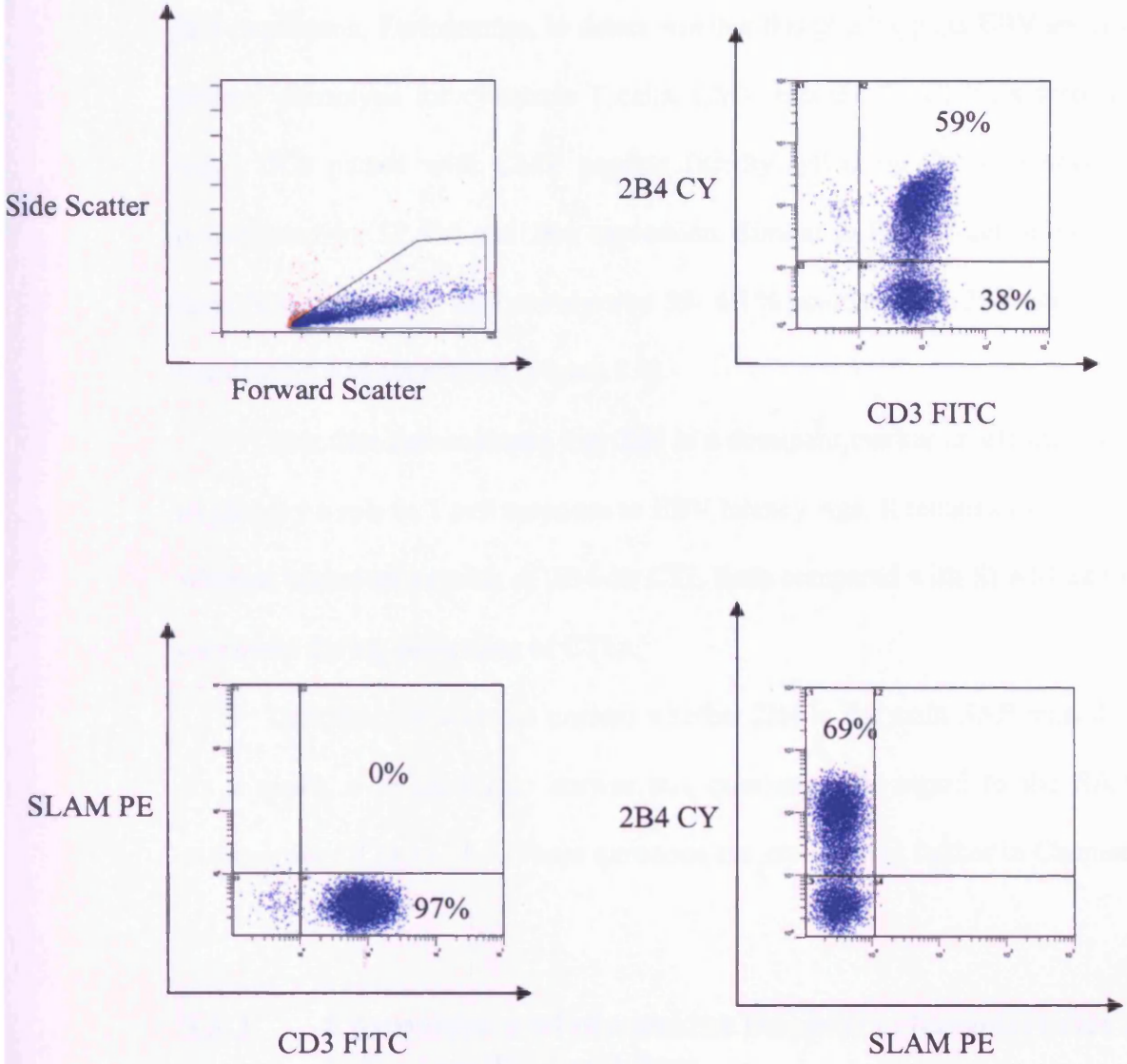


Figure 5.4 SLAM and 2B4 surface expression of CMV- T cell lines

The surface expression of SLAM and 2B4 by CMV- T cell line from a healthy individual was examined by flow cytometric analysis. Plots from a representative experiment are displayed and generated by gating on cells with a high forward and side scatter then on CD3 positive population. Quadrants were placed according to their isotype controls (not shown). CMV- T cell line expressed a high level of 2B4 and negative for SLAM.

XLP patients and healthy donors confirmed that SAP is not necessary for SLAM and 2B4 expression. Furthermore, to detect whether this phenotype is EBV specific or is a general phenotype for cytotoxic T cells, CMV specific T cell lines were generated using DCs pulsed with CMV peptide (kindly gifted by Dr S. Mackinnon) and investigated for SLAM and 2B4 expression. Similar to EBV-T cell lines, the results from flow cytometric analysis showed 59- 69 % positivity for 2B4 and very low or negative SLAM expression (Figure 5.4).

This data demonstrates that 2B4 is a dominant marker an effector cells which might play a role in T cell response to EBV latency Ags. It remains to be investigated whether higher expression of 2B4 on CTL lines compared with SLAM expression is consistent during generating of CTLs.

In addition it remains unclear whether 2B4 is the main SAP related pathway. As a result, it is crucial to answer this question with regard to the SAP related pathway for CTLs in XLP. These questions are investigated further in Chapter 6.

5.3.3 Chemokine and chemokine receptors characteristics of EBV- specific T cell lines

Chemokines play well-established roles as attractants of naïve and effector T cells (Cyster, 1999; Campbell *et al,* 2000; Sallusto *et al,* 2000). It has been recently reported that chemokine families also play important roles in T cell differentiation along with cytokines such as IFN-γ (Luther *et al,* 2001). Moreover, studies of immune response to viruses particularly HIV demonstrated the importance of chemokines (Cocchi *et al,* 1995; Hadida *et al,* 1998; Littman, 1998). Therefore, chemokines may play a crucial role in the pathogenesis of XLP, which is well documented to be

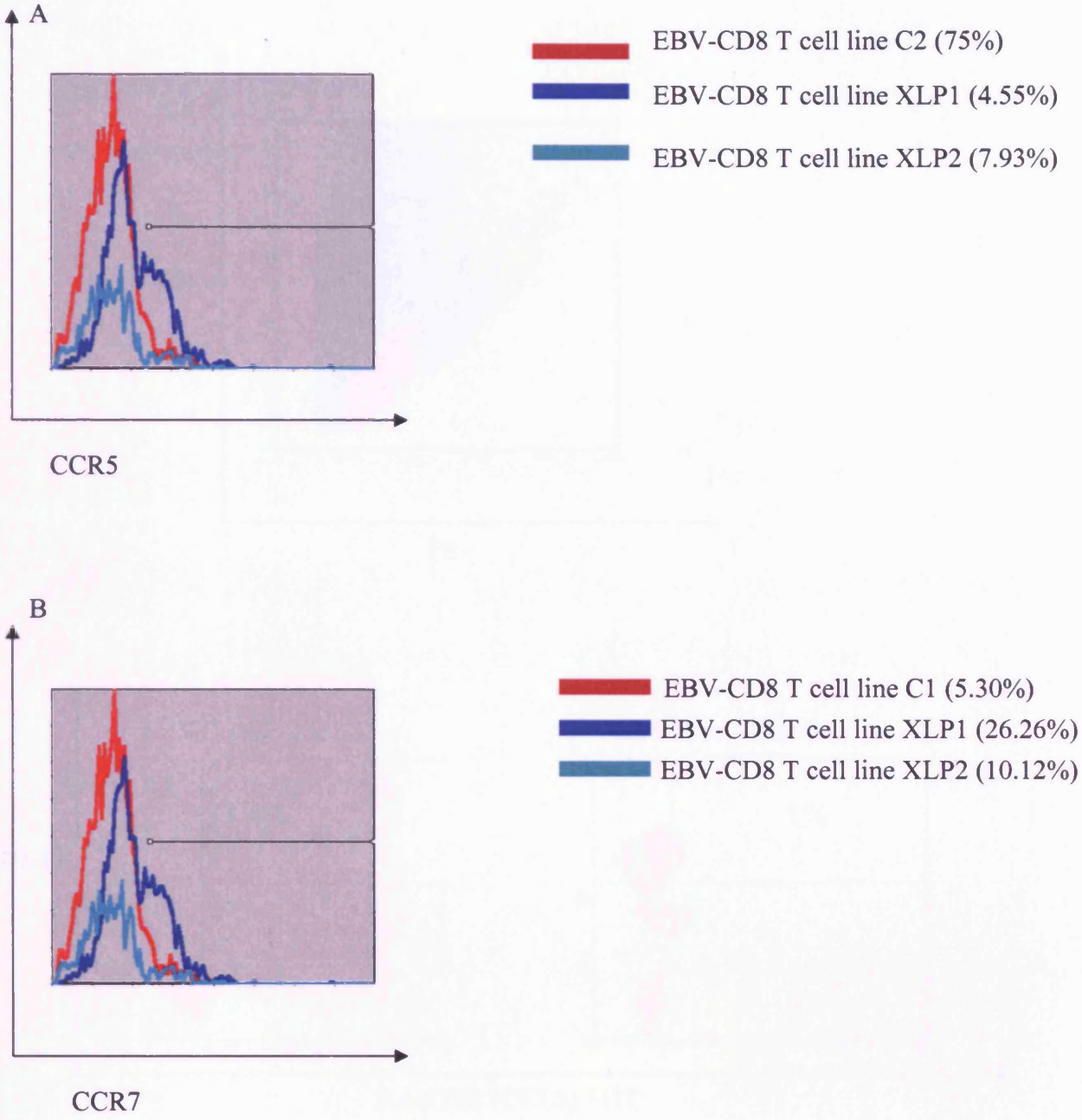


Figure 5.5 Surface CCR5 and CCR7 expression by EBV-CD8 T cell lines from C1 and XLP patients

The surface expression of CCR5 (A) and CCR7 (B) by EBV-CD8 T cell line from C1 and XLP1, 2 was examined by flow cytometric analysis. Histograms from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on CD3 positive population. The expression of CCR5 and CCR7 shown as overlay histogram.

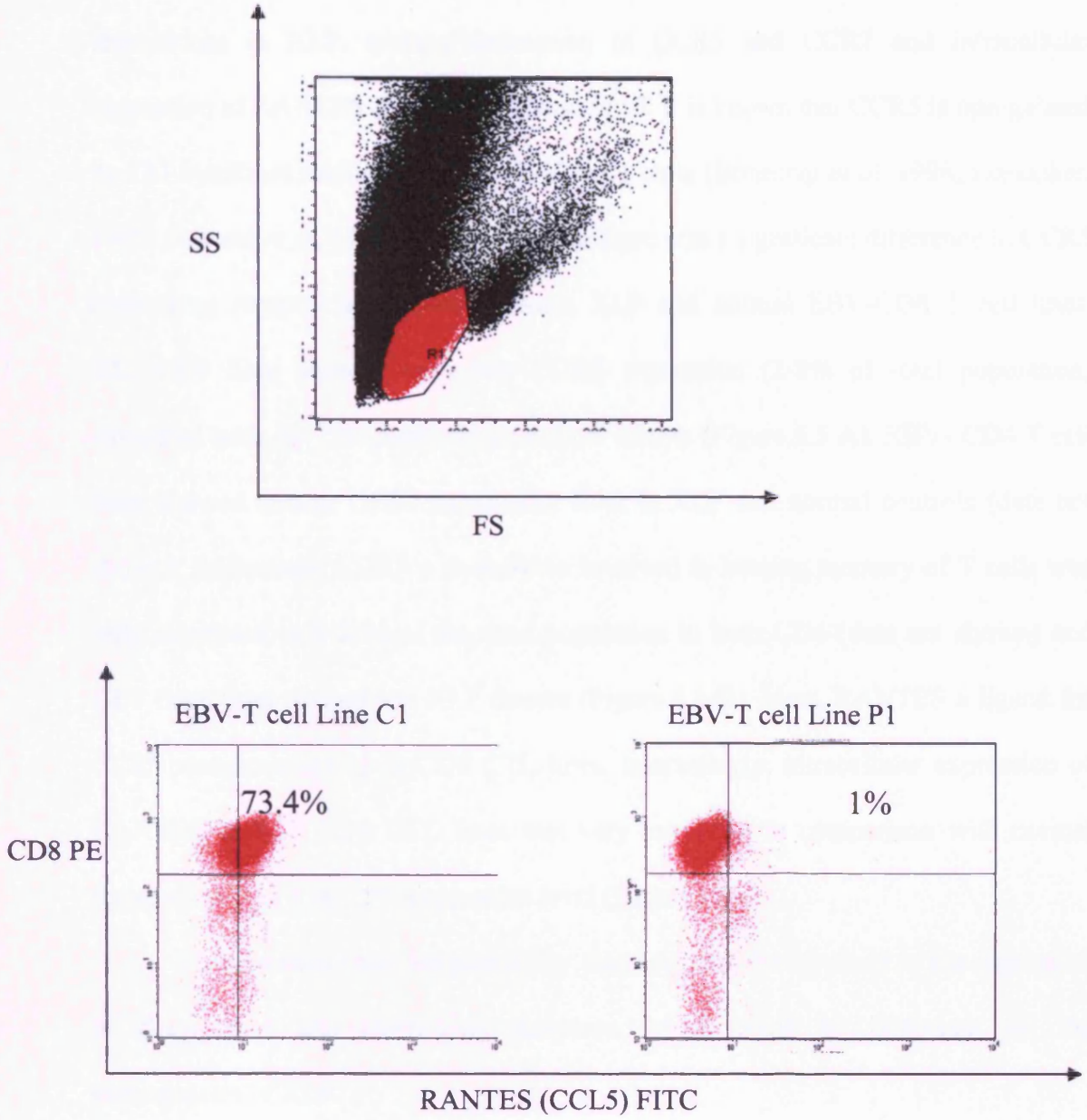


Figure 5.6 Intracellular expression of RANTES by EBV-CD8 T cell lines

Intracellular RANTES expression by EBV-CD8 T cell lines (XLP1 and C1) was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward scatter. Quadrants were placed according to their isotype controls (not shown). The expression of RANTES in XLP1 was very low in comparison with control (C1).

triggered by viral infections, especially EBV. Thus, to investigate the significance of chemokines in XLP, surface expression of CCR5 and CCR7 and intracellular expression of RANTES (CCL5) were examined. It is known that CCR5 is upregulated by Th1 cytokines such as IL-2 and IL-12 on T cells (Bonecchi *et al,* 1998; Loetscher, 1998; Sallusto *et al,* 1998). In EBV-T lines there was a significant difference in CCR5 chemokine receptor expression between XLP and normal EBV-CD8 T cell lines. XLP-CD8 lines showed very low CCR5 expression (2-8% of total population) compared with 50-75% positivity in normal donors (Figure 5.5 A). EBV- CD4 T cell lines showed similar CCR5 expression level in XLP and normal controls (data not shown). In contrast, CCR7 a chemokine involved in homing memory of T cells was only expressed in 5-26% of the total population in both CD4 (data not shown) and CD8 lines from normal and XLP donors (Figure 5.5 B). Next, RANTES a ligand for CCR5 was investigated in CD8 CTL lines. Interestingly, intracellular expression of RANTES in XLP CD8 CTL lines was very low (1%) in comparison with normal lines, which had a 70-75% expression level (Figure 5.6).

These results raise the possibility that SAP may be involved in the regulation of chemokines and chemokine receptors, which could be important for the pathogenesis of XLP.

5.4 Discussion

In normal individuals the immune response to EBV infection, the major pathological trigger in XLP, is dominated by the proliferation of CD8+ EBV-T cell lines and NK cells. The development of EBV driven lymphoma and fulminant IM in XLP would strongly suggest that defects exist in these effector populations (See Chapter one). In this study we show that it is possible to generate EBV-T cell lines

from XLP patient blood. Using different culture conditions we were able to generate T cell lines of both CD8+ and CD4+ phenotype from both normal and XLP patients. It has been shown T cells are normally capable of proliferating under different stimuli in SAP deficient murine models (Howei *et al,* 2002). Our results demonstrated that T cells from XLP patients show a comparable proliferation response to mitogen (PHA) or EBV-LCL. Sanzone *et al* (2003) recently showed that non-transformed T cell lines from two XLP patients failed to proliferate upon CD3 or CD3/28 stimulation but not with PHA, suggesting SAP is involved in early T cell activation. However, our results and a recent report by Plunkett *et al* (2005) support the idea that the early proliferation capacity of primary XLP T cells is intact. The different observations may be due to the investigational model (primary T cells versus established T cell lines) or the inter-individual differences between XLP samples that may have experienced a variable time course or type of exposure to infection (such as EBV).

Furthermore, although most cytotoxic T cell lines are CD8+, polyclonal expansion of T cells in response to viral antigens can result in CD4+ T cell lines and CD4+ cytotoxicity is well documented (Peggs *et al,* 2001; Sun *et al,* 2002). Cell surface marker expression profiles and intensity of expression were similar in both XLP and control groups. Interestingly, in both controls and XLP EBV-T cell lines there was very little SLAM expression observed while 2B4 was expressed at significant levels. To date, SLAM has been cited as the main partner for SAP binding and as an important co-stimulatory molecule in T cell responses to antigenic stimulation. Much of this work has been performed on murine T cells and the differential expression of SLAM and 2B4 in human effector cells has not been extensively studied. Our analysis shows abundant 2B4 expression in both control and XLP EBV-T cell lines, while SLAM expression was minimal. Furthermore, in our

studies, stimulation of EBV-T cell lines and PBMCs from normal individuals shows no significant enhancement in SLAM expression at least in the early response period (Chapter 7). Our observations would suggest that 2B4, not SLAM, is the major partner for SAP in EBV-T cell lines and, as previously observed in NK cells, signalling through 2B4 in XLP EBV-T cell lines may be defective.

Furthermore, all XLP lines showed a similar phenotype as compared to normal controls except for CCR5 expression in CD8 lines, which in XLP lines was minimal but expressed at high levels in normal EBV-CD8 T cell lines. CCR5 is chemokine receptor, which is up regulated by Th1 cytokines such as IL-2 and IL-12 through the STAT4 pathway (Bleul *et al,* 1997). CCR5 is expressed mainly on T cells with a Th1 phenotype (Loetscher, *et al* 1998) and recruits lymphocytes to sites of inflammation. As CCR5 upregulation is important in response to MIFα in DC and T cell interactions, this could play a role to initiate immune responses in APC process. More recently it has been reported that XLP patients show an abnormality in chemokine expression (Malbran *et al,* 2004). This may be as the result of the cytokine imbalance reported in XLP patients. However, the possibility of CCR5 mutations has not been ruled out in these patients as reported in HIV patients (Liu *et al,* 1996; Samson *et al,* 1996). More interestingly, the CD8 T cell lines from XLP patients showed a significant defect in RANTES (CCL5) production. It has been reported that RANTES (CCL5) magnifies the cytokine polarity of the T cell response and therefore may play a key role in the immune response to viral infection (Luther *et al,* 2001). In particular, chemokines are likely to play important roles in the pathophysiology of disease associated with EBV (Nakayama *et al,* 2004). EBV infection induces B cells to up regulate their constitutive expression of MIP-1α/CCL4, and RANTES, which are known to attract Th1 cells and activated cytotoxic T cells via CCR5 (Yoshie *et al,*

2001; Nishimura *et al,* 2002). Moreover, it has been demonstrated that T cells are a major source of RANTES production upon EBV infection *in vitro* (Jabs *et al,* 2002).

Together, this might demonstrate that due to lack of SAP function, or as a result of the reduction of IFN-γ response, there may be a consequent reduction of RANTES and CCR5 expression. This may play some role in the cellular pathogenesis or at least determine severity of IM in XLP patients. This result provides intriguing data regarding XLP pathogenesis and requires further investigation.

