Self-Inactivating Retroviral Vectors for Gene Therapy of X-linked Severe Combined Immunodeficiency

BY

SUSANNAH INES BAILEY

INSTITUTE OF CHILD HEALTH

UNIVERSITY COLLEGE LONDON

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

Declaration

I, Susannah Ines Bailey, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

X-Linked severe combined immunodeficiency (SCID-X1) is caused by mutations in the gene encoding the common cytokine receptor gamma chain, γc, resulting in profound defects in both cellular and humoral immunity. Although allogeneic bone marrow transplantation has proved highly successful, HLA-mismatched procedures are associated with significant morbidity and mortality. This disease is therefore a good candidate for gene therapy and sustained correction of 19 SCID-X1 patients have been reported in two clinical trials. However, the occurrence of severe adverse advents in one trial has highlighted the potential side-effects of retroviral gene transfer and reinforced the need to develop safer gene therapy vectors. A series of self-inactivating (SIN) gammaretroviral and lentiviral vectors for the treatment of SCID-X1 have consequently been developed. To reduce the potential for insertional mutagenesis mediated by the duplicated viral LTR sequences, alternative internal regulatory elements have been incorporated into the vector backbone. These include both endogenous (human elongation factor 1α – EFS) and viral (spleen focus forming virus - SFFV) promoters. *In vitro*, the SIN retroviral vectors were able to regulate γc expression on the cell surface of SCID-X1 cell lines and restore the lymphoid differentiation potential of *Il2rg*$^{-/-}$ haematopoietic progenitor cells. Functional correction of the immunological defect in the SCID-X1 mouse model was also achieved at similar levels for the both the SIN retroviral vectors and the LTR-regulated clinical vector. To further improve upon safety, lentiviral vectors were developed incorporating the endogenous human *IL2RG* promoter to regulate physiological expression of γc. *In vitro* and *in vivo* analysis of the promoter indicated a degree of haematopoietic tissue specificity and restoration of functional γc-receptor complexes was achieved following transduction of a SCID-X1 T cell line with a lentiviral vector incorporating this promoter; however phenotypic correction of the γc-deficient mouse was unsuccessful. These results demonstrate that SIN retroviral vectors for SCID-X1 are effective in restoration of the immune defect in the γc-deficient murine model. The SIN design together with an endogenous (EFS) promoter might provide a potentially less mutagenic but equally effective vector for gene therapy of SCID-X1.

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Abbreviations

α	Alpha
A	Ampoule
AAV	Adeno-associated virus
ADA	Adenosine deaminase
AML	Acute myeloid leukaemia
AMP^R	Ampicillin resistance gene
β	Beta
BM	Bone marrow
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complimentary DNA
CGD	Chronic granulomatous disease
CMV	Cytomegalovirus
CpG	Cytosine and guanine separted by a phosphate
cPPT	Central polypurine tract
Ci	Curie
Con A	Concanavalin A
CO_2	Carbon dioxide
cpm	Counts per minute
δ	Delta
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EF1α	Elongation Factor 1α
EFS	Short form of elongation factor 1α promoter
ELISA	Enzyme-linked immunosorbent assay

Env	Envelope
Eco	Murine ecotropic envelope
FACs	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Flt-3	Murine foetal liver tyrosine kinase 3 ligand
γ	Gamma
γc	Common cytokine receptor gamma chain
Gag	Group specific antigens
GALV	Gibbon ape leukaemia virus
GFP	Green fluorescent protein
Gy	Gray
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human Immunodeficiency Virus-1
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HSV	Herpes simplex virus
IL2RG	Common cytokine receptor gamma chain gene
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
ISO	Isotype control
JAK	Janus-activated kinase
kb	Kilobases
Kd	Dissociation constant
kDa	Kilo Dalton
LB	Luria-Bertani
LCL	Lymphoblastoid cell line
LMO2	LIM domain only 2
Lin^-	Lineage negative
LTR	Long terminal repeat
M	Molar

m	milli (10^{-3})
μ	micro (10^{-6})
MESV	Murine embyonic stem cell virus
MFI	Mean fluorescence intensity
MLV	Moloney murine leukaemia virus
MOI	Multiplicity of infection
MUD	Matched unrelated donor
n	nano
NK	Natural killer
OD	Optical density
Ψ	Packaging signal
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PRE	Post-transcriptional regulatory element
rag	Recombinase activating gene
RCR	Replication-competent retrovirus
RD114	Envelope of the endogenous feline type C virus
RRE	Rev-response element
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RSV	Rous sarcoma virus
S	Serine
Sca-1	Stem cell antigen
SCF	Murine stem cell factor
SCID	Severe combined immunodeficiency
SCID-X1	X-linked severe combined immunodeficiency
SH2	Src homology domain
SIN	Self-inactivating
SFFV	Spleen focus forming virus

STAT	Signal transducer and activator
TAE	Tris-acetate-EDTA
T-ALL	T cell acute lymphoblastic leukaemia
T cell	Thymus derived lymphocyte
TCR	T cell receptor
VSV-G	Vesicular stomatitis virus glycoprotein
v/v	Volume per volume
W	Tryptophan
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wild-type
w/v	weight per volume
VZV	Varicella zoster virus

Chapter One

Introduction

1. X-Linked Severe Combined Immunodeficiency

Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders characterised by a profound block in T cell differentiation. These diseases represent the most severe forms of primary immunodeficiencies, affecting approximately 1 child in every 75,000 live births (Fischer et al., 1997). In the absence of T cell-mediated immunity SCID patients are susceptible to opportunistic infections and fail to thrive; if left untreated these disorders are fatal within the first year of life (Fischer et al., 2005). Despite similar clinical manifestations, the molecular causes resulting in SCID are diverse and can be categorised into four different groups (Fischer et al., 2005): the first results from mutations in the gene encoding adenosine deaminase (ADA) leading to lymphocyte apoptosis due to the accumulation of toxic metabolites. The second group involves mutations in one of three genes (*IL2RG*, *JAK3* and *IL7Rα*) resulting in defective cytokine signalling, whilst defective rearrangement of antigen receptor genes due to mutations in the genes encoding either RAG-1, RAG-2 or ARTEMIS proteins form the third group. The last group of SCIDs result from mutations in the genes encoding CD45, CD3δ or CD3ε leading to defects in pre-TCR/TCR signalling.