Chapter 6

Expression of SLAM & 2B4 on Immune Cell Lineages

6.1 Introduction

Immune cells are activated as a result of productive interactions between ligands and various receptors. The result of this orchestrated response provides the balanced participation of several immune cell types. In immune cells, SAP is shown to associate with SLAM, 2B4, and NTB-A and other members of this group shown in transfected cell lines as mentioned previously. In this context, interaction of SAP with SLAM family members regulates activation of T and B cells, which may be important in the immune response to Ags such as EBV (Latour *et al,* 2004).

As discussed in section 1.6.2, SAP regulatory function seems to be complex. In particular, this is highlighted by its interaction with heterogeneously expressed SLAM family members in immune cells. Thus the pathogenesis of XLP may be more complex than previously thought. Investigation of SAP expression and its receptors in leading the dynamic processes of the immune response may shed some light on the pathogenesis of XLP and the role of the SAP pathway signalling machinery. It has been reported by several groups that SAP expression in human T and NK cells is regulated differently to that in murine cells (see section 1.4.3). Moreover, it has been reported that in the murine model, SLAM is upregulated upon T cell activation by non-specific stimulators but SAP at the mRNA level is down regulated (see section 1.6.2). Therefore, considering the importance of SAP in balancing function in T and NK cells, it is fundamental to look at regulation of SAP expression and the expression of its ligands particularly SLAM and 2B4. Thus in the following chapter SLAM-2B4 expression in different cell lineages in both the resting and activated condition will be discussed.

6.2 SLAM and 2B4 expression on immune cell lineages

SLAM and 2B4 expression on different immune cell lineages were described in Chapter 1 (see Table 3.1). However, there is little known about coexpression of SLAM family receptors on immune cells in the resting and active condition. Therefore, in the following section co-expression of SLAM and 2B4 pre and post stimulation is discussed.

6.2.1 SLAM and 2B4 expression on T cell subsets

SLAM and 2B4 expression on T cell subpopulations were investigated by flow cytometric analysis. As shown in Figure 6.1-2, SLAM expression on both resting CD4 and CD8 populations was low (1.5%-3%). However, 2B4 expression averaged 27% (15-30%) for CD8 cells and for CD4 cells an average of 9% (7-12%) expression was seen (Figure 6.1 and 6.2).

6.2.2 SLAM and 2B4 expression on B cells and BLCLs

EBV-BLCL lines showed double positive expression of both 2B4 and SLAM with 90-95% expression but 2B4 showed relatively low fluorescence intensity. Primary B cells showed very low SLAM expression and were negative for 2B4 (Figure 6.3).

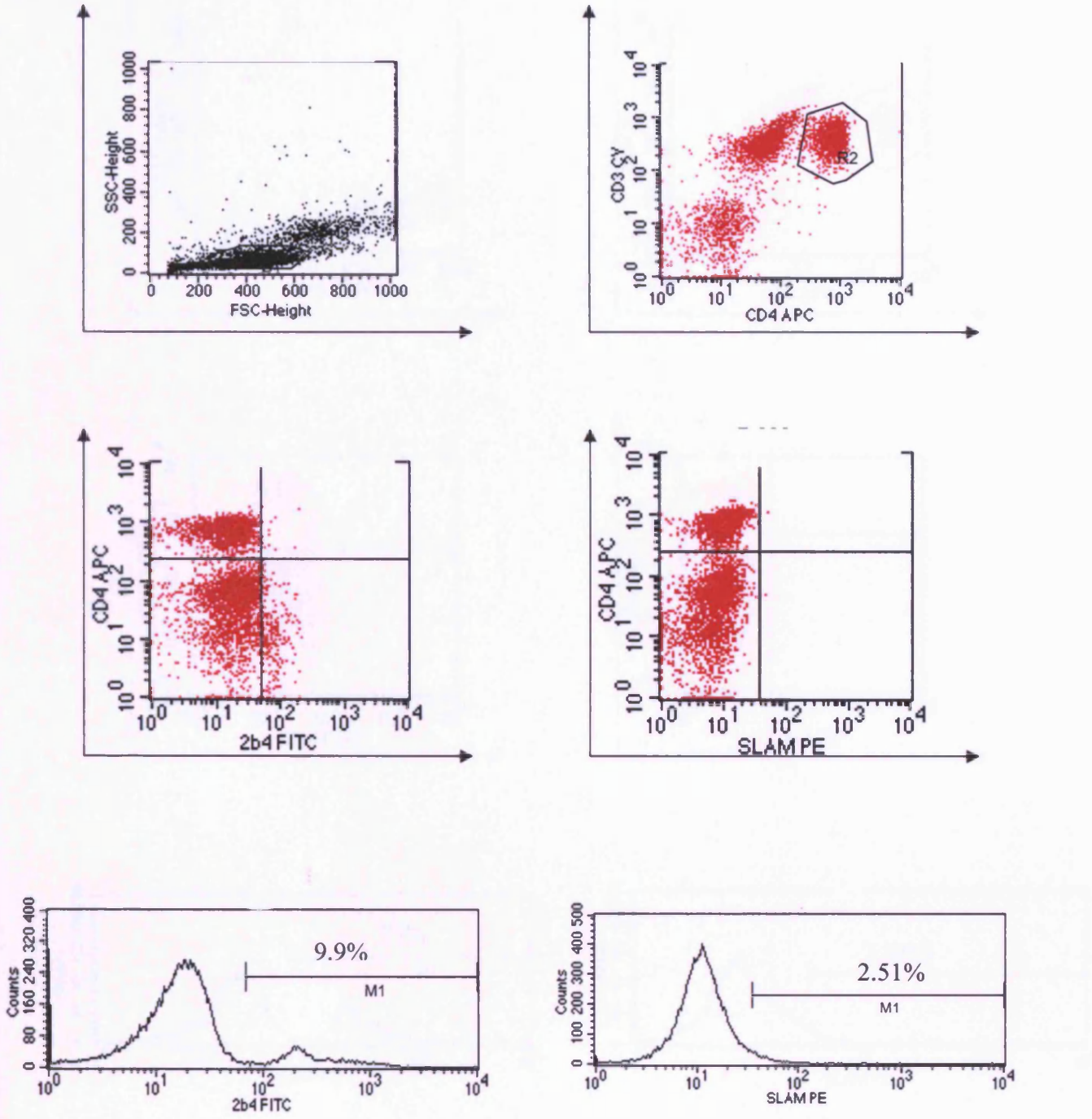


Figure 6.1 The surface coexpression of SLAM and 2B4 on CD4 T cells

The surface expression of SLAM and 2B4 by peripheral blood CD4 T cells was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on the CD3/4 double positive population. CD4 T cells expressed a low level of SLAM and a moderate level of 2B4 in unstimulated conditions.

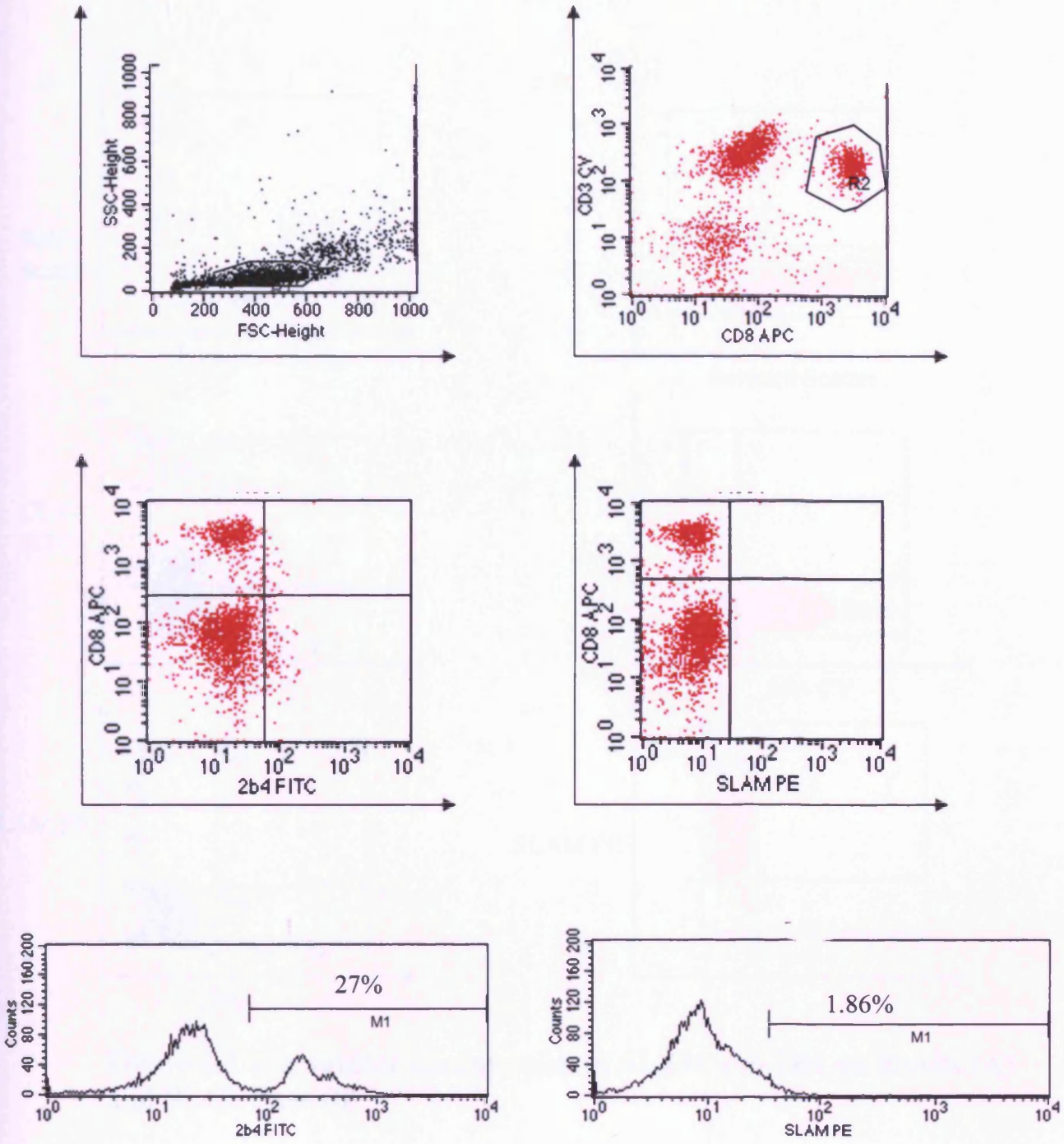


Figure 6.2 The surface coexpression of SLAM and 2B4 on CD8 T cells

The surface expression of SLAM and 2B4 by peripheral blood CD8 T cells was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on the CD3/8 double positive population. Quadrants were placed according to their isotype controls (not shown). CD8 T cells expressed a very low level of SLAM but a higher level of 2B4 in unstimulated conditions.

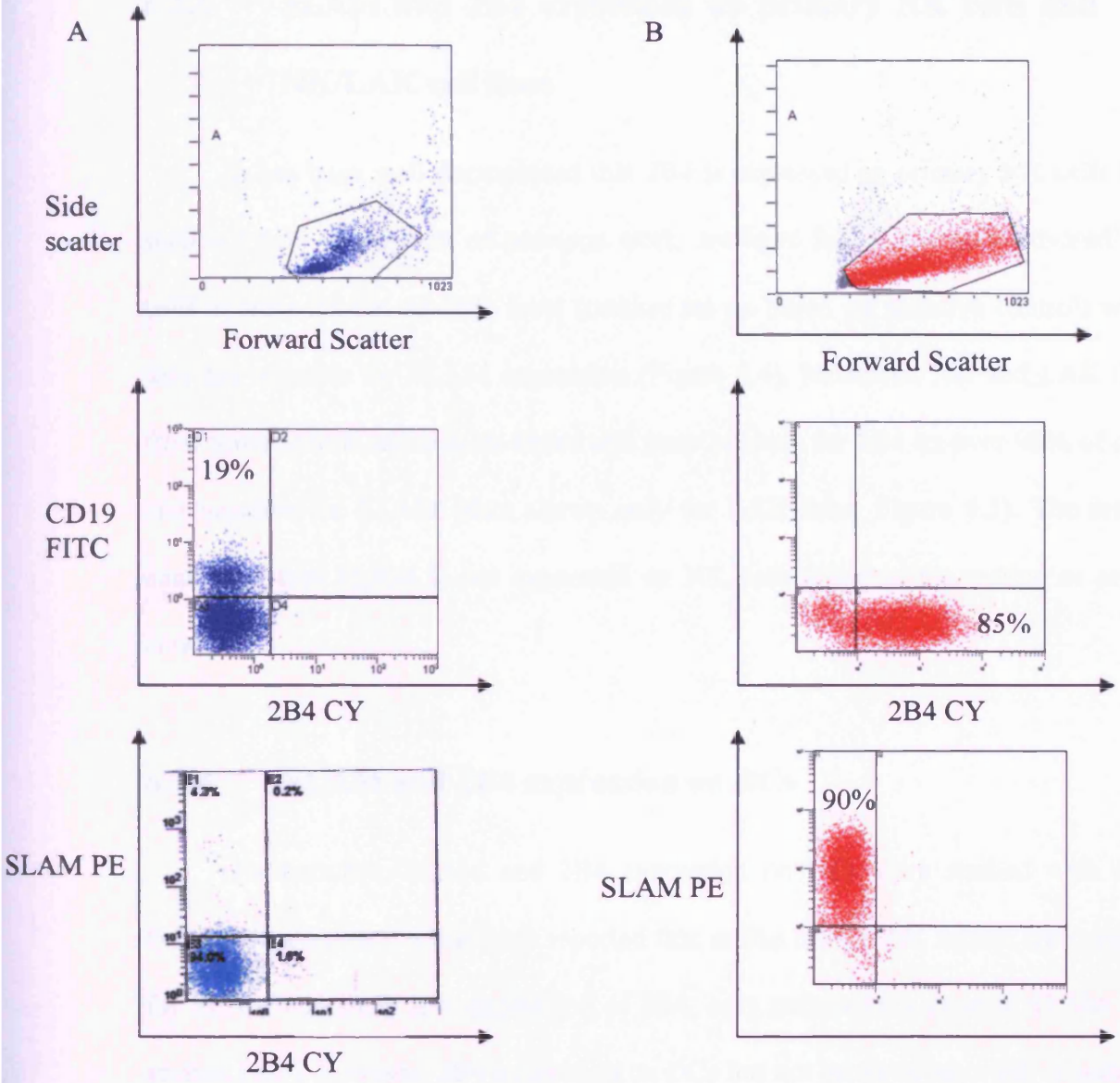


Figure 6.3 The surface coexpression of SLAM and 2B4 on B cells (A) and BLCL lines (B)

The surface expression of SLAM and 2B4 by peripheral blood B cells and BLCLs was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on the CD19 positive population. B cell expressed a very low level SLAM and 2B4 in unstimulated condition (panels group A). BLCL expressed a very high level of SLAM and 2B4 (panels group B).

6.2.3 SLAM and 2B4 expression on primary NK cells and NK/LAK cell lines

It has been well documented that 2B4 is expressed on primary NK cells (see section 1.6.1). In support of previous work, we have found that non-activated NK cells express 2B4 at 55-70% level (marker set up based on negative control) while they are negative for SLAM expression (Figure 6.4). Moreover, NK and LAK lines from three normal individuals tested and were positive for 2B4 on over 90% of cells and negative for SLAM (data shown only for LAK lines, Figure 6.5). The results confirmed that SLAM is not expressed on NK cells either in the resting or active condition.

6.2.4 SLAM and 2B4 expression on DCs

Furthermore, SLAM and 2B4 expression on DCs were studied with flow cytometric analysis. It has been reported that unlike monocytes, which are negative for SLAM and show low expression of 2B4, only mature DCs express SLAM (see section 1.61). However, 2B4 expression on DCs has not yet been reported. In order to answer this question, harvested DCs were analysed for 2B4 and SLAM. Immature DCs based on a CD11c positive phenotype (100%) and very low expression of CD86 (10.12%) a marker indicative of DC maturation, and mature DCs double positive for CD11c (100%) and CD86 (99%) were analysed. Interestingly, immature DCs were positive for 2B4 with 35-45% expression but were SLAM negative. After maturation over 48 hours, mature DCs showed 10-12% SLAM but were 75-76% 2B4 positive (Figure 6.6).

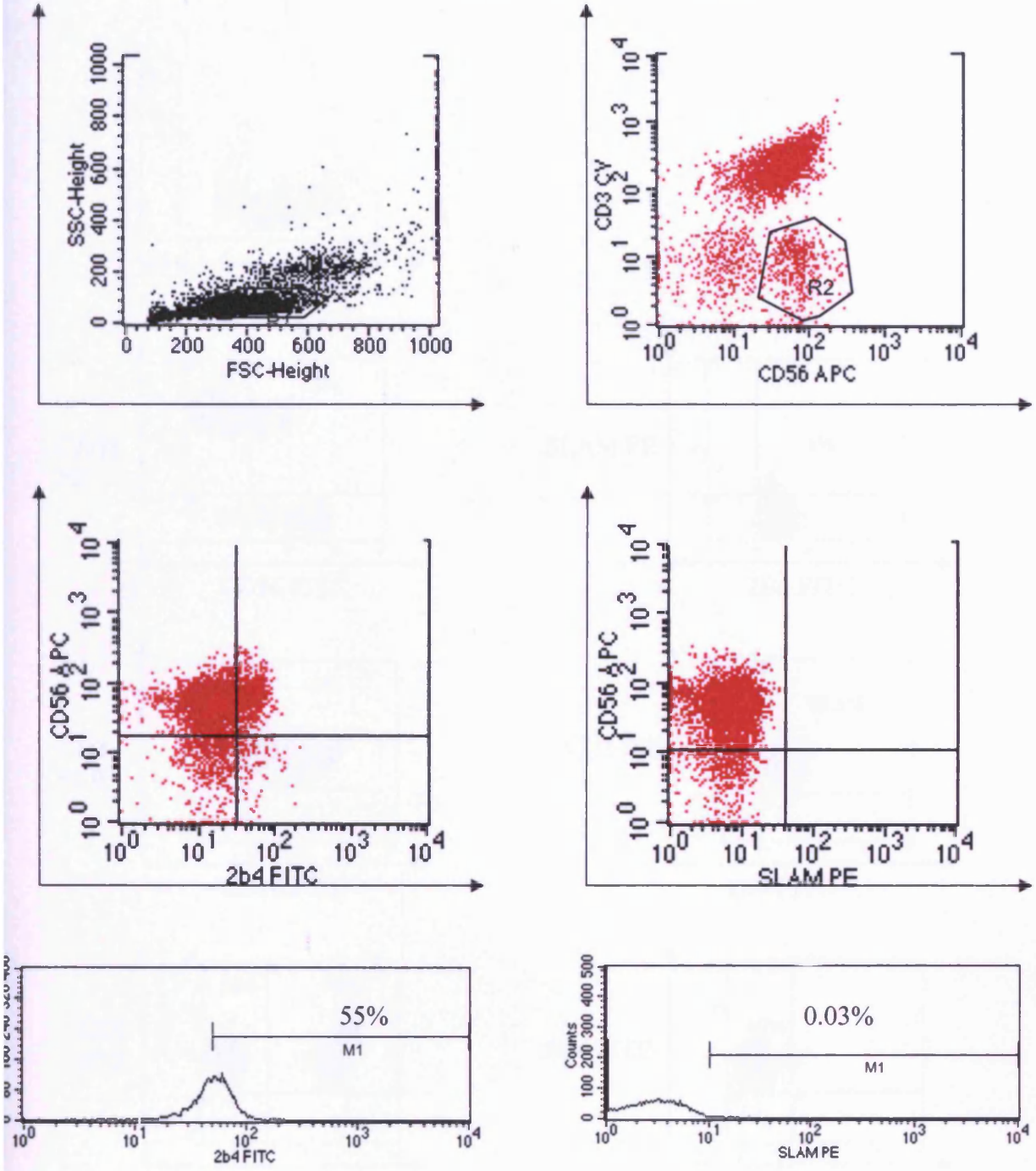


Figure 6.4 The surface coexpression of SLAM and 2B4 by NK cells

The surface expression of SLAM and 2B4 by peripheral NK cells was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on CD3 negative and CD56 positive population. NK cells expressed a very high level 2B4 (55%) (marker was based on negative control) and no expression of SLAM in unstimulated conditions.

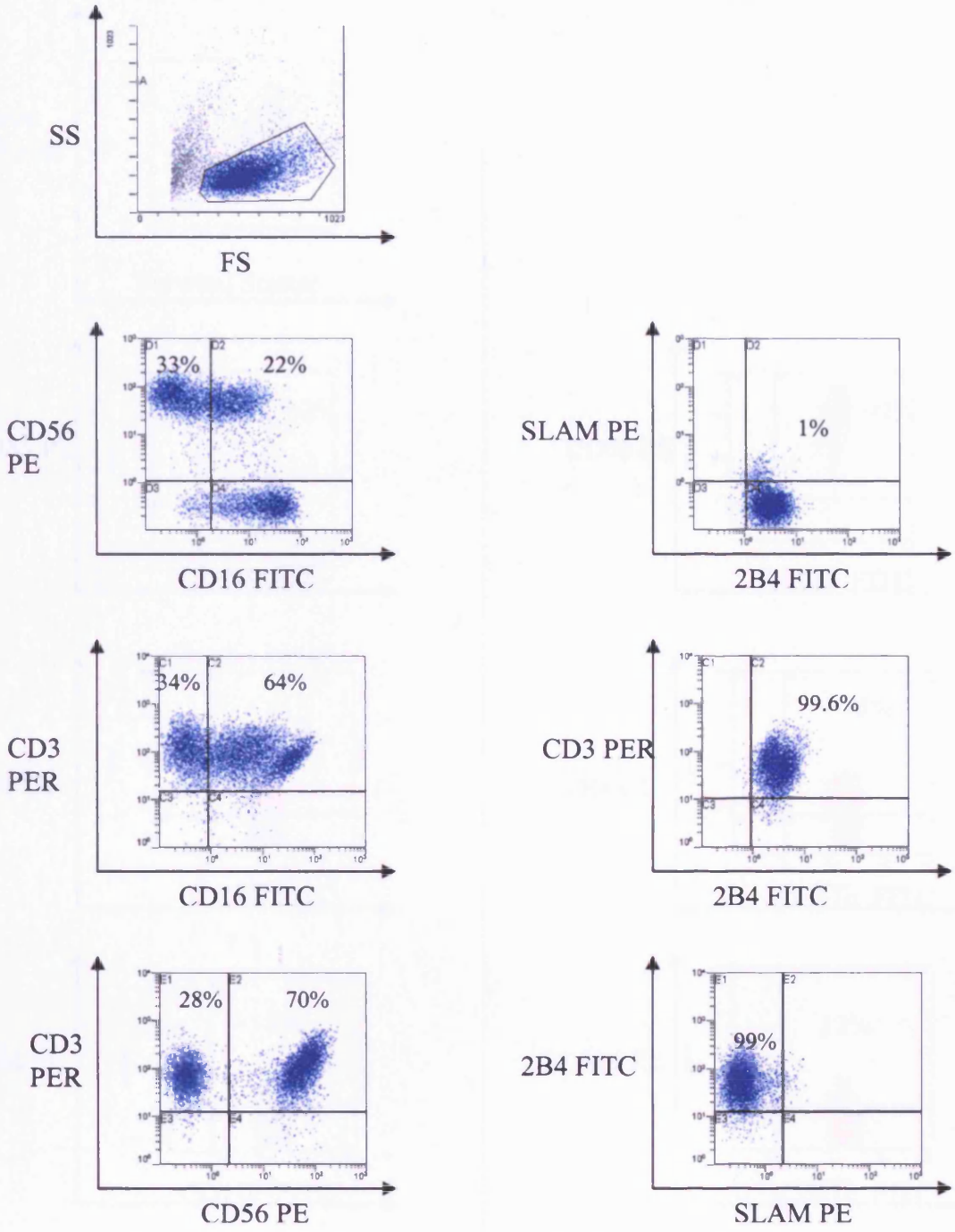
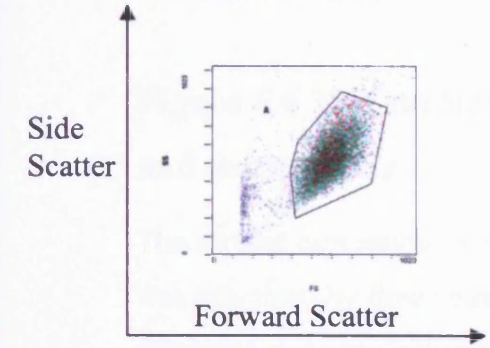
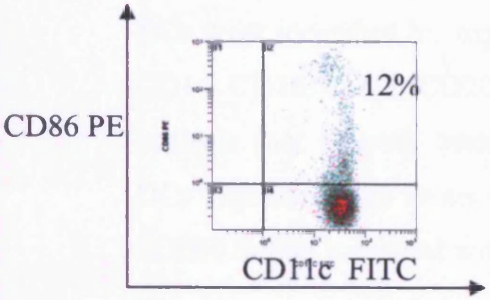


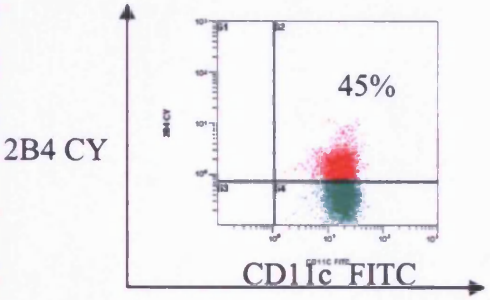
Figure 6.5 SLAM and 2B4 expression by LAK cell lines (CD3/56/16)

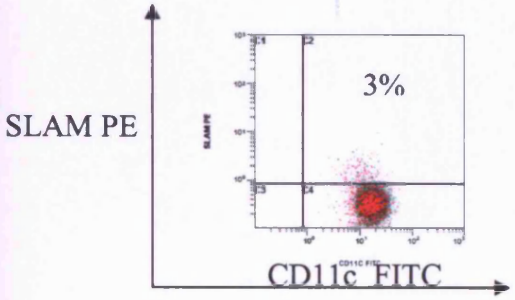
The surface expression of SLAM and 2B4 by LAK cell lines (presenting the results from C1) were examined by flow cytometry. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter. Quadrants were placed according to their isotype controls (not shown). The cells were positive for CD3/CD56/CD16. LAK cell lines expressed a very high level of 2B4 and were negative for SLAM expression.

Immature DCs

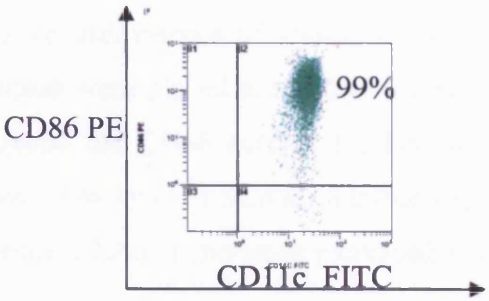


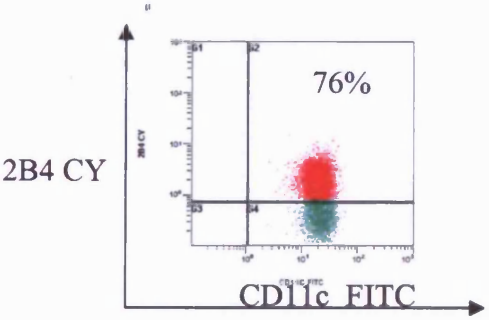


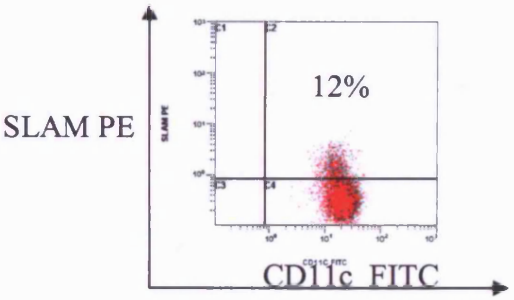




Mature DCs







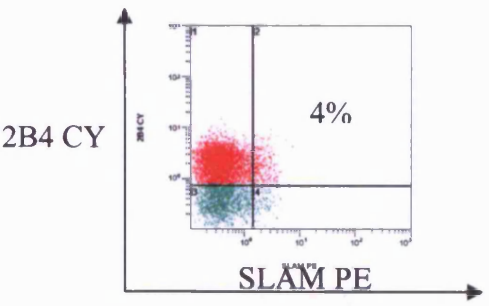


Figure 6.6 The surface coexpression of SLAM and 2B4 by immature and mature DCs

The surface expression of SLAM and 2B4 by normal immature and LPS-matured DC was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter. DCs were identified by expression of CD11c and absence of lineage markers: CD3, CD14, CD16, CD19, CD20, CD56. Quadrants were placed according to their isotype controls (not shown). Maturation upregulated the CD86 surface marker. Immature DCs expressed high levels of 2B4 and a very low level of SLAM. Surface expression of 2B4 further increased with maturation while SLAM expression increased slightly.

6.3 SLAM and 2B4 expression upon specific stimuli

It has been shown in humans that SLAM is up regulated upon CD3-CD28, Con A and PHA stimulation on T cells. Although initial reports showed that established CD4 Th1 clones show high SLAM expression with low expression on Th2 clones (Cocks *et al,* 1995), SLAM expression has not been comprehensively studied in primary T cells upon specific stimulation. To date, 2B4 expression has been mainly studied on NK cells but not on T cell populations. In this context there is little known about 2B4 expression in human T cells, although it has been suggested that 2B4 is up regulated after stimulation in murine cells (Sayos *et al,* 2000). The combination of SLAM/2B4 dual expression has not been investigated in T and NK cells, particularly their dynamic coexpression over time upon specific stimulation.

Therefore, after observing the predominance of 2B4 expression on EBV-specific T cell lines and on CD4 or CD8 T cells, we considered investigating the expression of these two markers under specific stimuli on different populations of T cells and NK cells.

SLAM and 2B4 expression upon CD3-CD28, CD3-SLAM, CD3-2B4, and autologous BLCLs stimulation at differing time points were investigated using flow cytometric analysis. Equal number of PBMCs from three normal donors were stimulated either with co-localised antibody (Ab) combination CD3/CD28, CD3/SLAM, CD3/2B4 or with autologous BLCL lines at a ratio of 10:1 cell (PBMCs/BLCL cells) were cultured in complete RPMI medium as described in Chapter 2. To check efficiency of activation, stimulated cells were analysed for CD69, an activation marker on T and NK cells. As shown in Figure 6.7, upon different antibody stimuli, T and NK cells showed constant activation during the stimulation period, with 40-67% of populations

positive for CD69 expression (data show expression after 12 hours). In contrast, cells when stimulated with BLCL showed rising CD69 expression after 6-12 hours of stimulation with over 95% CD69 positivity on both T and NK cells (data not shown). To consider the effect of the above stimuli on cell proliferation, stimulated cells were counted using Trypan blue to detect live cells. There was no significant difference in proliferation responses to specific stimuli (data not shown). Stimulated cells were then analysed with four colour flow cytometry for SLAM, 2B4, CD3, and one of CD4, CD8, CD56 parameters at time 0, 12, 24, and 48 hours after stimulation (some were checked after 6 hours or in case of stimulation with BLCL, checked after 72 hours). Results are presented based on CD8, CD4, and NK cell analysis and are discussed as follows.

6.3.1 SLAM and 2B4 expression on CD8 T cells

As described above, CD8 T cells express 2B4 at greater levels than SLAM in the resting situation. Interestingly, 2B4 upregulation was significantly higher and earlier than SLAM upregulation (Figure 6.8). However, SLAM was upregulated after CD3/28 stimulation reaching its highest level after 48 hours. Stimulation with BLCL induced no increasing of SLAM expression even after 72 hours, which correlates with our previous data from CTL lines. Double positive populations for SLAM and 2B4 on resting CD8 cells and upon different stimuli were very low except after CD3/CD28 stimulation, where an increase in 2B4 and SLAM double positive cells were observed after 24 hours. Significant 2B4 expression was observed regardless of the type stimulation, and only its expression upon CD3/2B4 stimulation at early time points was decreased but increased again after 12 hours. It may be due to internalization of

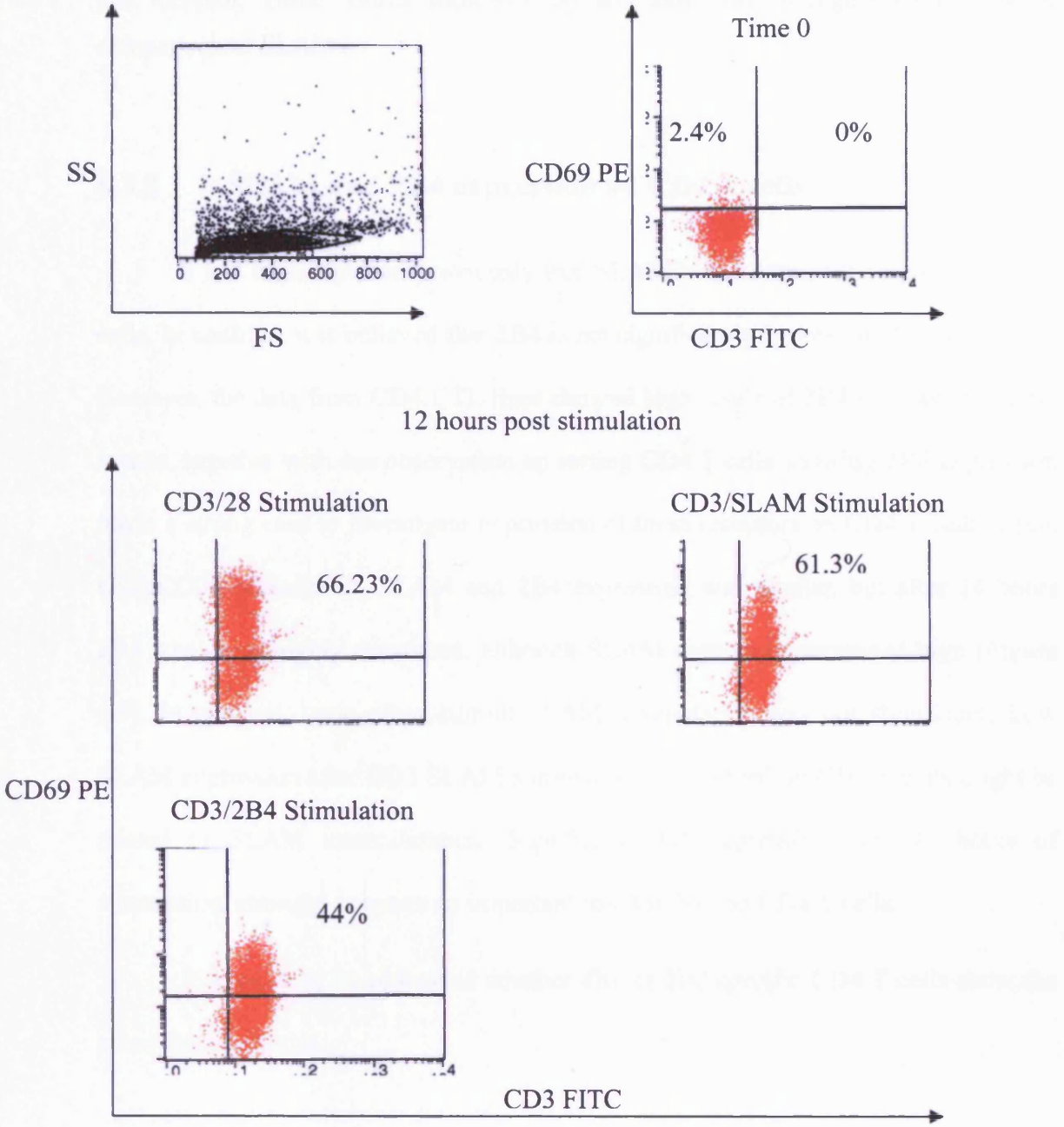


Figure 6.7 Surface expression of CD69 by activated T cell

The surface expression of CD69 by T cell was examined by flow cytometric analysis. Plots from a representative experiment are displayed and were generated by gating on cells with a high forward and side scatter then on CD3 positive population (not shown). Quadrants were placed according to their isotype controls (not shown). T cells were negative for CD69 expression at the time zero before stimulation. Upon different stimulation (CD3/28, SLAM, 2B4) CD69 expression upregulated between 6-12 hours (the results present the CD69 expression after 12 hours).

this receptor. These results showed early and sustained up regulation of 2B4 in comparison to SLAM.

6.3.2 SLAM and 2B4 expression on CD4 T cells

It has been reported previously that SLAM is the dominant receptor on CD4 cells. In contrast, it is believed that 2B4 is not significantly expressed on CD4 T cells. However, the data from CD4 CTL lines showed high levels of 2B4 expression. These results, together with our observation on resting CD4 T cells showing 2B4 expression made a strong case to investigate expression of these receptors on CD4 T cells. Upon CD3/CD28 stimulation, SLAM and 2B4 expression was similar, but after 24 hours 2B4 was more highly expressed, although SLAM expression remained high (Figure 6.9). In contrast, upon other stimuli SLAM upregulation was not significant. Low SLAM expression after CD3/SLAM stimulation as observed on CD8 T cells might be related to SLAM internalization. Significant 2B4 expression over 48 hours of stimulation, strongly suggests an important role for 2B4 on CD4 T cells.

It remains to be addressed whether Th1 or Th2 specific CD4 T cells show the same characteristics.