X-linked SCID (SCID-X1) is the most common form of SCID accounting for almost half of all cases. SCID-X1 results from mutations in the gene encoding the common cytokine receptor gamma chain, γc, a subunit of the cytokine receptors for interleukins 2, 4, 7, 9, 15 and 21 (Noguchi et al., 1993c; Noguchi et al., 1993b; Russell et al., 1993; Russell et al., 1994; Giri et al., 1994; Asao et al., 2001). The gene encoding the γc protein, *IL2RG*, is located on the X chromosome and is expressed constitutively in all haematolymphoid cells (Takeshita et al., 1992a; Orlic et al., 1997). The absence of γc signalling results in a classical phenotype characterised by the absence of T and Natural Killer (NK) cells, whilst B cells are present but poorly-functional ($T^-B^+NK^-$ SCID) (White et al., 2000).

1.1 Clinical Features of SCID-X1

The clinical presentation of the disease is characterised by early onset of infections, typically of the gut and respiratory tract, correlating with the loss of maternal antibodies and leading to diagnosis at approximately six months of age (Buckley, 2004). Due to mutations in the gene encoding γc, lymphocyte precursors are unable to respond to cytokine signals, leading to failure of T and NK lymphocyte development, whilst B cells are present but defective. Common opportunistic organisms include *Pneumocytis carinii* and *Aspergillus*, as well as viruses such as cytomegalovirus and adenovirus; these pathogens can cause recurrent infections, chronic diarrhoea and failure to thrive. Without recognition and treatment the condition is uniformly fatal for patients with classical SCID-X1, typically during the first year of life.

Histologically, the thymi from SCID-X1 patients resemble human fetal thymi at six to seven weeks gestation, prior to colonisation with T cell precursors. These thymi are markedly hypoplastic and lack cortex/medullar differentatiation; Hassall's bodies are also undetectable (Hale et al., 2004). Peripheral lymphoid organs in these patients are also found to be hypoplastic (Fischer et al., 1997).

1.2 The Molecular Pathology of SCID-X1

1.2.1 SCID-X1 is Caused by Mutations in *IL2RG*

The *IL2RG* gene, encoding the γc protein, was localised to the SCID-X1 locus on chromosome Xq13 through genetic linkage analysis (Noguchi et al., 1993c). Analysis of three unrelated SCID-X1 patients established association between mutations in this gene with development of the disease. Several studies have since analysed *IL2RG* mutations from numerous SCID-X1 males and their relatives (Clark et al., 1995; Hacein-Bey et al., 1996; Puck et al., 1997; Kumaki et al., 2000), and sequencing of this gene has been established as a diagnostic procedure for human SCID-X1.

IL2RG mutations have been identified in all eight exons and include point mutations, splice mutations, insertions and deletions (including deletions of complete exons and one deletion of the entire gene) (Clark et al., 1995; Hacein-Bey et al., 1996; Puck et al., 1997; Kumaki et al., 2000). These mutations abrogate γc function through destabilisation of *IL2RG* mRNA, disruption of functional motifs such as the WSXWS motif common to all members of the cytokine receptor superfamily, changes in protein conformation, premature protein truncation or alteration of the cytoplasmic portion of γc critical for signal transduction.

Up to January 2007, 198 unique mutations in the *IL2RG* gene resulting in SCID-X1 have been reported in the X-linked SCID mutation database (http://genome.nhgri.nih.gov/scid) with the highest proportion of cases resulting from single base-pair substitutions (figure 1.1). Although recurrent mutations have been observed, the majority of *IL2RG* mutations appear to be unique to one patient or family (Puck, 1996; Puck et al., 1997). Mutations in *IL2RG* are unevenly distributed, with five mutational 'hot spots' identified. Over a quarter of all mutations occur in exon 5, which contains the aforementioned WSXWS motif, upstream of which lie six CpG dinucleotides representing the most prominent mutational hot spot in *IL2RG* (Puck, 1996). Additional mutational hot spots in regions containing CpG dinucleotides are also found in exons 6 and 7.