6.3.3 SLAM and 2B4 expression on CD56 NK cells

It has been shown in murine cells that 2B4 is highly expressed on NK cells, and SLAM is only expressed at low levels on primary NK cells. Recently it has been reported that NTB-A (SF2000) and CRACC (CS-1) are expressed on NK cells. However, although 2B4 is expressed on primary NK cells and up regulates upon IL-2

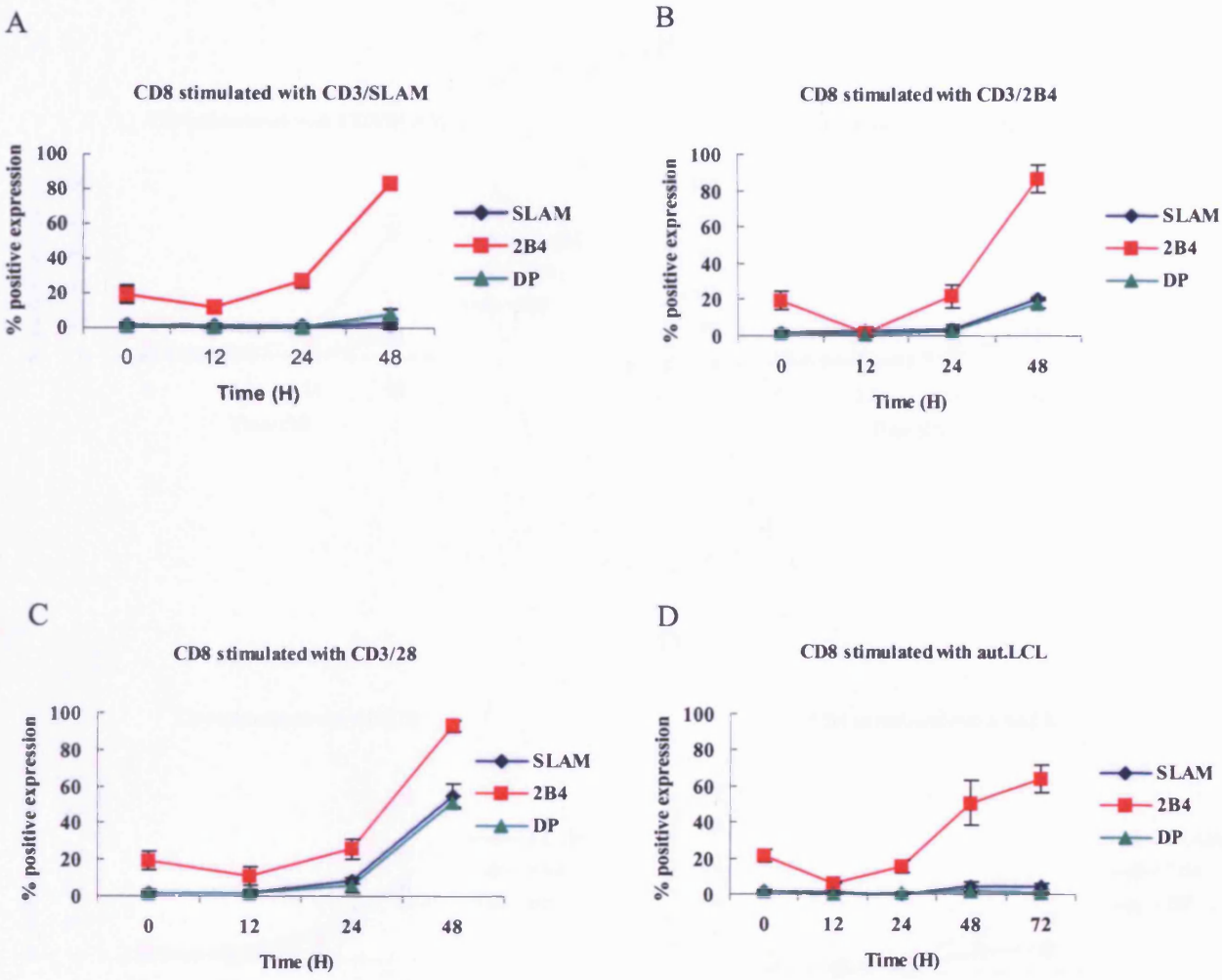


Figure 6.8 SLAM and 2B4 surface expression on CD8 T cells upon activation by different stimuli

SLAM and 2B4 expression on CD8 T cells was investigated after CD3-SLAM (A), CD3-2B4 (B), CD3-CD28 (C), and autologous BLCLs (D) stimulation at differing time points using flow cytometric analysis. Three healthy different individuals were studied twice each. The values for each experiment were derived from flow cytometric analysis of the data, and the average percentage (± SD) of expression for each individual and mean percentage for all three was calculated for each point. The percentage expression of SLAM, 2B4 and double positive (SALM+2B4) are shown in blue, red and green, respectively.

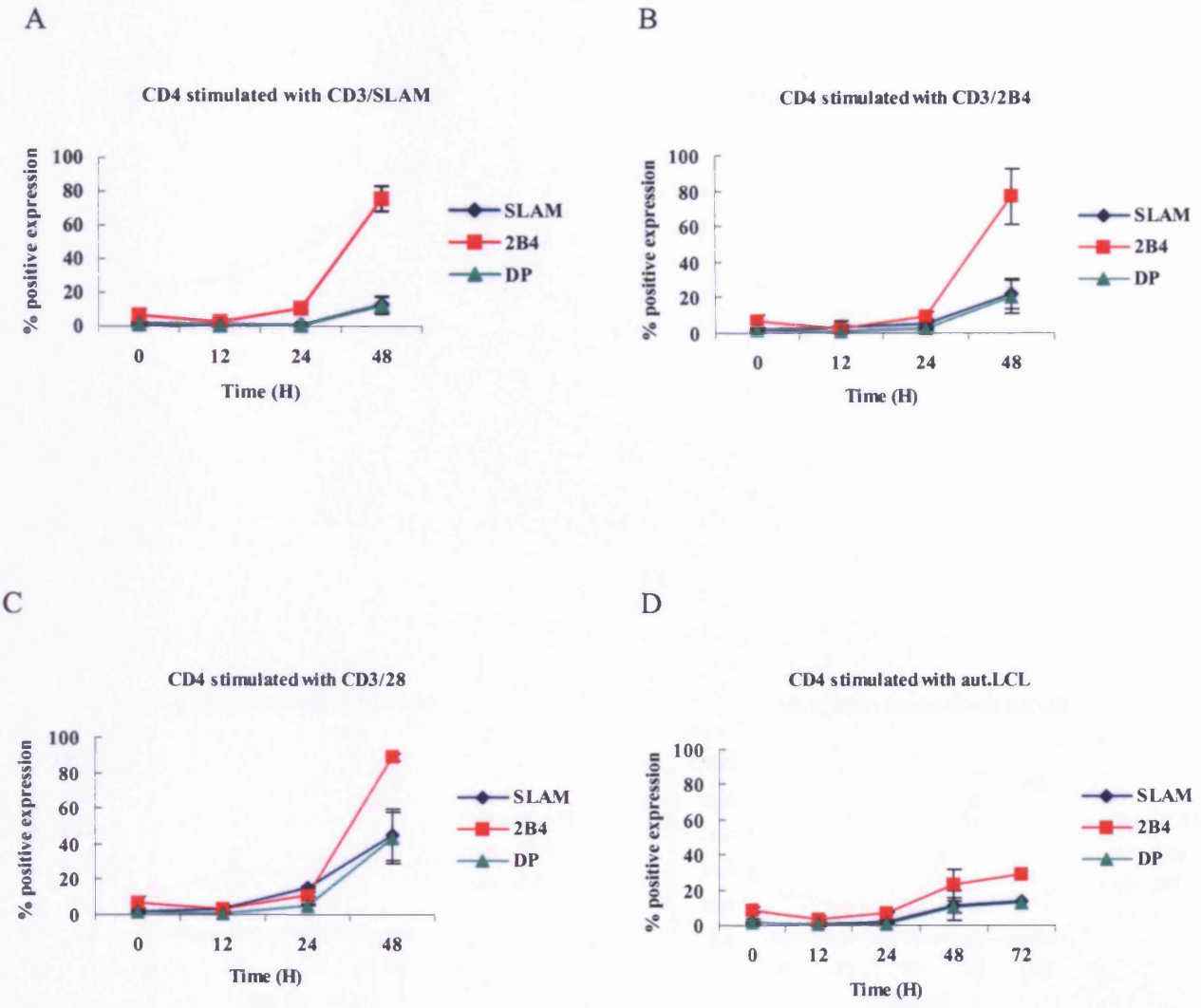
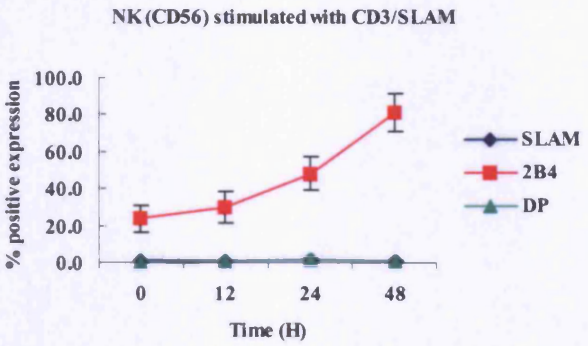


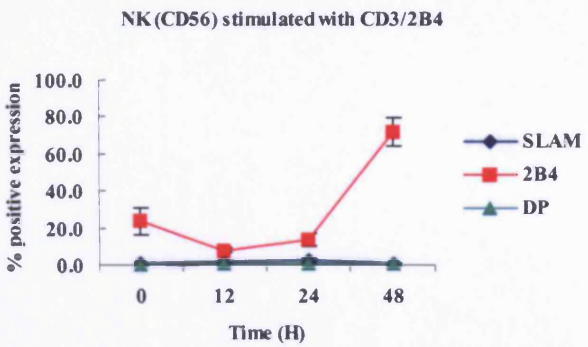
Figure 6.9 SLAM and 2B4 surface expression on CD4 T cells upon activation by different stimuli

SLAM and 2B4 expression on CD4 T cells was investigated after CD3-SLAM (A), CD3-2B4 (B), CD3-CD28 (C), and autologous BLCLs (D) stimulation at differing time points using flow cytometric analysis. Three healthy different individuals were studied twice each. The values for each experiment were derived from flow cytometric analysis data and the average percentage (± SD) of expression for each individual and mean percentage for three was calculated for each single time point. The percentage expression of SLAM, 2B4 and double positive (SLAM+2B4) are shown in blue, red and green, respectively.

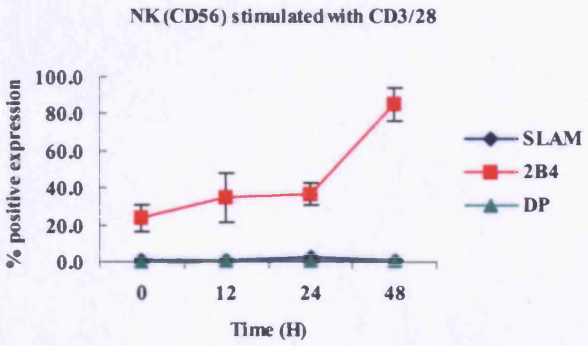
A



B



C



D

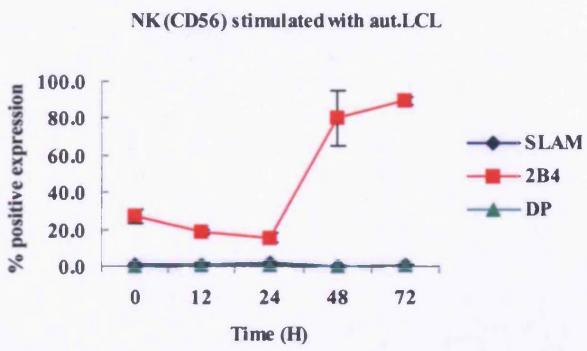


Figure 6.10 SLAM and 2B4 surface expression on NK cells upon activation by different stimuli

SLAM and 2B4 expression on NK cells was investigated during PBMCs stimulation after CD3-SLAM (A), CD3-2B4 (B), CD3-CD28 (C), and autologous BLCLs (D) stimulation at differing time points using flow cytometric analysis. Three healthy different individuals were studied twice each. The values for each experiment were derived from flow cytometric analysis data and the average percentage (± SD) of expression for each individual and mean percentage for three controls was calculated for each time point. The percentage expression of SLAM, 2B4 and double positive (SLAM+2B4) are shown in blue, red and green, respectively.

activation, it remains to be determined if SLAM and 2B4 are co-expressed on NK cells after various activations. SLAM was detected on 0.5-1% of resting NK cells with slight upregulation up to 3% on activated NK cells regardless of activation type. In contrast, 2B4 was up regulated upon all stimuli and highly expressed on CD56+/CD3- cells (Figure 6.10). Analysing CD56+/CD3+ cells showed the same expression for 2B4 and SLAM (data not shown). 2B4 internalisation was detected after α-2B4 stimulation on NK cells as observed by sudden decrease in 2B4 expression.

6.4 Discussion

Studies on SLAM and 2B4 expression showed, in contrast to SLAM, which is expressed at low levels on unstimulated T cells and not on other immune cell lineages, 2B4 has a high level of expression. It has been reported that monocytes are positive for 2B4 expression (Nakajima *et al,* 1999). Interestingly, we have found that 2B4 was expressed in immature DCs and was upregulated on mature DCs. In addition, 2B4 was expressed on LAK cell lines and EBV-LCLs suggesting a wider 2B4 distribution than thought previously. As described before (Chapter 1), 2B4 functions in cytotoxicity and T cells proliferation are well documented. However, the results shown here may suggest 2B4 plays a more diverse role in immune cell interaction.

Receptors of the SLAM family regulate immune cell function including regulating cytokine production and cytotoxicity. As has been addressed they are expressed heterogeneously and study of their co-expression under physiological conditions may be critical to understand the pathogenesis of the XLP. The results reported here on co-expression of 2B4 and SLAM upon specific stimuli suggest high

expression of 2B4 receptors as an early response on T and NK cells. It may suggest that SLAM probably is involved as part of an intermediate T cell response rather than in the very early response. However, the only condition where SLAM expression was higher than 2B4 was on CD4 T cells upon CD3/28 stimulation after 12 hours, which decreased after 24 hours. It reveals that regulation of SLAM family receptors expression is more complex than has been thought and the combination of different stimuli determines the outcome of cellular responses. In addition, there were double SALM/2B4 positive CD4 and CD8 T cell populations, whose percentage varied upon different stimulation. It would be very interesting to investigate the function and phenotype of these populations.

Moreover, SLAM and 2B4 showed significant internalization upon stimulation with combinations of CD3/SLAM and CD3/2B4 respectively. More recently, it has been shown that CD229 (Ly9) a family member of SLAM family is rapidly internalized on T cells after antibody ligation. Although CD229 extracellular domains are slightly different from rest of SLAM family (it has 4 extra cellular domains instead of two domains), it is possible other members of this family may mimic a similar pattern (Del Valle *et al,* 2003). Internalisation may be crucial step in formation of signal synapse involving SLAM and 2B4 in immune cell lineages.

In brief, the results presented here require more fundamental investigations and reconsideration of the role of SAP and its receptors involvement in immune cell activation, the results of which may help us to understand the pathogenesis of the XLP.

Chapter 7

Immunocellular Function of XLP Patients

7.1 Introduction

An adequate immune response to EBV requires a coordinated humoral and cell mediated immune response including Th, Tc and NK cells to eradicate and control EBV infection. Although the exact pathogenesis of XLP is poorly understood, it has been suggested that the abnormalities of the immune system of XLP patients may be due to defects of Th cells, CTLs, and NK cells (see section 1.10). So far it has been shown that the hallmark of XLP is a dysregulated, ineffective host response against EBV in the majority of cases with a formal demonstration that a T cell defect is central to the pathophysiology. However, the most supportive data of this hypothesis are based on a murine model, which was shown to exhibit an Th1/Th2 imbalance, particularly increased IFN-γ production upon TCR and SLAM stimulation either in combination or alone. More recently, it has been shown in a murine system that SAP has an essential role in maintaining late B cell help and long term humoral immunity by effective CD4 T cell function.

In humans, the T cell paradigm in the pathogenesis of XLP is controversial and not well documented. It remains to be investigated further whether the Th1 and Th2 cytokine profile in XLP is similar to that observed in mice, particularly in response to specific stimuli. Furthermore, as shown in previous chapters SLAM expression is not necessarily dominant either on EBV-T cell lines nor on activated T cells and instead higher levels of 2B4 expression have been demonstrated. It remains to be addressed whether SLAM or 2B4 has the dominant role in controlling the Th1 profile of XLP patients and in particular IFN-γ secretion and how these functions are regulated by SAP.

In XLP it has been suggested that the inability of the immune system to control EBV-infected B lymphocyte proliferation may be due to defects of cytotoxic activity by CTL and/or NK cells. Studies on NK cells from XLP patients show failure of 2B4 mediated cytotoxic killing of CD48 expressing target cells and EBV-LCLs (see Chapter 1). This defect is thought to arise from the delivery of an inhibitory signal through the 2B4/CD48 interaction since disruption of this association restores normal cytolytic function (Parolini *et al,* 2000). However, as discussed in Chapter 1, the results of studies on specific T cell immunity to EBV are contradictory. In particular T cell cytotoxicity against EBV remains elusive. In these studies cytotoxicity due to TCR specific EBV antigen recognition has not been addressed comprehensively because of technical difficulties. In *SAP*-deficient murine models, although CTL function was not studied specifically it was shown that following challenge with LCMV (lymphocyte choriomeningitis virus) the numbers of tetramer-positive CD8 cells were three to fivefold higher in the spleens of *SAP* deficient mice in compared to wild type mice (Wu *et al*, 2001). It was concluded that CD8 T cells were effective at eliminating the virus in SAP-deficient mice by measurement of virus load. In contrast, XLP patients who were EBV seropositive were shown to have persistent high numbers of EBV-infected B cells in the peripheral blood and other lymphoid tissues indicating a defective cell mediated immune response (Okano *et al,* 1990). Together, current data from XLP patients and *SAP* deficient mice models are contradictory with regard to CTL function.

To understand the cellular defects in XLP, we investigated the Th1/Th2 profiles of primary PBMCs by ELISPOT assay in response to TCR stimulation with SLAM, 2B4, CD28, CD3 or combinations thereof and specific Ag stimulation (BLCL). To determine Th1 and Th2 profiles, IFN-γ and IL-5, IL-10 were

investigated, respectively, whereas in XLP only IFN-γ was studied because of lack of access to patient material. We also used EBV autologous specific T cell lines from XLP patients and studied the functionality of these effector T cells with IFN-γ profiles and cytotoxic activity. Finally, using a retrovirus gene delivery system encoding hSAP cDNA, we demonstrated reconstitution of cytokine secretion and cytotoxic function in patient EBV-T cell lines, thereby confirming SAP dependent cellular defects in XLP.

7.2 Cytokine production by PBMCs from XLP patients and normal individuals

7.2.1 IFN-γ production in PBMCs

Disturbances in cytokine secretion may play an important role in the pathogenesis of XLP and recent studies on SAP deficient murine strains demonstrated increased secretion of IFN-γ in response to viral infection and TCR and SLAM stimulation (see section 1.10.2). IFN-γ release in patients with XLP has been previously studied but the existing data is controversial.

Therefore, IFN-γ production in cells from XLP patients in comparison to normal individuals was studied in response to a variety of different stimuli. In initial experiments responses in PBMCs from three XLP (P1, P2 and P3) individuals (with defined genetic lesions; see Table 5.1) were analyzed by ELISPOT assays and compared to responses seen in control samples (C). Following PMA stimulation, cells from XLP patients showed high levels of IFN- γ production with profiles similar to these observed in normal individuals, suggesting an intact capacity to produce IFN- γ (Figure 7.1). Cells were then activated with more specific stimuli. In these specific receptor stimulation experiments we observed significant differences in IFN- γ

production between controls and patient responses. After stimulation with CD3, CD3/2B4 and 2B4, significantly less IFN- γ secretion was observed in patient samples compared to controls (Fig 7.2 A and B). One exception to this was P3 (who was EBV seronegative at the time of analysis), who showed an increased IFN-γ response following CD3 stimulation ($p>0.05$). His responses to CD3/2B4 and 2B4 were similar to the other patients. Cells were then stimulated with autologous EBV immortalized LCLs and again the IFN- γ was decreased in all patients, although in P3 who was EBV seronegative and where no spots were observed, this may be expected due to lack of memory to EBV (Fig 7.2 B). Cells were also stimulated with CD28, CD3/CD28, SLAM or CD3/SLAM but no differences were observed between control individuals and XLP patients (data not shown).