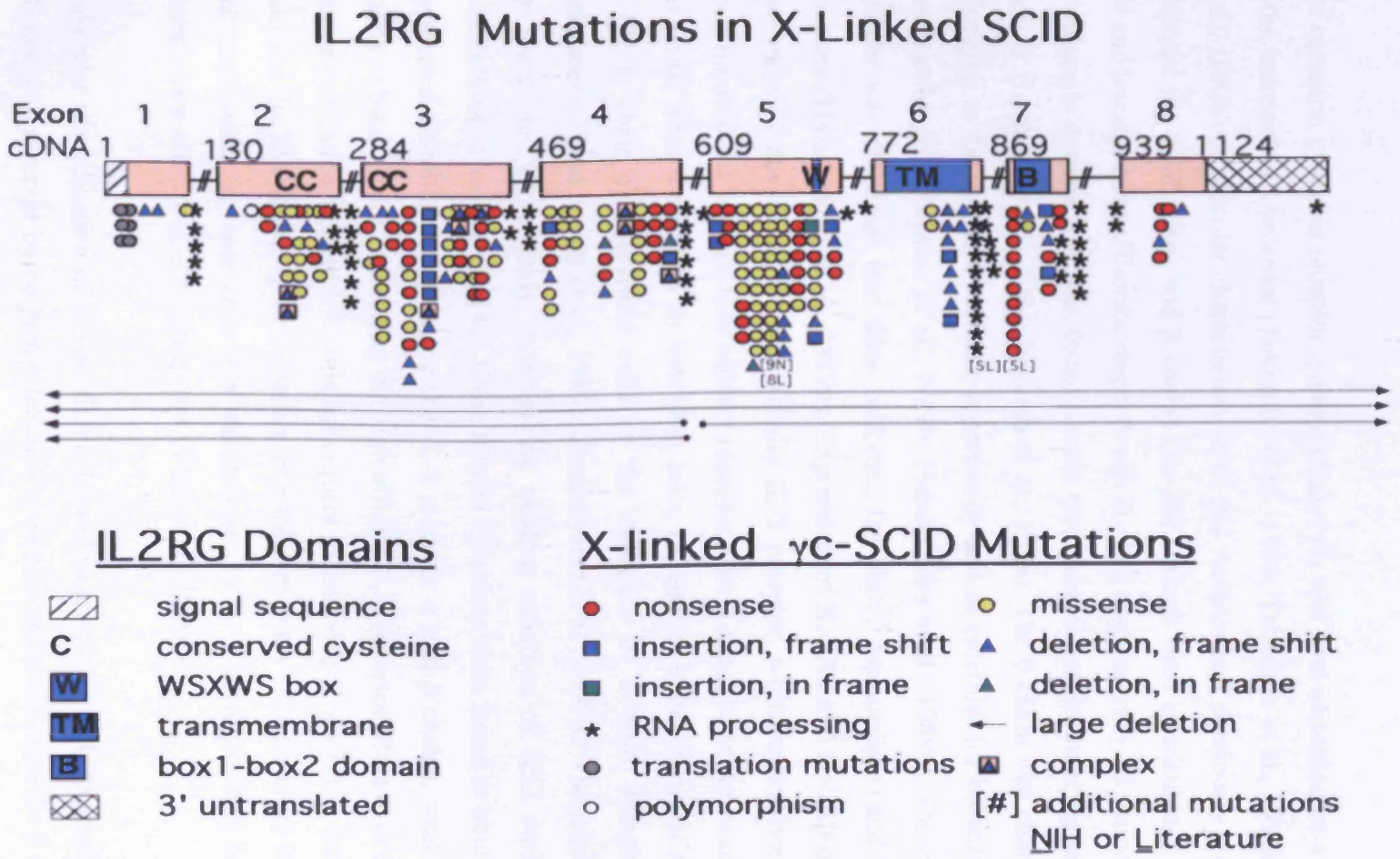

Figure 1.1. *IL2RG* Mutations in SCID-X1. (Figure from the X-Linked SCID Mutation Database, National Human Genome Research Institute, National Institutes of Health)

1.2.2 The Common Cytokine Receptor Gamma Chain – γc

The common cytokine receptor gamma chain (γc) was first identified as a component of the interleukin 2 receptor (Takeshita et al., 1990; Takeshita et al., 1992b; Takeshita et al., 1992a). Molecular characterisation of this receptor had previously identified two subunits; the α (IL-2Rα) and β chains (IL-2Rβ) which bind interleukin 2 (IL-2) with low and intermediate affinities, respectively. IL-2Rα contains only 13 amino acids in its cytoplasmic domain and was found unable to transduce intracellular signals mediated by IL-2 (Leonard et al., 1984; Nikaido et al., 1984). The β chain was characterised as belonging to the cytokine receptor superfamily and as essential for intracellular signal transduction (Hatakeyama et al., 1989b; Hatakeyama et al., 1989a). The interleukin 2 receptor was classified into three isoforms, the high-, intermediate- and low-affinity receptors (Hatakeyama et al., 1989b). Expression of IL-2Rα and IL-2Rβ on lymphoid lines induced the functional high-affinity IL-2 receptor, whilst expression of IL-2Rβ alone induced the intermediate affinity receptor. The β chain however, was found non-functional when expressed on fibroblast cells, suggesting either essential modification of the β chain in lymphoid cells or the existence of a third, lymphoid-specific component (Hatakeyama et al., 1989b; Hatakeyama et al., 1989a). The latter hypothesis was supported by a study investigating binding affinities of IL-2 molecules with substitutions at residue Gln141. These mutant cytokines were found to bind normally to transfected fibroblasts expressing the IL-2 receptor α and β chains, whilst they were unable to bind T cells expressing the high affinity IL-2 receptor (Zurawski et al., 1990). Analysis of cancer patient NK cells subsequent to receiving *in vivo* IL-2 therapy further endorsed the likelihood of the existence of a third receptor subunit, since increased IL-2Rβ expression on these cells was unaccompanied by a corresponding high level of intermediate affinity IL-2 binding sites (Voss et al., 1990).

Molecular identification of γc was first achieved in lymphoid cells expressing the high affinity IL-2 receptor by co-precipitation of a 64 kDa molecule, with the β chain, in the presence of IL-2 (Takeshita et al., 1990; Takeshita et al., 1992b). Cloning and characterisation of γc revealed a protein consisting of 347 amino acid residues, with sequences typical of the cytokine receptor superfamily proteins, such as IL-2Rβ. Both

the β and γ chains contain two pairs of conserved cysteines located within neighbouring exons, and the conserved WSXWS motif in their extracellular domains (see figure 1.2) (Noguchi et al., 1993a). The cytoplasmic domain of γc was determined to be considerably shorter than that of IL-2Rβ, however the presence of two subdomains of the Src homology region 2 (SH2), which can bind phosphotyrosine residues of some phosphoproteins, suggested a putative role in signal transduction.

1.2.3 The IL-2 Receptor System

IL-2 is a T cell-derived cytokine which, through binding to the IL-2 receptor, plays a pivotal role in immune responses by inducing lymphocyte proliferation and activation (Smith, 1989). The identification of all three receptor subunits allowed definitive characterisation of the IL-2 receptor complexes (figure 1.2). Combinations of expression plasmids for IL-2Rα, IL-2Rβ and γc were stably transfected into a fibroblast cell line and the resultant clones analysed for binding of IL-2 (Takeshita et al., 1992a). Expression of the α subunit alone resulted in the low-affinity receptor (Kd = 10^{-8} M), previously characterised as unable to transduce IL-2 mediated signals (Greene et al., 1985); whilst the β or γ chains alone were unable to bind IL-2 molecules. The intermediate-affinity receptor (Kd = 10^{-9} M), constitutively expressed on NK cells and a subset of resting lymphocytes (Siegel et al., 1987; Tsudo et al., 1987), was found to consist of IL-2Rβ and γc, whereas the high-affinity receptor (Kd = 10^{-11} M) is formed by simultaneous expression of all three subunits (Leonard, 1996). Both the intermediate- and high-affinity IL-2 receptors are capable of transducing IL-2 signals.

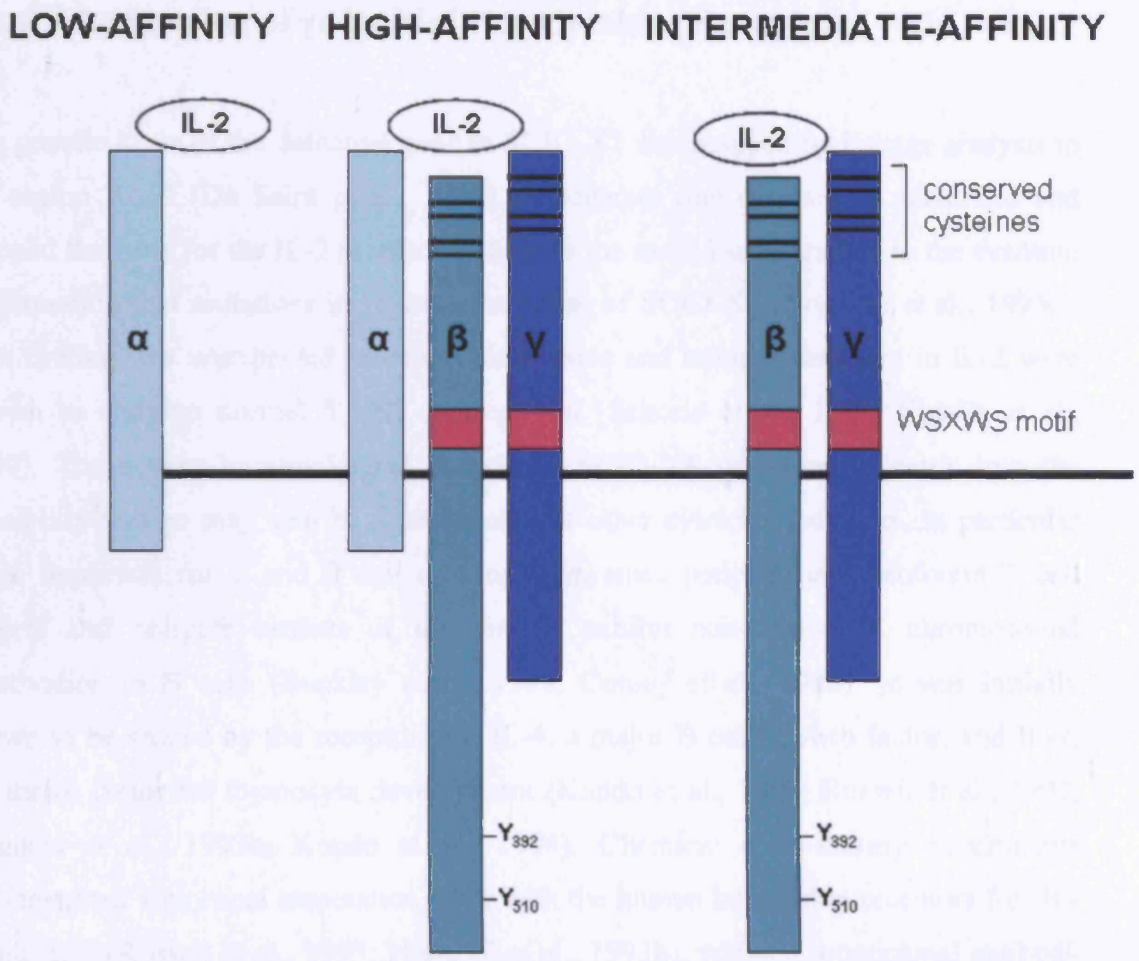

Figure 1.2. Schematic of the IL-2 receptor isoforms. The three isofoms of the IL-2 receptor: the low-affinity receptor (composed of the α chain), the high-affinity receptor (composed of the α, β and γ chains) and the intermediate-affinity receptor (composed of the β and γ chains). Only the high- and intermediate-affinity receptors are able to transduce IL-2 signals. The two pairs of conserved cysteine residues and WSXWS motif, both typical of the cytokine receptor superfamily, are illustrated for the β and γ chains. The locations of tyrosines (Y392 and Y510) required for docking and activation of Signal transducer and activator (STAT) proteins on the cytoplasmic domain of IL-2Rβ are indicated. (Figure adapted from Leonard, W.J. *Annu. Rev. Med.* **47**:229-39 (1996))