7.2.2 Th2 cytokines production in PBMCs

Th2 cells induce humoral and allergic responses and downregulated inflammation through production of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 cytokines. It has been reported that in murine CD4 Ag-specific clones ligation of SLAM by anti-SLAM Abs redirected Th2 responses to a Th1 or Th0 phenotype. More recently, it has been demonstrated that SAP deficient murine T cells are unable to produce Th2 responses following challenge with pathogens or upon TCR activation (see section1.10.2). These studies may suggest that SLAM and SAP modulate Th2 responses. However, the role of SLAM family receptors and SAP in Th2 profiles has not been studied on primary human T cells, and in particular in response to specific stimuli. To investigate this matter, we studied IL-5 and IL-10 production in cells from

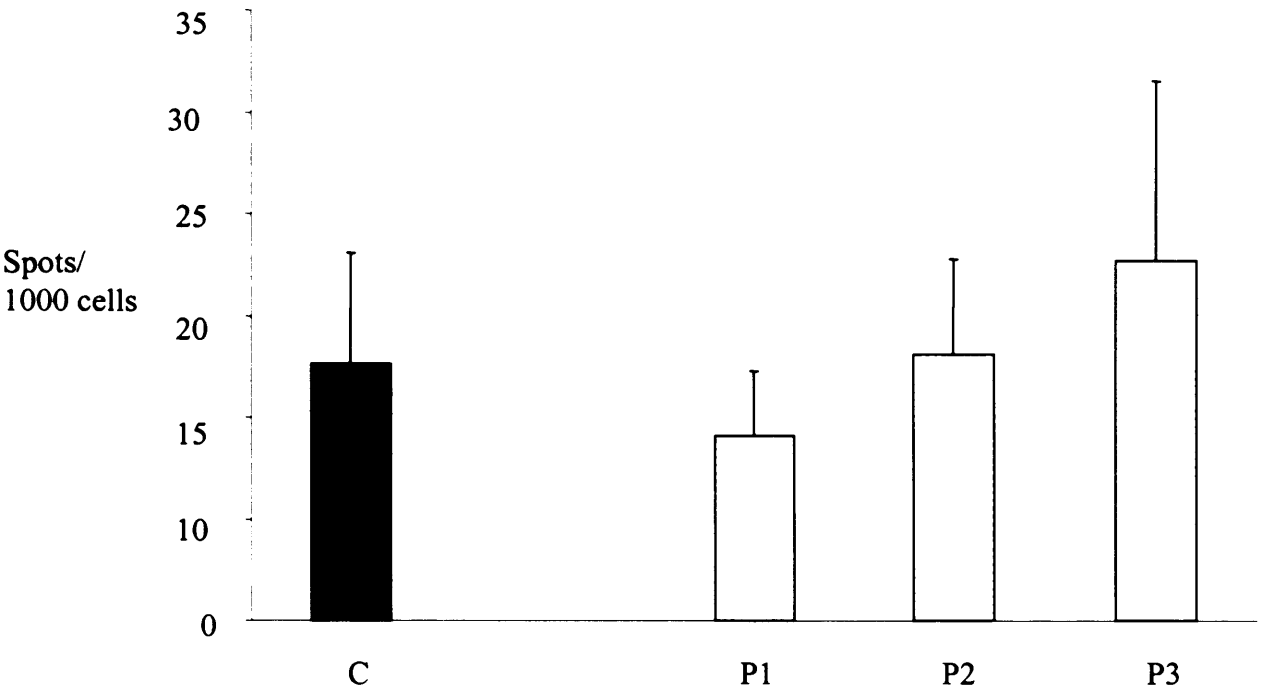
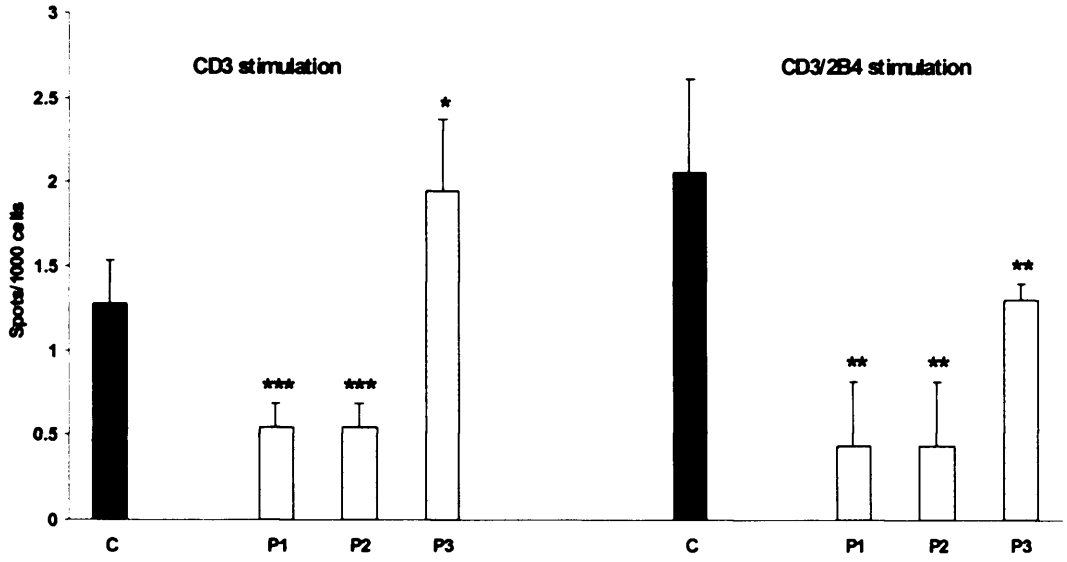


Figure 7.1 IFN-γ expression by PBMCs from controls and XLP patients following stimulation with PMA

ELISPOT analysis was used to determine IFN-γ expression following stimulation of PBMCs from controls (C) and XLP patients (P1, P2 and P3) with PMA. Control values for each experiment were derived from pooled data on a number of control individuals so that each experiment has a single control value, mean and standard deviation. Three different control individuals were studied each in triplicate and therefore the control mean is obtained from 9 data points. Each mean patient response and variance was then compared against the control response for each experiment.

A


CD3 stimulation
CD3/2B4 stimulation
Spots/1000 cells
3
2.5
2
1.5
1
0.5
0
*
***
***
**
**
**
C
P1
P2
P3
C
P1
P2
P3

B

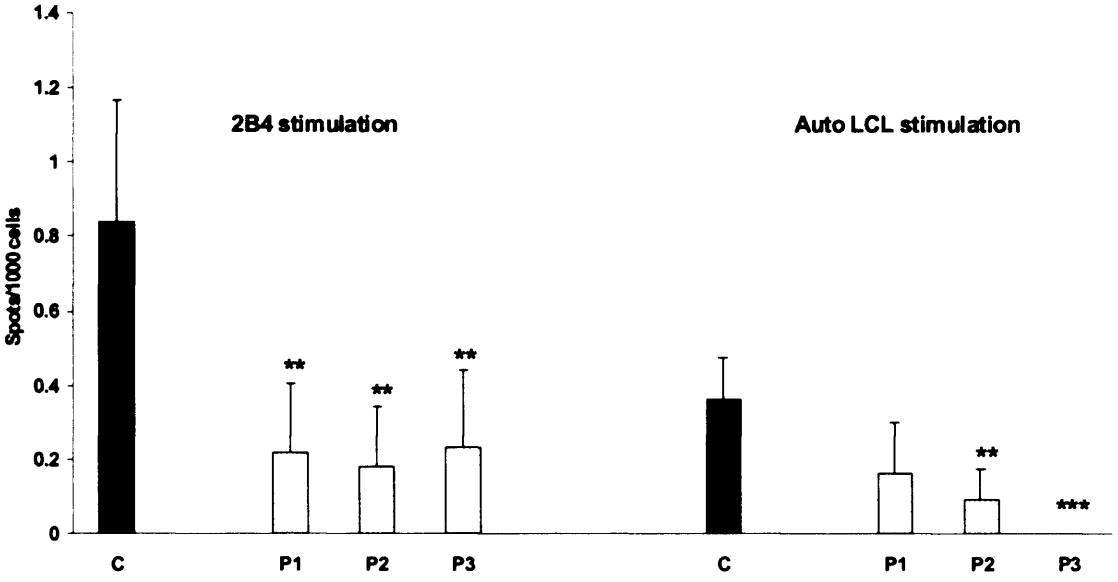

2B4 stimulation
Auto LCL stimulation
Spots/1000 cells
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
**
***
C
P1
P2
P3
C
P1
P2
P3

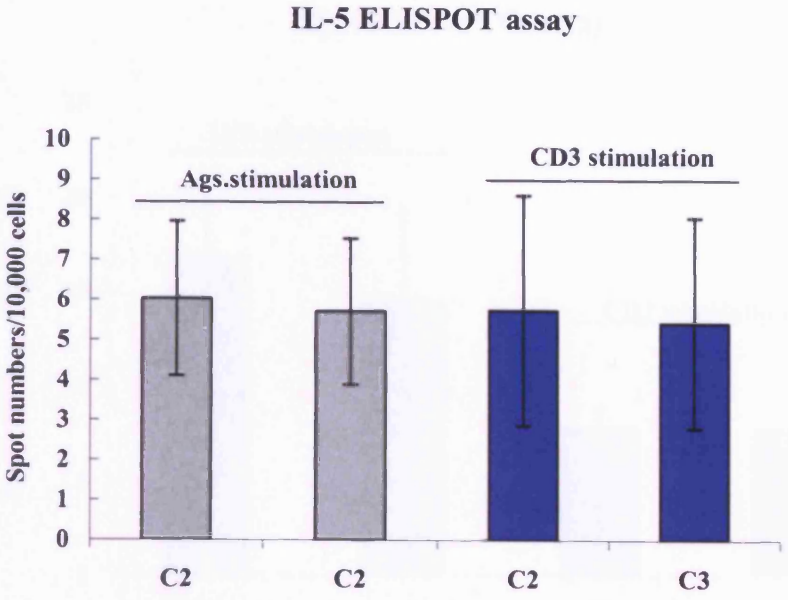
Figure 7.2 IFN-γ expression by PBMCs from controls and XLP patients following specific stimulation

ELISPOT analysis was used to determine IFN-γ expression following stimulation of PBMCs from controls (C) and XLP patients (P1, P2 and P3) with CD3 or CD3/2B4 (A), 2B4 alone or autologous LCL stimulation (B). Control values for each experiment were derived from pooled data on a number of control individuals so that each experiment has a single control value, mean and standard deviation. Three different control individuals were studied each in triplicate and therefore the control mean is obtained from 9 data points. Each mean patient response and variance was then compared against the control response for each experiment and analysed for statistical significance using a one tail unpaired T-test assuming, unequal variance between the two study groups. Significant differences are indicated by asterisks (no asterisk, no statistical significance; *, $p<0.05$: **, $p<0.01$; ***, $p<0.001$).

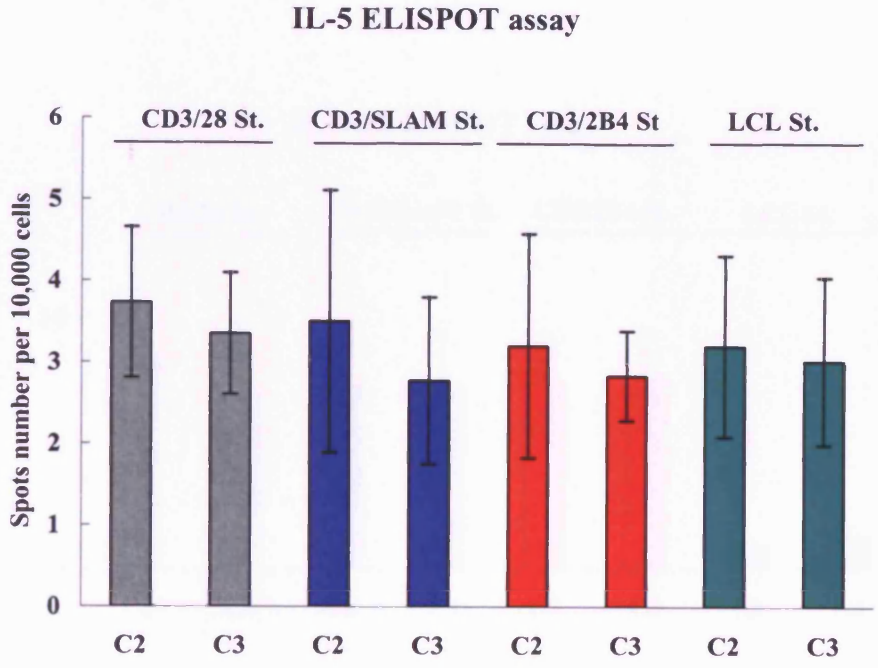
normal donors in response to a variety of different stimuli. IL-5 is often co-expressed with IL-4 and anti-CD3 induces production of both cytokines in humans. This cytokine is responsible for eosinophil differentiation and activation and is involved with helping B cells in IgM, IgE production along with IL-4 (Coffman *et al*, 1998). In contrast to IL-5, IL-10 was identified as a Th2 cytokine synthesis inhibitory factor, which has cytokine inhibitory function and inhibits Th1 cytokine production particularly from T and NK cells. T and B cells are the sources of IL-10 production mainly.

In this study, responses in PBMCs from two normal individuals (C2,C3) were analysed with ELISPOT assays. Responses to Ags or LPS were used respectively for IL-5 and IL-10 as positive control (see chapter 2). Stimulation with CD3 induced IL-5, which was equivalent responding to Ags. In contrast, IL-10 production upon CD3 stimulation was less than response to LPS (Figure 7.3 A, C). Cells were stimulated with CD3 and combination of CD28, SLAM or 2B4 showed a slightly decrease in IL-5 and no any change in IL-10 production (Figure 7.3 B, D). Stimulation with autologus BLCL only decreased IL-10 production and not IL-5. Upon CD28, SLAM and 2B4 stimulation alone, no IL-5 and IL-10 secretion was observed (data not shown). Put together, the results showed SLAM or 2B4 and TCR activation in human might modify some of Th2 cytokines response.

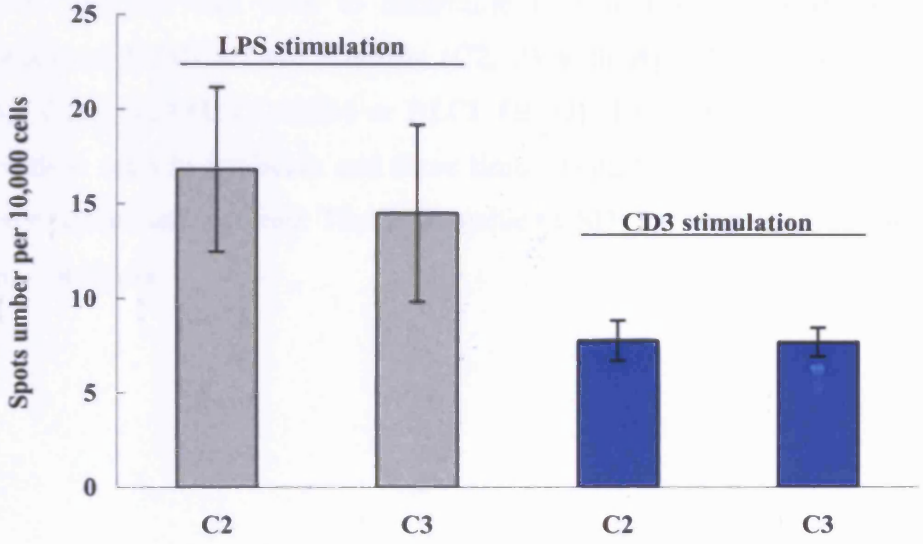
A


IL-5 ELISPOT assay
Spot numbers/10,000 cells
10
9
8
7
6
5
4
3
2
1
0
Ags.stimulation
CD3 stimulation
C2
C2
C2
C3

B


IL-5 ELISPOT assay
Spots number per 10,000 cells
6
5
4
3
2
1
0
CD3/28 St.
CD3/SLAM St.
CD3/2B4 St
LCL St.
C2
C3
C2
C3
C2
C3
C2
C3

C


IL-10 ELISPOT assay
LPS stimulation
CD3 stimulation
Spots umber per 10,000 cells
25
20
15
10
5
0
C2
C3
C2
C3

D

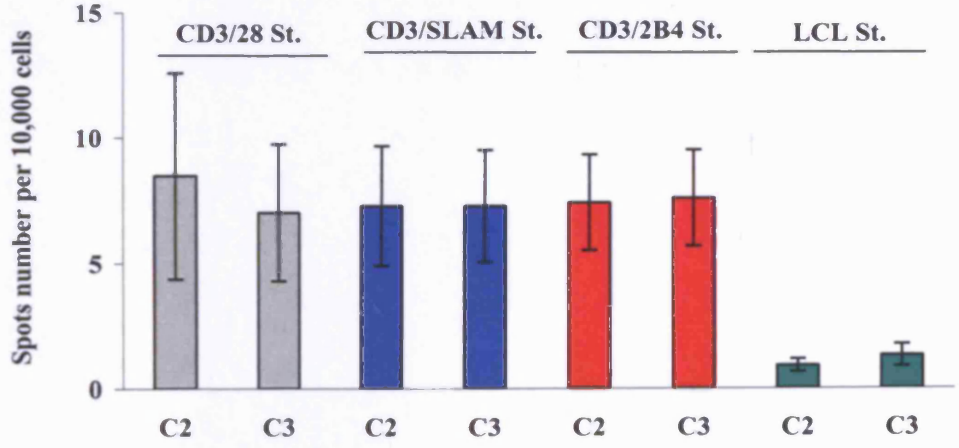

IL-10 ELISPOT assay
CD3/28 St.
CD3/SLAM St.
CD3/2B4 St.
LCL St.
Spots number per 10,000 cells
15
10
5
0
C2
C3
C2
C3
C2
C3
C2
C3

Figure 7.3 IL-5 and IL-10 expression by PBMCs from healthy controls following specific stimulation

ELISPOT analysis was used to determine IL-5 and IL-10 expression following stimulation of PBMCs from controls (C2, 3) with Ags, LPS or CD3 (A, C) and CD3/28, CD3, SLAM, CD3/2B4 or BLCL (B, D). Two different control individuals were studied each in triplicate and three times. Data from three experiments on two volunteer donors is presented. The mean value (± SD) for each condition was used for statistical analysis.

7.3 EBV-T cell line function in XLP and reconstitution of defects following SAP gene transfer

It has been postulated that the cellular pathogenesis of XLP may reside in abnormal function of EBV-T cell lines and in their inability to control EBV infection. Previous reports, performed before the identification of the SAP gene defect, are inconclusive with certain studies demonstrating that cytokine secretion and cytoxicity are abnormal and others suggesting that both functions are intact. We also examined whether introducing expression of the SAP gene into EBV-T cell lines from XLP patients (known to lack SAP expression) could restore these observed functional defects. We transduced EBV-T cell lines from XLP patients P1 and P2 (CTLP1 and CTLP2) with a retroviral vector encoding the SAP cDNA and the reporter eGFP (transduced cells designated S+CTLP1 and S+CTLP2). Transduced EBV-T cell lines were analyzed and showed the same phenotypic characteristics as the parent cell lines (data not shown). The expression of eGFP and SAP in the transduced cell lines was confirmed by flow cytometric and immunoblot analysis, respectively (Figure 7.4A-B). The percentage of cells transduced was approximately 45% and these cells were purified by FACS for use in the reconstitution experiments described below.