1.2.4 The Sharing of γc by Multiple Cytokine Receptors

The genetic locus of the defective gene in SCID-X1 was mapped by linkage analysis to the region Xq13 (De Saint et al., 1987). A separate line of research identified and mapped the gene for the IL-2 receptor γ chain to the same locus, leading to the eventual confirmation that mutations in γc were the cause of SCID-X1 (Noguchi et al., 1993c). This finding was unexpected however, since mice and humans deficient in IL-2 were known to undergo normal T cell development (Schorle et al., 1991; Chatila et al., 1990). The severe immunological defects in SCID-X1 prompted research into the possibility that γc may also be a component of other cytokine receptors, in particular those important for T and B cell development, since patients have profound T cell defects and obligate carriers of the disease exhibit non-random X chromosomal inactivation in B cells (Buckley et al., 1986; Conley et al., 1988). γc was initially shown to be shared by the receptors for IL-4, a major B cell growth factor, and IL-7, the major factor for thymocyte development (Kondo et al., 1993; Russell et al., 1993; Noguchi et al., 1993b; Kondo et al., 1994). Chemical cross-linking experiments demonstrated a physical association of γc with the human interleukin receptors for IL-4 and IL-7 (Russell et al., 1993; Noguchi et al., 1993b), whilst a monoclonal antibody specific for γc was found to inhibit IL-4 induced growth of murine lymphoid cell lines (Kondo et al., 1993). Subsequent research also identified γc as a critical component of the receptors for IL-9, IL-15 and IL-21, enabling further understanding of the molecular basis of the severe SCID-X1 phenotype (Russell et al., 1994; Giri et al., 1994; Asao et al., 2001).

1.2.5 The Biology of the γc-Dependent Cytokines - Causative Relationship between defective γc and SCID-X1

IL-2 was initially described as a potent T cell growth factor *in vitro*, however as previously discussed, lymphoid development is essentially normal in IL-2 deficient mice and humans (Schorle et al., 1991; Chatila et al., 1990). Ageing mice deficient in IL-2 develop autoimmune systems such as haemolytic anaemia and inflammatory

bowel disease (Sadlack et al., 1993; Sadlack et al., 1995), and recent research has indicated that the main, non-redundant role for IL-2 is in the production of regulatory T cells (T_{reg}) and maintenance of peripheral tolerance (Malek and Bayer, 2004). Consequently, the lack of T cell development in SCID-X1 seemed likely due to aberrant IL-7 signalling, since mice deficient in IL-7 or the IL-7 receptor exhibit defective T and B cell development (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). This was further supported by the identification of SCID patients with defective IL-7Rα expression, exhibiting a $T^-B^+NK^+$ phenotype (Puel et al., 1998).

The block in B lymphoid development observed in IL-7 or IL-7Rα deficient mice indicates a disparity between the crucial role of this cytokine in mice and humans with regards to B cells. SCID-X1 patients have normal or elevated numbers of B lymphocytes; these cells are however non-functional, resulting in low immunoglobulin levels. Whilst this may be partially explained by a lack of T cell help, an intrinsic defect is indicated by non-random X chromosome inactivation in terminally differentiated B cells in SCID-X1 carrier females (Conley et al., 1988). Furthermore, a significant number of patients continue to require immunoglobulin therapy, post-bone marrow transplant, after reconstitution of their T cell compartments (Buckley et al., 1999). IL-4 is an important cytokine for mature B cell function, essential for Ig class switching to IgG1 and IgE (Paul, 1991). Similarly, the most recently identified γc-dependent cytokine, IL-21, is also implicated in B cell function; mice lacking the IL-21 receptor were found to have impaired IgG1 production and elevated levels of IgE (Ozaki et al., 2002). Double knockout *IL-4*$^{-/-}$ *IL-21R*$^{-/-}$ mice exhibit a phenotype which closely resembles that of B cells from SCID-X1 patients, leading to the conclusion that this defect might result from the combined loss of IL-4 and IL-21 mediated signalling (Ozaki et al., 2002).

The absence of NK cell development in SCID-X1 is attributed to defective IL-15 signalling. IL-15 was first identified as a T cell growth factor with similar biological properties to IL-2, owing to the shared use of IL-2Rβ and γc receptor subunits. Treatment of mice with an antibody to IL2Rβ resulted in the selective long-term elimination of NK cells, a defect not seen in IL-2 deficient animals, implicating IL-15 in the development and/or differentiation of these cells (Tanaka et al., 1993). An obligate role of IL-15 in NK cell development was confirmed following observations

that this lymphoid lineage is completely absent in IL-15 and IL-15Rα deficient mice (Kennedy et al., 2000; Lodolce et al., 1998). Analysis of these mice also revealed a role for IL-15 in homeostasis of naïve and memory $CD8^+$ T cell populations.

The exact contribution of defective IL-9 signalling in the development of SCID-X1 remains unclear. IL-9 is active on a variety of haematopoietic cell types, including T lymphocytes, and in particular is a potent mast cell growth factor (Hultner et al., 1990; Townsend et al., 2000). Mice deficient in this cytokine undergo normal T cell development, however they exhibit excessive mucus production and mast cell proliferation, abnormalities not observed in patients with SCID-X1 (Townsend et al., 2000). Dysregulation of IL-9 has also been implicated in the development of cancers; mice over-expressing IL-9 develop thymic lymphomas, underlining the ability of thymocytes to respond to this cytokine (Renauld et al., 1994; Lauder et al., 2004). The creation of double-knockout mice deficient in IL-9 and a second γc-dependent cytokine might elucidate a redundant role for this cytokine in T cell development.

1.2.6 γc-Dependent Cytokine Signalling

As discussed above, signals mediated by the γc-dependent cytokines and their receptors are critical in the development and regulation of the haematopoietic system. There are several major signalling pathways implicated to be activated by these cytokines, one of the most important being the Janus-activated kinase (JAK)/ Signal transducer and activator of transcription (STAT) pathway.

Cytokine receptor molecules lack intrinsic kinase activity and therefore signal via the JAK family of cytoplasmic tyrosine kinases. Four different JAK kinases have been identified – JAK1, JAK2, JAK3 and TYK2, they constitutively associate with cytokine receptors and all, except JAK3, are ubiquitously expressed (Leonard and O'Shea, 1998). JAKs associate with cytokine receptors through binding to regions termed Box1 and Box2, located in the membrane proximal cytoplasmic region (Murakami et al., 1991). Deleting these domains abolishes cytokine signalling and mutations within this region in human *IL2RG* result in SCID-X1 (Miyazaki et al., 1994). The cytokine-

specific receptor subunits (such as IL-2Rβ and IL-4Rα) associate with JAK1, whilst γc associates with JAK3, an inducible kinase whose expression is restricted to haematopoietic cells (Miyazaki et al., 1994). The discovery of the specific association between γc and JAK3 prompted the hypothesis that mutations in the gene encoding this kinase would result in immunodeficiency analogous to SCID-X1. Patients with SCID due to mutations in *JAK3* were subsequently identified and approximately 7% of all SCID cases result from JAK3 deficiency (Buckley, 2004).

Seven mammalian STAT proteins have been identified - STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. They act as cytoplasmic latent transcription factors and serve as substrates for phosphorylation by JAK proteins (Leonard and O'Shea, 1998). In particular STAT5a and STAT5b are postulated to play an important role in T lymphocyte proliferation, and these proteins are activated by all of the γc-dependent cytokines except IL-4 (see table 1.1) (Yao et al., 2006).

The JAK/STAT pathway is one of the most rapid cytoplasm-to-nucleus signalling mechanisms (figure 1.3) (Puel et al., 1998). Cytokine binding on the cell surface brings together receptor subunits and their associated JAKs, leading to JAK activation and phosphorylation of key tyrosine residues in the receptor-specific chains. The phosphorylated tyrosines serve as docking sites for STAT proteins, which bind via SH2 domains in their C-termini, and are in turn phosphorylated at a conserved tyrosine leading to homo- or heterodimerisation. Dimerised STAT molecules translocate to the nucleus where they regulate transcription of target genes, either through direct interaction with semi-palindromic motifs (known as GAS motifs), or in combination with other transcriptional co-activators (Imada and Leonard, 2000).

Other signalling pathways known to be activated by the γc-dependent cytokines are the phosphoinositide 3-kinase (PI3K)/Akt pathway, implicated in IL-2, IL-4 and IL-7-mediated signalling, and the RAS-mitogen-activated protein kinase (MAPK) pathway (Kovanen and Leonard, 2004).