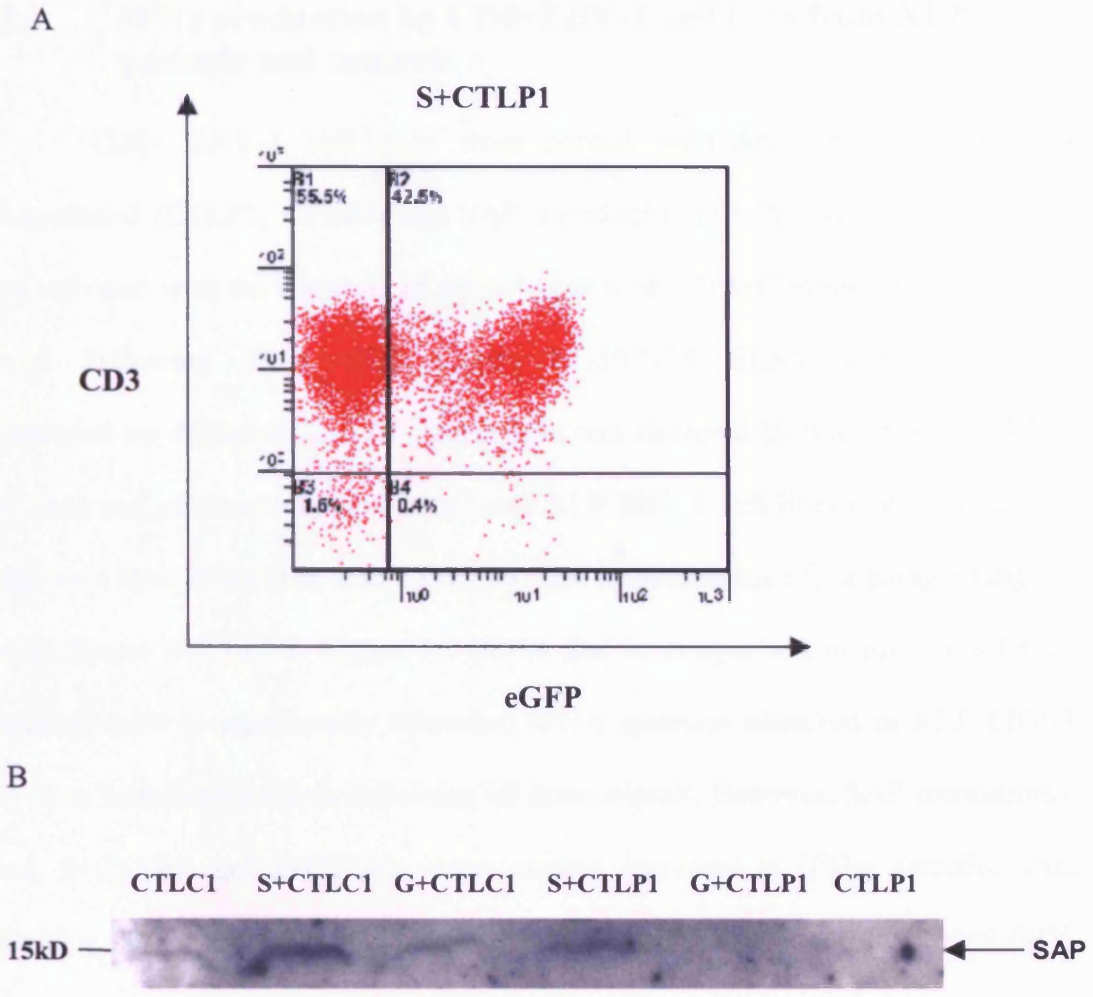


Figure 7.4 eGFP and SAP expression following retroviral transduction of EBV-T cell lines from XLP patients

(A) Flow cytometric analysis of EBV-T cell lines from XLP patient P1 following transduction with eGFP/SAP encoding retroviral construct shows 42% expression of eGFP in the EBV-T cell line from patient1 (S+CTLP1).

(B) Immunoblot analysis using an anti-SAP antibody shows a 15kD protein expressed in EBV-T cell lines from a control (CTLC1) and in cells transduced using the eGFP/SAP retroviral vector (S+CTLC1) and a vector encoding eGFP only (G+CTLC1). The 15kD protein is also seen in XLP EBV-T cell lines transduced with the eGFP/SAP retroviral vector (S+CTLP1) but not in EBV-T cell lines transduced with the vector encoding eGFP only (G+CTLP1) or in untransduced EBV-T cell lines (CTLP1).

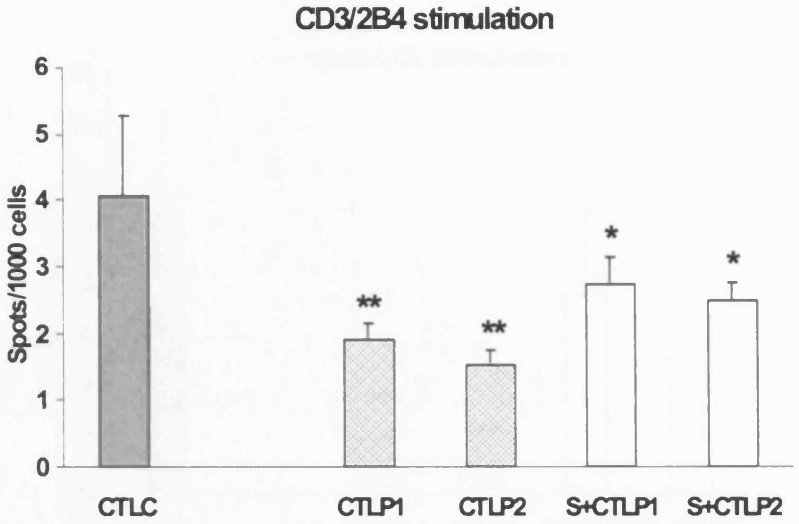
7.3.1 IFNγ production by CD8+EBV-T cell lines from XLP patients and controls

CD8+ EBV-T cell lines from normal individuals (C), XLP patients untransduced (CTLP1, CTLP2) and SAP transduced (S+CTLP1, S+CTLP2) were then activated with the same set of stimuli used in the PBMC experiments described above. Following PMA, CD3, CD28, CD3/CD28, SLAM and CD3/SLAM stimulation no difference in IFN- γ secretion was observed between control EBV-T cell lines and untransduced and transduced XLP EBV-T cell lines (data not shown). Cells were then stimulated with CD3/2B4, 2B4 or autologous LCLs using a range of target:effector cell ratios. Figure 7.5 shows that in comparison to the control CTL response, there is significantly decreased IFN-γ secretion observed in XLP EBV-T cell lines from both patients following all three stimuli. However, SAP reconstituted lines, S+CTLP1 and S+CTLP2, show marked increases in IFN-γ secretion that, following 2B4 or autologous LCL stimulation are no different from the control EBV-T cell line response. The most dramatic reduction in IFN-γ secretion and reconstitution of function is seen following autologous LCL stimulation (Figure 7.5C). As a further control, XLP EBV-T cell lines transduced with eGFP alone did not show any differences in IFN- γ secretion suggesting that the process of retroviral transduction *per se* did not affect cytokine secretion (data not shown).

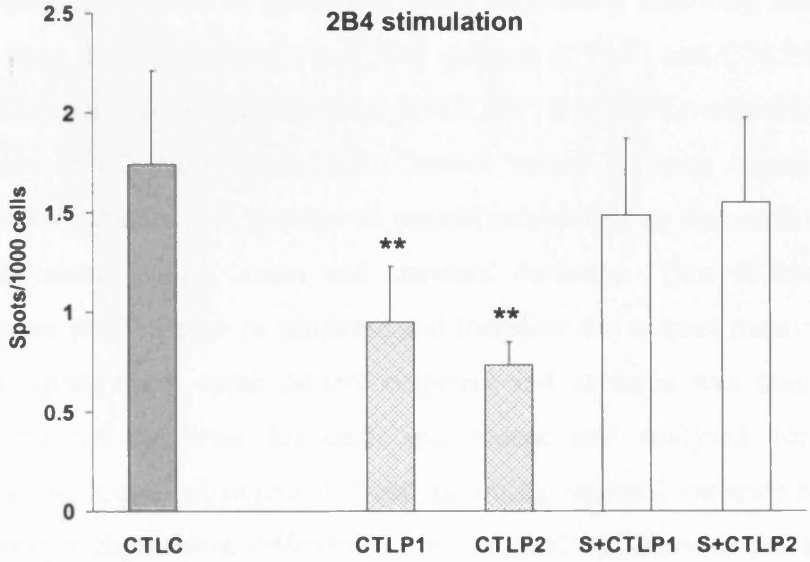
7.3.2 Cytotoxic activity of unreconstitute and reconstitute EBV-T cell lines from XLP patients and normal individuals

We next tested the ability of XLP EBV-T cell lines to kill autologous LCLs using a standard LDH release assay (Figure 7.6). EBV-T cell lines from both XLP

A


CD3/2B4 stimulation
Spots/1000 cells
6
5
4
3
2
1
0
**
**
*
*
CTLC
CTLP1
CTLP2
S+CTLP1
S+CTLP2

B


2B4 stimulation
Spots/1000 cells
2.5
2
1.5
1
0.5
0
**
**
CTLC
CTLP1
CTLP2
S+CTLP1
S+CTLP2

C

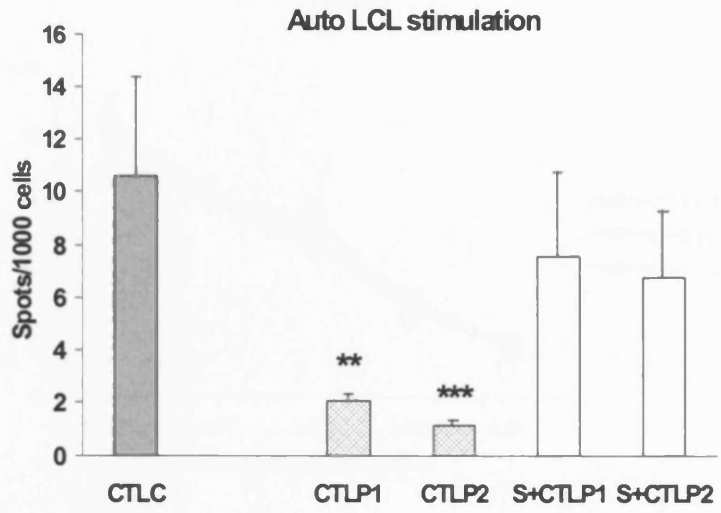
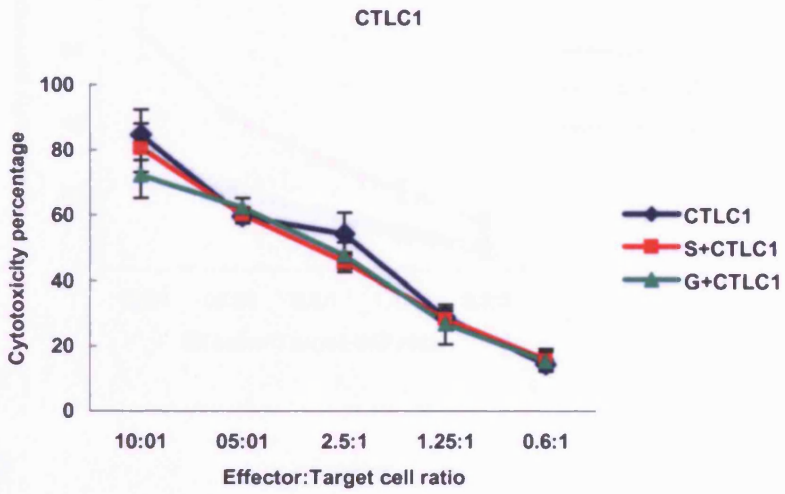


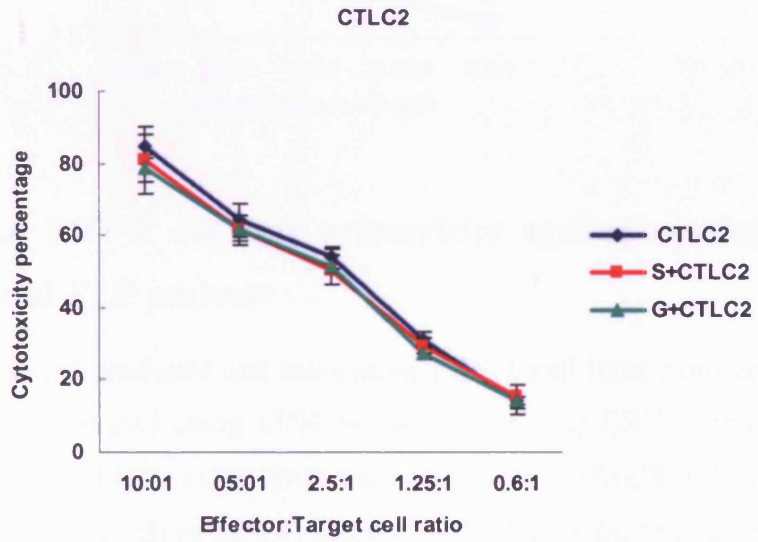
Figure 7.5 IFN-γ expression by EBV-T cell lines from controls and XLP patients following stimulation

ELISPOT analysis was used to determine IFN-γ expression following stimulation of EBV-T cell lines from controls (C) and XLP patients (CTLP1 and CTLP2) and SAP transduced XLP patient EBV-T cell lines (S+CTLP1, S+CTLP2) with (A) CD3/2B4, (B) 2B4 alone or (C) autologous LCL. Control values for each experiment were derived from pooled data on a number of control individuals so that each experiment has a single control value, mean and standard deviation. Two different control individuals were studied each in triplicate and therefore the control mean is obtained from 6 data points. Each mean patient response and variance was then compared against the control response for each experiment and analysed for statistical significance using a one tail unpaired T-test assuming, unequal variance between the two study groups. Significant differences are indicated by asterisks (no asterisk, no statistical significance; *, $p<0.05$: **, $p<0.01$; ***, $p<0.001$).

A


CTLC1
Cytotoxicity percentage
100
80
60
40
20
0
10:01
05:01
2.5:1
1.25:1
0.6:1
Effector:Target cell ratio
CTLC1
S+CTLC1
G+CTLC1

B


CTLC2
Cytotoxicity percentage
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80
60
40
20
0
10:01
05:01
2.5:1
1.25:1
0.6:1
Effector:Target cell ratio
CTLC2
S+CTLC2
G+CTLC2

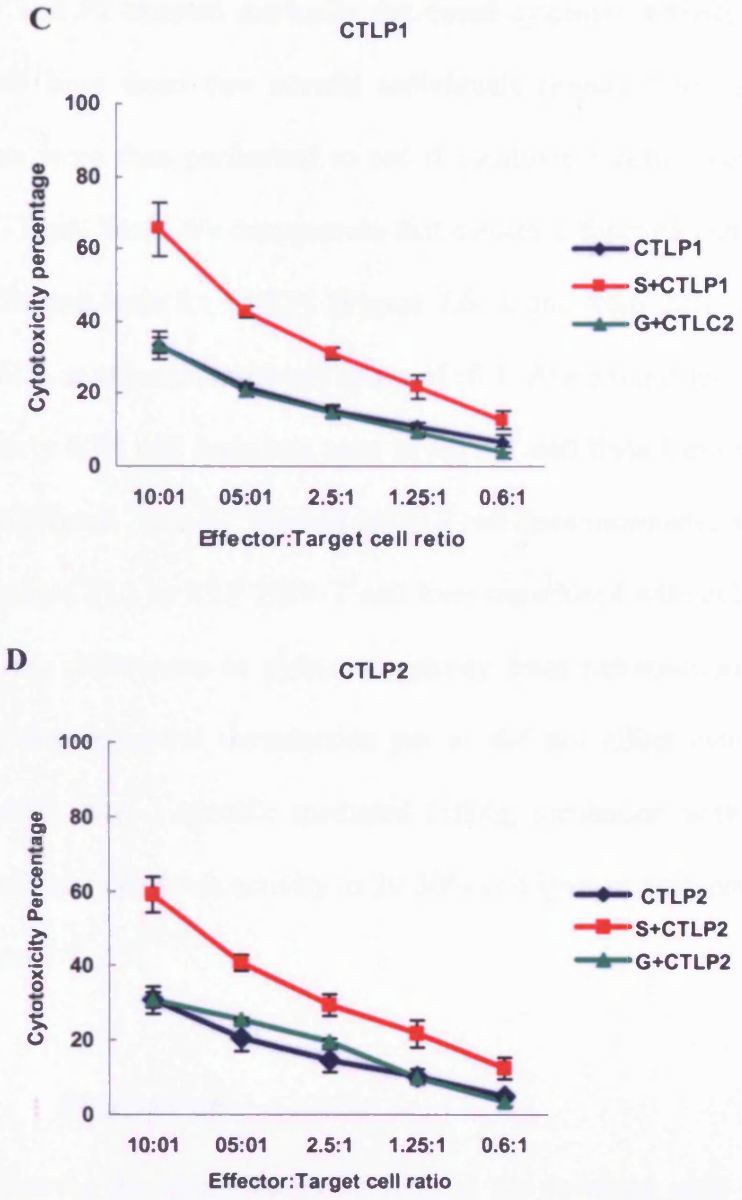

Figure 7.6 EBV-T cell line cytotoxicity against autologous LCL in controls and XLP patients

Cytoxicity of untransduced and transduced EBV-T cell lines from controls and XLP patients was measured using LDH release assays. (A) EBV-T cell lines from C1, untransduced (CTLC1), eGFP/SAP vector transduced (S+CTLC1) and eGFP alone vector (G+CTLC1), (B) as for (A) using CTLC2, (C) as for (A) using CTLP1, (D) as for (A) using CTLP2.

patient P1 and P2 showed markedly decreased cytotoxic activity in comparison to EBV-T cell lines from two normal individuals (Figure 7.6). SAP reconstitution experiments were then performed to see if cytotoxic function could be restored to XLP EBV-T cell lines. We demonstrate that cytotoxic function can be increased from 30% to 60% cell lysis for CTLP1 (Figure 7.6C), and from 20% to 60% for CTLP2 (Figure 7.6D), at effector:target cell ratios of 10:1. At similar effector:target cell ratios approximately 80% cell lysis was seen in EBV-T cell lines from normal individuals C1 and C2 (Figure 7.6A-B). Normal EBV-T cell lines transduced with SAP or eGFP (S+CTL or G+CTL) or XLP EBV-T cell lines transduced with eGFP (G+CTLP) did not show any differences in cytotoxic activity from untransduced cell lines, again suggesting that retroviral transduction *per se* did not affect cytotoxic activity. To confirm MHC class I specific mediated killing, incubation with an MHC class I antibody reduced cytotoxic activity to 20-30% cell lysis in both normal and S+CTLP lines (Figure 7.7).

7.4 Discussion

Following the identification of SAP as the defective gene in XLP, there has been considerable progress in the understanding of the SAP protein structure, its interaction with cell surface molecules and more recently with proximal tyrosine kinases such as Fyn T (Chan *et al,* 2003; Latour *et al,* 2003). However, the cellular pathogenesis of XLP in humans remains poorly understood, especially given the different clinical phenotypes of the disease. Murine models of SAP deficiency suggest a tendency to a dysregulated Th1 responses with T cell activation and increased IFN-γ production, but it is clear from studies of other immunodeficiencies that abnormalities observed in murine models do not always accurately reflect human disease

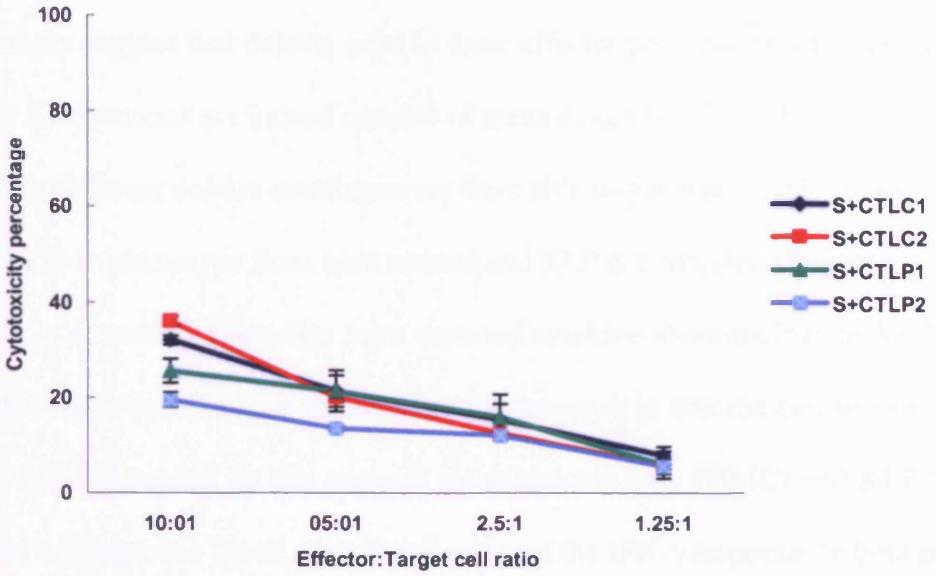

Figure 7.7 Blocking EBV-T cell line cytotoxicity against autologous LCL in controls and XLP patients

Incubation of eGFP/SAP vector transduced EBV-T cell lines from both controls and XLP patients (S+CTLC1, S+CTLC2, S+CTLP1, S+CTLP2) with W6/32 antibody. Cytoxicity of untransduced and transduced EBV-T cell lines from controls and XLP patients was measured using LDH release assays.