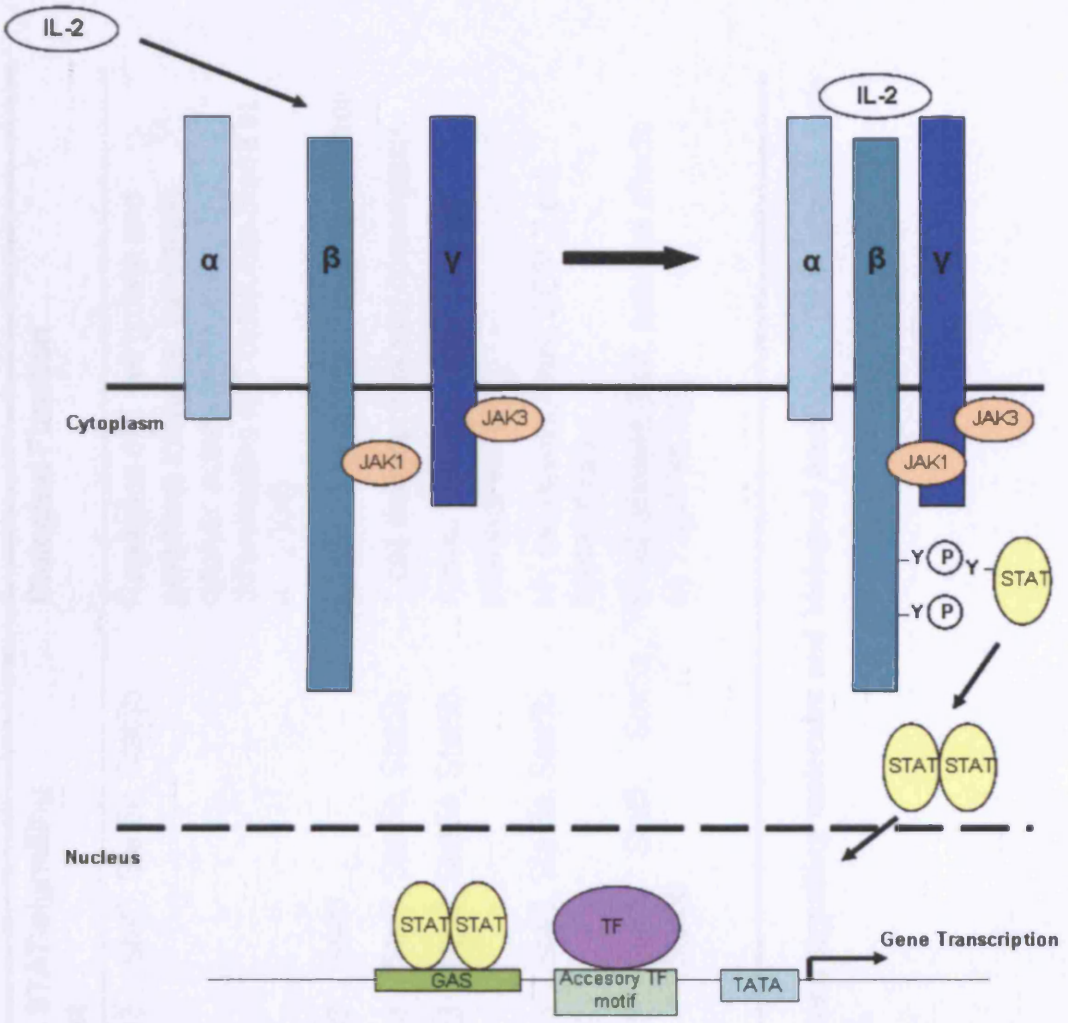

Figure 1.3. IL-2 signalling via the JAK/STAT pathway. Binding of IL-2 induces the high-affinity IL-2 receptor, leading to activation of JAK1 and JAK3. The activated JAK molecules phosphorylate key tyrosine residues on the cytoplasmic region of IL-2Rβ allowing docking of STAT proteins. The recruited STAT proteins are in turn phosphorylated enabling dimerisation, translocation to the nucleus and DNA-binding. STAT proteins may initiate transcription in the presence of accessory transcription factors (TF). The TATA box enables binding of the basal transcription machinery. (JAK – janus-activated kinase; STAT – signal transducer and activator of transcription; GAS – γ-interferon activated sequences). (Figure adapted from Leonard, W.J. *Nat. Rev. Immunol.* **1**:200-208 (2001))

Cytokine	Receptor	Jak- and STAT-signalling molecules		Biological Function
IL-2	IL-2Rα, IL2-Rβ, γc	Jak1, Jak3	Stat3, Stat5a, Stat5b	Regulation of T cell growth and peripheral tolerance; increasing cytolytic activity of NK cells; Th2 differentiation of T cells(Cote-Sierra et al., 2004)
IL-4	IL-4Rα, γc	Jak1, Jak3	Stat6	B cell differentiation, Th2 differentiation
IL-7	IL-7Rα, γc	Jak1, Jak3	Stat3, Stat5a, Stat5b	T cell development and homeostasis
IL-9	IL-9Rα, γc	Jak1, Jak3	Stat3, Stat5a, Stat5b	Airway mucus production, mast cell proliferation
IL-15	IL-15Rα, IL2Rβ, γc	Jak1, Jak3	Stat3, Stat5a, Stat5b	NK cell development, $CD8^+$ T cell homeostasis
IL-21	IL-21Rα, γc	Jak1, Jak3	Stat1, Stat3, Stat5a, Stat5b	B cell differentiation, potential effects on T and NK cells

Table 1.1.The γc-dependent cytokines - their receptors, signalling molecules and biological functions. (Table adapted from Leonard, W.J. *Immunol. Rev.* **202**:67-83 (2004))

1.3 Conventional Treatment of SCID-X1 - Haematopoietic Stem Cell Transplantation

In 1968 the first successful allogeneic bone marrow transplantation for SCID-X1 was reported (Gatti et al., 1968). Many patients with SCID-X1 have since been treated by haematopoietic stem cell transplantation (HSCT) with long-term survival rates of greater than 90% for transplants from genotypically-matched family donors (Cavazzana-Calvo et al., 2005; Antoine et al., 2003). This high success rate is due in part to the lack of T and NK cells in patients with SCID-X1, allowing engraftment of donor cells in the absence of myelosuppressive conditioning. The majority of patients however lack an HLA-identical related donor and consequently haploidentical HSCT or transplants from matched unrelated donors (MUD) are their only alternative. Similar mean three-year survival rates within a cohort of patients receiving either MUD or genotypically-matched HLA-identical transplants have been achieved, however the survival rates following haploidentical HSCT have been lower (Antoine et al., 2003). These transplants have been performed since 1981, when techniques to deplete human marrow of T cells became available (Reisner et al., 1983). The overall success rate for haploidentical transplants is lower than that for HLA-matched procedures, with mean three-year survival rates of approximately 75% (Haddad et al., 1998; Buckley et al., 1999; Antoine et al., 2003). Recipients of haploidentical transplants are susceptible to infections during the time required for mature, functional T cells to develop (approximately three to four months) and mortality is also associated with toxicity of the conditioning regime used to enhance engraftment of donor stem cells.

One frequent limitation of HSCT is persistence of B cell deficiency. A significant proportion of patients fail to develop donor-derived B lymphocytes and hence continue to require immunoglobulin (Ig) replacement therapy post-transplant (Haddad et al., 1998; Buckley et al., 1999).

1.4 Gene Therapy

Gene therapy aims to treat a disease through restoring, modifying or replacing a defective gene. Translation of this technology to success in the clinic was initially disappointing, with many clinical gene therapy trials conducted throughout the 1990s with only limited success (Scollay, 2001); major obstacles being inadequate gene delivery systems, poor transgene expression and induction of an immune response to the gene-modified cells or vector (Nathwani et al., 2005).