(Khan *et al,* 1995). It is evident from a number of reports that human NK cell defects are present in this condition, but there is very little clear functional data on specific T cell populations (Nkajima *et al,* 2000; Parolini *et al,* 2000; Tangye *et al,* 2000). In this study the use of EBV-T cell lines from XLP patients represents a physiological human T cell effector population. The lack of cellular transformation or species differences provides a relevant model for examining defects in this condition.In normal individuals the immune response to EBV infection, the major pathological

trigger in XLP, is dominated by the proliferation of CD8+ EBV-T cell lines and NK cells. The development of EBV driven lymphoma and fulminant IM in XLP would strongly suggest that defects exist in these effector populations. In this study we show that XLP patients are indeed capable of generating EBV-T cell lines.
Using different culture conditions we were able to generate T cell lines of both CD8+ and CD4+ phenotype from both normal and XLP patients (see Chapter 5).

A number of studies have reported cytokine abnormalities in XLP. However, in the majority of studies undertaken, the response to specific cell surface stimuli has not been addressed. In this analysis we stimulated both PBMCs and XLP EBV-T cell lines with specific T cell stimuli and assessed the IFN-γ response. In both populations, differences were observed in response to CD3, CD3/2B4, 2B4 or autologous LCLs and not in response to PMA, CD28, CD3/CD28, SLAM or CD3/SLAM. The lack of response to CD3/SLAM or SLAM alone would be in keeping with the low levels of SLAM observed in these cell lines.

Following CD3 stimulation alone, P1 and P2, who are EBV experienced, demonstrated significant downregulation of IFN-γ production. This is in keeping with observations made by Sanzone *et al.* where EBV experienced XLP patients were analysed (Sanzone *et al,* 2003). However, P3 (who was EBV-seronegative at the time of analysis) shows an exaggerated IFN- γ response that is significantly elevated from controls and is similar to the CD3 stimulation T cell responses observed in SAP deficient mice (Czar *et al,* 2001; Wu *et al,* 2001). Importantly stimulation via 2B4 or CD3/2B4 in P3 shows a downregulated response and is similar to P1 and P2. These observations albeit in only one patient, suggest that SAP deficiency in the EBV inexperienced individual augments CD3 stimulation which after EBV infection is downregulated. The role of SAP downstream of CD3 triggering without co-

stimulatory input is unclear but these data suggest a regulatory role for SAP, which needs further investigation in larger numbers of patients.

In addition, it is possible that combination of CD3 and 2B4/SLAM stimulation regulates Th2 response IL-5 and IL-10 in normal individuals as we showed here. However, it remains unclear whether in human T cells it is a direct function of 2B4 or SLAM signalling in Th2 regulation as it has been recently shown in *SAP* and *SLAM* deficient murine models (Cannons *et al,* 2004; Wang *et al,* 2004).

In our study, differences in XLP and normal EBV-T cell lines following CD3/2B4 or 2B4 activation suggest that activation through this specific pathway is important in EBV-T cell line function and, given the similarities in the cell surface phenotype between the two T cell populations, point to defective SAP expression as being responsible for aberrant transduction of the 2B4 signal. Similar findings after stimulation by autologous LCLs are consistent with these findings. Cell surface LCL/EBV-T cell line interactions include homotypic association between SLAM molecules (though in this study low SLAM expression negates this pathway) and also between 2B4 on EBV-T cell lines with its ligand CD48 which is abundantly expressed on LCLs. Disruption of the signal arising from this interaction results from the lack of SAP expression in XLP EBV-T cell lines probably leading to decreased IFN- γ expression and to the observed failure of cytotoxicity of autologous LCLs.

Our studies on human cells from EBV experienced individuals demonstrate a decrease in IFN- γ production in response to specific stimuli and contrast significantly with murine data. A possible explanation for this discrepancy may be the fact that in murine studies, animals were examined at an early age and only after limited antigenic exposure. It would be interesting to repeat these studies on mice exposed to repeat or chronic antigen stimuli. This would better mimic the situation in XLP,

which was initially described as a 'progressive' immunodeficiency and where repeated immunological insults and more specifically with EBV may result in a gradual deterioration in immune function.

We demonstrate that human EBV-T cell lines from two patients with a molecular diagnosis of XLP and lack of SAP expression show significant defects in direct killing of autologous LCLs. Previous studies have suggested this using indirect assays such as LCL growth regression. That this defect occurs via the 2B4/CD48 pathway is supported by the abundance of 2B4 rather than SLAM expression and also by the demonstration of 2B4/CD48 abnormalities in NK cell cytotoxicity in XLP (Nakajima *et al,* 2000). The molecular detail of this pathway is unclear but it has been shown that 2B4 associates with the LAT (linker for activation of T cells) leading to tyrosine phosphorylation of both molecules. Whether this interaction is SAP dependent has yet to be defined. It is also evident that other receptors are involved in mediating cytoxicity in T cells. NTB-A is a recently identified member of the SLAM family and can bind SAP following phosphorylation of its cytoplasmic tail (Bottino *et al,* 2001). NTB-A behaves in an analogous manner to 2B4 in NK cells triggering cytotoxicity but is also expressed in T cells suggesting that the observed defects in XLP EBV-T cell lines may arise from abnormal signaling downstream of a number of different surface receptors. The final events in these pathways may be the inability to form the cytolytic machinery such as release of granzyme or perforin molecules and expression of such proteins in XLP EBV-T cell lines is currently being investigated. More recently it has been shown that the lytic effectors perforin and granzyme-B are normal in SAP-deficient CTL derived from XLP patients (Dupre *et al*, 2005).

The cytokine expression and cytotoxicity defects are SAP dependent since introduction of SAP by gene transfer restored functionality. A marked increase was

seen in both patients for both cytotoxicity and cytokine assays. Although the restoration of function was not entirely equivalent to control samples, mock transfected cells did not show any reconstitution.

Retroviral transfer of the SAP gene into EBV-T cell lines was achieved during the repeated stimulation of T cells by autologous LCLs and makes CTLs an attractive target for gene transfer. Although the studies were principally conducted to show that the defects were SAP dependent, the restoration of function suggests that SAP gene transfer in XLP may hold some therapeutic value. In cases without a matched sibling donor, gene corrected CTLs could be generated and stored for future use if lymphoma or fulminant IM were to arise in XLP patients. However, much further work would be necessary to show that constitutive SAP expression under the control of a viral LTR does not lead to adverse effects. The possibility of SAP expression under the control of a T lineage restricted or endogenous SAP promoter could also be explored.

To date functional cytotoxic T cell defects in XLP have not been carefully defined, especially in response to EBV infection. Using this autologous EBV-LCL/EBV-T cell line model we clearly demonstrate that both cytokine production and cytolytic activity are severely impaired as a result of SAP deficiency. In combination with previously described NK cell defects this may explain the failure to control EBV infection and predisposition to B cell lymphoma which occurs in one third of XLP patients and which indeed was the presenting feature of XLP patient P2. One may also speculate that the tendency to fulminant IM (as in P1) and hemophagocytosis may possibly arise from the initial CTL and NK cell failure leading to further dysregulated immune responses.

Chapter 8

Discussion

8.1

SAP is the defective gene in XLP, a disease, which results in dysregulated immune responses. Many features of the disease are unexplained, including excessive susceptibility to EBV infection, cellular and humoral immune dysfunction, late haematopoietic malignancy and further diverse phenotypes. However, evidence unravelling the molecular defect responsible for XLP in recent years has led to critical new insights into the pathophysiology of this disease. It has been demonstrated that SAP is involved in regulation of the immune response particularly in T and NK cells. In particular, SAP interacts with the SLAM family of immune cell receptors. Whereas the functions of these receptors are only beginning to be elucidated, they seem to play a major role in XLP pathogenesis (see Chapter 1). More recently, development of XLP murine model has made it possible to investigate the immune function of SAP *in vivo* and *in vitro* following challenge with various pathogens. However, one limitation of the *SAP* deficient murine models is that mice are not susceptible to EBV infection and therefore are unlikely to be useful in explaining the molecular and cellular features of the SAP defect in humans. This is also debate particularly when CTL and NK cells function consider as major and crucial players in immune response to EBV infection in humans. Moreover, it is becoming clear that SLAM family receptors are widely involved in immune cell function and attributing all molecular features of XLP to the SLAM pathway is likely to be an oversimplification.

The main aim of this thesis, therefore, was to investigate the cellular pathogenesis of XLP in humans in particular to explore the function of EBV-CTLs and to develop an *ex vivo* re-constitutional model using a retroviral gene transfer system. As a subsidiary aim, it was planned to characterise and profile the cytokine-phenotype of lymphocyte lineages in XLP patients in a more detailed manner.

8.2 The role of SAP in immune cell functions

Appropriate immune responses rely on the functions of immune cells controlled by the signalling of complex and heterogenous receptor families. The balance between the signalling pathways determines the specificity of immune response and regulates its intensity and duration. Adaptors play crucial roles to maintain this balance and orchestrate the intracellular pathways. SAP is an example of a modulator protein, which is expressed predominantly in T and NK cells. Defective SAP in XLP syndrome highlights the important function of these adaptors in the pathophysiology of immune responses (Latour *et al*, 2004). The unique structure of SAP represents a family of adaptors, including EAT-2, that have the ability to interact with a variety of immune cells receptors from the SLAM family.

SLAM family receptors conduct a variety of functions including the modulation of cytokine production and cytotoxicity (see Chapter 1). In addition, it has been reported that expression of human SLAM facilitates infection of lymphoid cells by measles virus infection (Hsu *et al*, 2001). While the roles of this family are only beginning to be understood, the clinical and pathology findings of XLP suggest the functions of SAP-SLAM family receptors are vital in immune responses, in particular to EBV infection in humans. EBV infection has several aspects, which are different from other viral pathogens, particularly modulation of cytokine production and B cell immortalization as described in Chapter 1. In this context, SAP function may provide an essential role in the immune surveillance against EBV. SAP enables SLAM family receptors to mediate their diverse functions through facilitating tyrosine phosphorylation of their cytoplasmic tails. Depending on the tyrosine- based motifs present in their intra-cytoplasmic domain, SLAM family receptors can recruit distinct sets of SH-2 domain-containing effectors that lead to different signals with distinct

biological consequences (Figure 8.1). Typically, SAP makes a triple complex and promotes the recruitment and activation of Src-related tyrosine kinase (PTK) Fyn and its interaction with SLAM in murine T cells (Latour *et al,* 2001; Chan *et al,* 2003; Latour *et al,* 2003; Davidson *et al,* 2004). These observations suggest that SAP acts like a modulator and differently from all signalling molecules such as ZAP70 and Btk described in Chapter 1. Moreover, the unique structure of SAP makes it different from other adaptors involved in signalling such as the SLP-76 adaptor family including B cell linker (BLNK) or Dok family. Although this adaptor family, like the SAP family, are not receptors or kinase, they exhibit more complex structure and bind to SH2 domain-containg partners. For instance, upon BLNK interaction with Syk, BLNK becomes rapidly phosphorylated on multiple N-terminal tyrosine residues, which act as binding sites to recruit the SH2 domain containing proteins PLCγ2, Btk, Vav, Grb2 and Nck into signalling complex (Goitsuka *et al,* 1998; Kurosaki *et al,* 2000). Therefore BLNK acts as a molecular scaffold to link the pre-BCR (B cell receptor) activated Syk kinase to regulators of multiple downstream signalling pathways (Schebesta *et al,* 2002).

Recent biochemical investigations suggested that SAP deficiency might cause an abnormal TCR signalling cascade in human T cells (Nakamura *et al,* 2001; Sanzone *et al,* 2003). Studies on T cells from XLP patients showed that early TCR-induced signals such as tyrosine phosphorylation of PLC-γ and calcium fluxes were increased in SAP-deficent T cells, whereas T cells from a *SAP* deficient murine

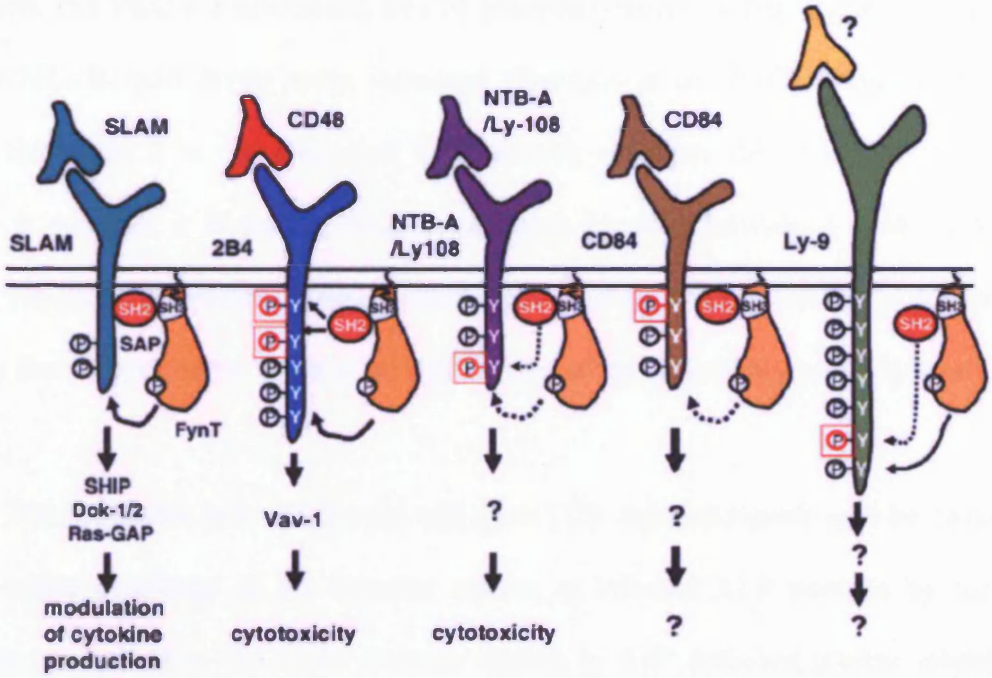

Figure 8.1 Regulation of SLAM family receptors by SAP

A model of SLAM-related receptor regulation by SAP is presented. SAP binds to tyrosines in the intra-cytoplasmic domain of SLAM family receptors (the consensus binding sites are boxed in red), thereby allowing recruitment and activation of FynT. This leads to tyrosine phosphorylation of the receptor, and recruitment and activation of downstream effectors that activate cellular responses (as specified in the figure). The presence of distinct sets on tyrosine-based motifs in the cytoplasmic domain of the various SLAM-related receptors enables these receptors to transduce different signals and mediate unique functions. (Taken from Latour *et al,* 2004).

model showed normal calcium mobilization and ERK activation upon TCR activation, but PKC-θ recruitment, Bcl-10 phosphorylation, IκB-α degradation, and nuclear NF-κB1/p50 levels were increased (Sanzone *et al,* 2003; Cannons *et al,* 2004). However, it is not clear that SAP directly regulates the TCR downstream signals or whether it is part of SAP's regulatory function interacting with SLAM family receptors pathways, although interaction of SAP with cytoplasmic proteins such as Dok1 reported by Sylla *et al* may be part of this regulatory axis (Sylla *et al,* 2000).

The difference between human and mice TCR-induced signals may be caused by excessive challenge of the immune system in affected XLP patients by EBV infection, compared to the naïve immune system in *SAP* deficient murine models. However, it cannot be excluded that the different phenotypes in human and mice may be caused by differences in signalling pathways controlled by SAP function. It is notable that recent data demonstrated SAP deficiency in murine T cells appears to selectively affect NF-$\kappa\beta$ members (Cannons *et al,* 2004). For example c-Rel required for IL-2 production is not affected by lack of SAP in T cells. Interestingly, this pattern has been reported in Fyn $^{-/-}$T cells, supporting the assumption that SAP-Fyn interaction is necessary for the PKC-θ/NF- $\kappa\beta$ pathway (Cannons *et al,* 2004). Whereas, recruitment of Fyn by SAP has not been reported in humans and also given the preference of other PTK members such as Lck in human T cells, it is therefore possible that SAP may play different roles in controlling T cell activation in humans and mice and deserves further exploration.

8.3 Th1/Th2 profile in XLP human and mice

SAP deficiency causes abnormalities in Th1/Th2 cytokine profiles as described in Chapter 1. It has been demonstrated that there are different patterns of cytokine production in human and SAP deficient mice. Whereas these mice were shown to exhibit an augmentation of Th1 responses (IFN-γ production) and reduced Th2 responses (especially IL-4 production), human T cells from XLP patients failed to produce Th1 cytokines such as IFN-γ and IL-2, although the Th2 profile has not been investigated in these patients (Nakamura *et al,* 2001; Sanzone *et al,* 2003). Our results presented in Chapter 7 not only reveal Th1 response failure (IFN-γ) in primary T cells and EBV- T cell lines in agreement with the previous reports (Yasuda *et al,* 1991; Nakamura *et al,* 2001), but also we have been able to show this defect is partial as highlighted upon stimulation through specific receptors (Sharifi *et al,* 2004; this thesis). The fact that stimulation with PMA and ionomycin overcome the defects of SAP-deficient T cells observed upon TCR stimulation (with anti-CD3 Ab) strongly suggests that SAP is involved in proximal T cell signalling. Moreover, in our patients IFN-γ production was lower than normal levels upon TCR stimulation, except in an EBV seronegative XLP case that had showed a significant increase of IFN-γ production in comparison to normal controls. Whether this is related to EBV seronegative condition or cytokine polymorphisms is not clear and this may emphasise the complexity of cytokine imbalance in humans affected with XLP when compared with *SAP* deficient murine models. More recently, it has been shown that TCR stimulation (with anti CD3/28 or lectin) of SAP-deficient CD8 T cell-lines (autologous and allogenic) from three XLP patients lead to normal production of IL-2, IL-4 and IFN-γ (Dupre *et al,* 2005). This controversy remains to be addressed probably with a larger sample size and more well defined methods.

Intriguingly, the studies on *SAP* deficient murine models consistently show aberrant TCR response conducting Th1/Th2 production (Czar *et al,* 2001; Wu *et al,* 2001; Latour *et al,* 2001). The precise mechanism by which the SAP signalling pathway modulates production of cytokines by T cells is not well documented. However, it seems that FynT, SHIP-1, Dok family adaptors and or RAS/GAP are implicated in this effect. The most recent reports showed that CD4 T cells from non-infected *SAP* Knock out mice exhibiting a slightly increased IFN-γ production in comparison to controls, while IL-2 production was normal (Cannons *et al,* 2004; Davidson *et al,* 2004). The Fyn $^{-/-}$ T cells presented the same phenotype as seen in SAP $^{-/-}$ T cells. Moreover, the profound defect in Th2 cytokine secretion (IL-4 production) in SAP-deficient T cells appears to be independent of the effects of IFN-γ level. The defect in Th2 response has been shown in Fyn $^{-/-}$ T cells and also in T cells from SAP^{R78A} Knockin mice (a mouse model in which the Fyn T binding site of SAP, R78, was replaced by an alanine in the germ line) suggesting the impaired TCR-mediated signals are SAP-Fyn dependent (Cannons *et al,* 2004; Davidson *et al,* 2004). SAP-deficient CD4 T cells are able to polarize and secrete normal levels of both Th1 and Th2 cytokines (Latour *et al,* 2001; Wu *et al,* 2001; Cannons *et al,* 2004). However, the affected CD4 T cells showed down-regulation of GATA-3 and not T-bet expression level (GATA-3 and T-bet are master regulators of Th2 and Th1 differentiation, respectively). Thus, murine SAP-deficient CD4 T cells have a specific defect in GATA-3 induction in response to TCR-mediated signals.

Furthermore, the recent description of mice lacking SLAM expression revealed that TCR-induced secretion of IFN-γ was only slightly increased by CD4 T cells whereas Th2 response (IL-4 production) was profoundly impaired (Wang *et al,* 2004). It questions the previous reports in mice showing that SLAM is important in

Th1 cytokine regulation with an inhibitory outcome. This along with the recent reports suggests that the triple complex of SAP-Fyn-SLAM is a major regulator in Th2 response and not Th1 response (Cannons *et al,* 2004; Davidson *et al,* 2004). These findings suggest that SLAM is an activator of IL-4 production. This is most perplexing, as CD4 T cells upon anti-SLAM stimulation did not produce IL-4 cytokine. However, an explanation for these models could be co-localization of SLAM with the TCR upon T cell activation (Howie *et al,* 2002).

It is notable that the additional SLAM-related receptors expressed in T cells, including 2B4, Ly-9 and NTBA, are candidates, which should be studied and could be incorporated in this model. It should be pointed out that the recruitment of Fyn to SLAM by SAP has not been shown with other SLAM family receptors. These receptors may play different roles in T cell population function and homeostasis.

According to evidence based on *SAP* deficient murine models, it seems probable that the immunological abnormalities in XLP are caused by a defect in Th2 response (IL-4) rather than Th1 responses. However, although Th2 response defect may explain dysgammagloubinemia phenotype of XLP, this Th2 paradigm is unlikely to address the uncontrolled FIM and late haematopoietic malignancy features of XLP. Firstly, the cytokine IFN-γ plays a central role in resistance of the host to EBV infection via a direct antiviral effect as well as through modulation of the immune system response (Morrison *et al,* 2001). Therefore, considering the essential role of the Th1 response in pathophysiology of EBV infection, defects in Th1 response as seen in XLP patients (Chapter 7) remain unexplained by data from murine studies. Secondly, we have been able to show that the SLAM responses likely to be a late response, and also that its expression in comparison to 2B4, is relatively low on T cell populations (Chapter 6). This contradicts to the idea that the pathophysiology of SAP

defect is predominantly related to SAP-Fyn-SLAM pathway, which assumes that SLAM upregulation is the main receptor in signals regulated by SAP. Future work is needed to investigate how SAP modulates other SLAM family receptors such as CD84/CS1/NTBA/2B4 that are expressed in T cells. Lastly, it is important to note that the defects in antibody production in response to viral infections observed in *SAP* $^{-/-}$ murine model were a late defect (Crotty *et al,* 2003) and not related to B cell function. Therefore, severe Th2 impairment reported in the murine model is unlikely to explain the late defect of B cells function. SAP may have a role at least in some B cell population and homeostasis of Igs production, a role which is under estimated and not effectively addressed by Th2 imbalance theory.

It has been suggested that SLAM family receptors, particularly SLAM, are involved in Th1 cytokine production and not the Th2 response in humans (see Chapter 1). However, the Th2 cytokine profile has not been comprehensively studied in XLP patients particularly in response to various stimuli. As it has been shown in Chapter 7, SLAM and 2B4 may have an inhibitory effect on Th2 cytokine production (IL-5 and IL-10 production) by human primary T cells and this requires further investigation. Also it is important to investigate whether the lack of SAP affects the Th2 cytokine balance in XLP patients and if dysregulation of Th2 cytokine production contributes to XLP pathogenesis in humans.

8.4 CTL function in XLP patients

EBV infection triggers the pathological presentation in 90% of XLP cases. T and NK lymphocyte roles in cytotoxic function and cytokine homeostasis are crucial in the immune response to EBV infection (Rickinson *et al,* 1997). The Th2 response failure theory demonstrated by murine models may not completely explain the

observed defects in these cell populations. The preliminary results reported by Wu *et al* indicated that NK and CTL function in *SAP* $^{-/-}$ mice are normal during acute phase of infection (Wu *et al,* 2001). However, the function of these lymphocyte populations in the murine model has not been investigated in detail.

The results reported in Chapter 7 demonstrate SAP dependent defects in T cells cytotoxicity in XLP patients, where the defect was corrected by *SAP* gene transfer (Sharifi *et al,* 2004). This is consistent with observations made by Sanzone *et al* where reconstitution of allogenic T cell lines from two XLP patients using retroviral vector encoding the *SAP* gene has completely restored abnormal signalling events (Sanzone *et al,* 2003). The impaired EBV- T cell function, including Th1 response defects suggest that these abnomalities may participate in the pathophysiology of the disease. Firstly, this is supported with evidence from XLP patients who are unable to eliminate the EBV virus from the blood and other organs. Secondly, the feature of tumourgenesis in XLP is in favour of T and NK cell surveillance defects in these patients. Thirdly, the data presented on abnormalities of NK cell function in XLP patients (Sullivan *et al,* 1980; Harada *et al*, 1982; Nakajima *et al*, 2000; Parolini *et al*, 2000; Tangye *et al*, 2000) confirmed that the cellular pathogenesis of XLP is not only caused by Th1/Th2 imbalance, but that impaired cytotoxicity of NK cells along with CTLs could play a major role in the clinical manifestations of XLP due to SAP deficiency. Although, *2B4*-deficient mouse NK cells were found to exhibit increased NK cell- mediated cytotoxicity and IFN-γ production (Lee *et al*, 2004), this may highlight the differences in pathology between XLP patients and *SAP*-deficient murine models.

The failure of EBV-specific T cell line cytotoxicity is unlikely to be SLAM dependent, as the cytotoxic T cells were mainly 2B4 positive (Sharifi *et al*, 2004;

Dupre *et al*, 2005). In T cells, triggering of 2B4 does not lead to T cell activation by itself, but it can augment antigen-specific T cell cytotoxicity (Nakajima *et al*, 1999; Lee *et al*, 2003). In addition, the CD48-2B4 interaction is important for the regulation of CD8 T cell proliferation (Kambayashi *et al*, 2001). SAP is essential for 2B4 signalling, probably by preventing SHP-2 and possibly SHP-1 binding to 2B4 and also by recruiting Fyn to 2B4 (Tangye *et al*, 1999; Eissmann *et al*, 2005). However, the mechanism of the cytotoxicity defect of XLP EBV-specific T cell lines in our study remains elusive. A recent study has demonstrated that SAP-deficient CTLs from patients with XLP exhibit a specific lytic defect against autologous and allogeneic EBV-positive B cells; the defect was 2B4 dependent due its role in lytic synapse formation (Dupre *et al*, 2005). It has been suggested that SAP regulates 2B4 activity by controlling lytic synapse formation (Roda-Navaroo *et al*, 2004). Effective lytic activity requires the formation of a lytic immunological synapse with immediate central polarization of perforin-containing lytic granules at the effector T cell: target cell contact (Stinchcombe *et al*, 2001; McGavern *et al*, 2002). The absence of SAP may cause a defect in the assembly of the lytic immunological synapse. Dupre *et al* have been able to show that CTLs from three XLP patients exhibit impaired polarization of both lytic granules and lipid rafts at the contact point of CTLs with target cells (Dupre *et al*, 2005). Interestingly, the defect was restricted to EBV-positive target cells with CD48 expression, whilst the polarization of lytic granules (perforin and GM1) and lytic activity against the K562 targets were not affected in SAP-deficient CTL (Dupre *et al*, 2005). This supports previous studies on NK cells from XLP patients, which show failure of 2B4-mediated cytotoxic killing of CD48-expressing target cells and EBV-immortalized LCLs (Nakajima *et al*, 2000; Parolini *et al*, 2000; Tangye *et al*, 2000).

In most viral infections, specific CTL activity arises within 3-4 days after infection, peaks by 7-10 days, eliminating virions during this period (Ramshaw *et al*, 1997). Since humoral immune response defects may be a late defect in XLP patients, the function of CTL and NK in elimination of EBV infected B cells would be essential in these patients. It has been reported that the rapid elimination of B cells immediately following EBV exposure in two XLP patients using the anti-CD20 antibody rituximab, provided recovery from viral infection symptoms and reduction of EBV circulating viral load in addition to reduction of post EBV infection morbidity such as FIM or lymphoma (Milone *et al*, 2005). Although these patients exhibit an increased numbers of CD8 T cells, the T cell dysfunction has not been investigated directly. However, the effect of eliminating infected B cells using a monoclonal antibody, demonstrates the importance of the CTL and NK cell eliminating function in XLP pathogenesis.

Although there is substantial evidence supporting CTL dysfunction as a crucial role in XLP pathogenesis, the molecular aspects of XLP pathology in these cells remain uncertain, particularly the relationship of SAP to SLAM family receptors expressed in these cells such as 2B4, CS1, NTBA.

8.5 Feasibility of gene therapy for XLP

Although rare, primary immunodeficiencies are in many ways ideally suited to the application of somatic gene therapy. This technology has delivered promising outcomes in treatment of various types of immundeficienies disorders in recent years, mostly targeting haematopoietic stem cells (Bordignon *et al*, 1995; Cavazzana-Calvo *et al*, 2000; Hacein-Bey-Abina *et al*, 2002; Gaspar *et al*, 2004). The lack of success of conventional treatment for XLP makes the development of a gene therapy strategy for

XLP an attractive proposition. The disease is monogenic and the coding sequence of SAP cDNA spans only 400bp and is readily incorporated into retroviral vector constructs. In addition the major cellular deficit lies in T and NK cell populations, which are easily accessible. More recently it has been shown that SAP is a crucial regulator on NKT cell ontogeny in humans and mice (Nichols *et al,* 2005). Therefore, other lymphocyte populations such as NKT and LAK cells may be potential targets for XLP gene therapy.

Advances in the genetic modification of T lymphocytes have led to the development of therapeutic approaches including suicide gene therapy after allogeneic HSCT (Heslop *et al,* 1996; Bonini *et al,* 1997; Qasim *et al,* 2002), and ADA-SCID gene therapy (Blaese *et al,* 1995; Muul *et al,* 2003). It also has been used for rapid selection of antigen-specific T lymphocytes (Koehne *et al,* 2000). However, many issues have been raised such as the impact of T cell gene therapy on their functionality including cytotoxicity (Duarte *et al,* 2002; Sauce *et al,* 2002), T cell subpopulations and T cell receptor repertoire (Dietrich *et al,* 1997; Movassagh *et al,* 2000; Qasim *et al,* 2003). For instance, it has been reported that the transduction protocol using anti-CD3 antibody and IL-2 to activate T cells leads a significant reduction in EBV-reactive T cells (Sauce *et al,* 2002).

In this study we have tried to investigate the feasibility of an in vitro gene therapy approach targeting EBV-specific T cells as a potential treatment for XLP. We have been able to develop a T cell transduction protocol using only Ags (autologous BLCLs) stimulation with reliable efficiency with a retroviral vector encoding SAP (Chapter 4). Although both retroviral and lentiviral vectors showed good transduction efficiency with non-specific stimulation (PHA, IL-2, CD3/28), the transduction efficiency of lentiviral vectors was limited when we used a Ags stimulation protocol

only (Chapter 4). Recent data suggests that lentiviral vectors cannot efficiently transduce primary T cells without prior stimulation, such as IL-7 pre-stimulation, despite their unique ability to transduce different cell types (Verhoeyen *et al,* 2003).

We have been able to demonstrate that the transduction of EBV-specific T cells with retroviral vector constructs using Ags stimulation protocol, maintains the cytotoxicity and IFN-γ production abilities of transduced T cells (Chapter 7). Most importantly we have showed that the in vitro EBV-specific T cell model for XLP gene therapy shows promising results and can reconstitute the cytotoxicity and IFN-γ production in EBV-specific T cell lines (Chapter 7; Sharifi *et al,* 2004). Other groups have also recently shown reconstitution of SAP expression by retroviral vectors encoding SAP led to significant restoration of TCR signalling defects in T cell lines derived from two XLP patients (Sanzone *et al,* 2003). In addition our retroviral vector constructs encoding SAP have been used by Plunkett *et al* in a study on the telomerase activity in primary CD8 T cells of XLP patients and again have shown stable SAP expression (Plunkett *et al,* 2005).

However, the next step to measure the feasibility of the EBV-specific T cell model for XLP gene therapy is to develop an in vivo model. The SCID (RAG-γc) mouse strain model is an ideal model to study the in vivo behaviour of engrafted human tumour cells and primary lymphocytes (Goldman *et al,* 1998). Intraperitoneal inoculation of EBV-LCLs and administration of autologous EBV-specific T cells to this type of mice results in the eradication of EBV driven lymphoproliferative disease (Lacerda *et al,* 1996). Therefore, this model provides a relevant physiological model to assay the ability of SAP reconstituted EBV-specific T cells.

The insertional mutagenesis has been reported subsequently of HSC retroviral transduction in mice and human (Li *et al,* 2002; Hacein-Bey-Abina *et al,* 2003).

Retroviruses integrate essentially at random into the host genome which could induce malignancies as has been reported in mice and rhesus monkeys through repeated infection that cause multiple synergizing mutations in the same cell (Nathwani *et al,* 2004). The tumorgenesis following integration is known to arise by activation of oncogene sequences occuring as a result of promoter/ enhancer insertion, and/ or tumour suppressor gene (TSG) inactivation (Neil *et al,* 1987). However, alteration of gene expression following proviral insertion also has been demonstrated in mice, which can lead to lethal ebmryonic outcomes (Jaenisch *et al,* 1983).

In the French gene therapy trial for SCID-XI, three boys have developed T cell leukaemia (Hacein-Bey-Abina *et al,* 2003). T cells originated from a single transduced cell, in which the oncoretroviral genome inserted in the vicinity of LIM domain only 2 (LMO-2) locus, in a manner that enabled the retroviral promoter to mediate aberrant expression of LMO-2 in these cells. The LMO-2 gene encodes a transcription factor that is required for normal haematopoiesis; however, when aberrantly expressed, it is associated with childhood leukaemia (McCormack *et al,* 2004). However, although insertional mutagenesis is a rare incidence (Kohn *et al,* 2003), with replication –incompetent retrovial vectors, it is not clear whether this is as a consequence of wild type promoter or combination with encoded transgene such as γc.

Thus, the murine model also could provide useful information regarding transgenic mutations in targeted cells and safety issues following introduction of new gene by retroviral vectors. Whether the promoter in retroviral vector affects the chance of insertional mutagenesis is not clear and replacement the SFFV promoter with a tissues specific promoter such as CD28 or CD3 promoter may reduce the chance of mutagenesis.

8.6 Future work

8.6.1 SAP and SLAM & 2B4 expression

SAP and the SLAM family are expressed heterogeneously in immune cells. The results presented in Chapter 6 add further information on SLAM and 2B4 expression in primary immune cell lineages. SAP expression in LAK cells and recently in NKT shown by Nichols *et al* suggest a wider regulatory function for SAP (Chapter 1; Nichols *et al*, 2005). Moreover, there is uncertainty regarding regulation of SAP expression particularly in its promoter region and in signalling cascades both of which require further investigation.

We have been able to study co-expression of SLAM and 2B4 on different immune cell lineages. The expression of 2B4 on DCs and CD4 T cells may suggest a more complex function of this receptor in immune cells rather than controlling cytotoxicity and IFN-γ production and may also point to critical roles in Ag presentation and B cell help. In addition, stimulation studies of T (CD4/8) and NK cells with a variety of stimuli showed 2B4 expression is more dominant than SLAM expression in the early response and SLAM is expressed as a late response upon TCR stimulation with combination of CD28, 2B4 and SLAM (Chapter 6). Although other groups showed that SLAM up regulates upon lectin stimulation or CD3 in combination with high level of IL-2 in CD4 T cells *in vitro*, the co-expression of these receptors and the effect of different methods has not been fully investigated.

8.6.2 Chemokines role in XLP

Chemokines are important in a wide range of physiological functions including, chemotaxis, angiogenesis, haematopoiesis, embryonic development, and metastasis (Gerard *et al,* 2001; Oppermann, 2004). It has been well documented that

they play crucial roles in directing cell movements necessary for the initiation of T cell immune responses (Luther *et al*, 2001). More recently, the chemokines profiles have been investigated in common variable immunodeficiency (CVID) showing a low CCR7 expression profile (Holm *et al,* 2004) and in XLP patients demonstrating reduction of circulating CCR4 T cells (Malbran *et al,* 2004). With the results reported in Chapter 5, we have been able show low CCR5 and RANTES expression profiles in two XLP patients. Whether this phenotype is secondary to Th1/Th2 imbalance profile in XLP patients or is due to direct effect of SAP signalling impairment should be addressed in further studies. The chemokine profile in XLP may also help to classify the XLP disorder as has been suggested in CVID (Holm *et al*, 2004).

8.6.3 New targets for gene therapy in XLP

NK cells are a very attractive target for immune cell and gene therapy. The defect in NK cells derived from XLP patients is well documented. Therefore, SAP reconstitution of NK cells with viral or non-viral vectors may provide an alternative treatment in those patients with FIM or malignant lymphoma clinical phenotypes. However, the transduction of primary NK cells has not been successful so far and developing an appropriate vector and stimulation protocol would be crucial steps towards this application.

Successes in targeting X-linked SCID and ADA deficiency with HSC gene therapy suggest that similar approaches may be effective at least for other forms of primary immunodeficiency (reviewed by Gaspar *et al*, 2003). However, the major consideration in HSC gene therapy in XLP would be heterologous SAP expression in immune cells. SAP reconstitution of HSC using a gene therapy approach in SAP

deficient murine models and also of human HSCs from normal individuals may provide valuable information to investigate efficiency of SAP reconstituted HSC in correction of XLP phenotype, as well as SAP over expression effect on immune cell functions and phenotype. So far the HSC gene therapy model has not been used either in murine or human systems.

8.6.4 Application of new technologies in study of XLP

XLP remains a difficult disease in terms of its molecular and cellular pathogenesis. Although the T cell paradigm suggested by Purtilo still holds true, it may represent an oversimplification. The recent studies into the XLP pathogenesis mainly after developing the *SAP* deficient mice have been beneficial to give a clear understanding of the disease process and to shed some light into the complexity of SAP function in immune system. With the advent of gene microarrays and protein mass spectrometry (proteomics) it becomes possible to examine thousands of gene and proteins involved in a particular disease in comparison with normal controls. Already the proteomics approach (SELDI-Ciphergen Co) has been used in many different areas including haematological disorders (Kummo *et al,* 2004; Hanna *et al,* 2005). These technologies would be great tools to establish genomic and proteomic fingerprints in XLP patients with different clinical phenotypes as well as in different lymphocyte subpopulations. They would produce high output data, which if effectively interpreted will help us in making a platform to develop better diagnostic assays and treatment procedures.

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Appendix

Publications

These enclosed three publications have resulted from this work.

SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease

Reza Sharifi, Joanna C. Sinclair, Kimberly C. Gilmour, Peter D. Arkwright, Christine Kinnon, Adrian J. Thrasher, and H. Bobby Gaspar

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The impact of telomere erosion on memory CD8$^+$ T cells in patients with X-linked lymphoproliferative syndrome

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Review

Table II. Diagnostic criteria for XLP.