The first approved clinical gene therapy trial was initiated in 1990 to treat adenosine deaminase deficiency (ADA), a form of SCID resulting from a defective enzyme critical in the salvage pathway for purine biosynthesis (Anderson, 1992). Clinical improvement was observed in patients following intravenous infusion of gene-corrected autologous T lymphocytes, however, due to only transient transgene expression, regular infusions of gene-modified cells continue to be required. A major setback to the field occurred in 1999 following the death of a patient suffering from partial ornithine transcarbamylase deficiency (OTC) enrolled in a pilot safety gene therapy study. The patient suffered a fatal inflammatory response following systemic delivery of an adenoviral vector to the liver; representing the first death directly attributable to gene therapy (Raper et al., 2003).

Since 1989 over eleven hundred gene therapy clinical trials have been initiated worldwide (Journal of Gene Medicine clinical trials database), with therapies being developed to treat many diseases including haemophilia, cardiovascular disease, cystic fibrosis and cancer (Young et al., 2006). Almost 70% of all gene therapy trials target a form of cancer and in 2004, the use of an adenoviral vector was licensed in China as a routine treatment for head and neck squamous cell carcinoma (Peng, 2005).

The morbidity and mortality associated with haploidentical bone marrow transplants together with the high incidence of failure of donor B cell engraftment created a need for an alternative treatment for SCID-X1 patients for whom no genotypically-matched family donor is available, and led to the application of gene therapy for this disease. The SCID-X1 clinical trials (detailed below) represent the first major success achieved

using this form of treatment and highlight its potential as a long-term or even permanent cure of a hereditary disease. Similarly encouraging results have since also been achieved in gene therapy clinical trials to treat adenosine deaminase deficient-SCID and chronic granulomatous disease (CGD) (Aiuti et al., 2002; Gaspar et al., 2006; Ott et al., 2006).

1.5 Gene Therapy for SCID-X1

Several features of SCID-X1 make the disease an ideal candidate for gene therapy. Firstly, it is a monogenic disorder and well-characterised at the molecular level. Secondly, correction of the gene-defect in haematopoietic precursors should confer a strong growth and survival advantage which obviates the need for pre-conditioning. This concept is supported by partial immunological reconstitution observed in a patient following a spontaneous mutation reversion in a single T lymphocyte precursor (Stephan et al., 1996), demonstrating that the correction of even a small number of lymphoid precursors may confer significant therapeutic benefit. Furthermore, an *in vivo* competitive study between normal and γc-deficient bone marrow cells in mice provides evidence of selective repopulation of lymphoid lineages from normal γc-expressing bone marrow cells (Otsu et al., 2000b).

Efficacy of gene therapy for SCID-X1 has been demonstrated both *in vitro* and *in vivo*. Using retroviral-mediated gene transfer, γc expression and function has been restored in patient B cell lines (Hacein-Bey et al., 1996). Gene-corrected SCID-X1 cord blood $CD34^+$ cells and haematopoietic stem cells were also demonstrated to differentiate into NK and T lymphocytes respectively in appropriate culture conditions (Cavazzana-Calvo et al., 1996; Hacein-Bey et al., 1998). Reconstitution of γc-deficient mice with gene-modified haematopoietic stem cells verified the feasibility of *in vivo* correction of the immunodeficiency. Three groups independently reconstituted T, B and NK cell lineages in these animals; no indication of toxic effects were observed and the corrected phenotype was stable for up to 11 months (Soudais et al., 2000; Lo et al., 1999; Otsu et al., 2000a).

1.5.1 French Gene Therapy Trial

The first clinical gene therapy trial for SCID-X1 was initiated at the Necker Hospital in Paris (Cavazzana-Calvo et al., 2000; Cavazzana-Calvo et al., 2005). Ten SCID-X1 infants under the age of one were enrolled between March 1999 and May 2002, under the criteria that they lacked an HLA-identical donor. The protocol involved the *ex vivo* transduction of patient's $CD34^+$-selected bone marrow cells. These cells were first pre-stimulated with cytokines to induce proliferation, followed by three one-day cycles of transduction with an amphotrophic gammaretroviral vector. After completion of the four-day procedure, the autologous cells were infused back into the patient intravenously in the absence of myeloablative conditioning (Cavazzana-Calvo et al., 2000). In all but one of the patients, infusion of γc-corrected $CD34^+$ cells resulted in the emergence of T and NK lymphocytes (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002; Cavazzana-Calvo et al., 2005). Mature T cells were detectable in the periphery at ten to twelve weeks post gene therapy, and T cell counts reached normal levels within three months in seven of the patients. The remaining two patients received fewer transduced $CD34^+$ cells per kilogram of body weight than the other participants in the trial and were found to have undergone only partial T cell reconstitution.

It was demonstrated that the reconstituted T cells were polyclonal and elicited antigen-specific proliferative responses *in vitro* following patient immunisation. Clearance of varicella zoster virus (VZV) infection in several patients established T cell functionality *in vivo*. Sustained active thymopoeisis is indicated by the persistence of $CD45RA^+$ naïve T cells and detectable TCR excision circles, 3-5 years post-gene therapy. Ultrasonograms also revealed the development of normal-sized thymi in these patients. Restored NK cell populations in the patients were found to be low in number but were nevertheless functional. Similarly only a low proportion of γc-expressing B cells were detected, however enough immunoglobulin was produced to negate the requirement of Ig therapy in the majority of patients.

Retroviral integration site analysis revealed common insertion sites shared by T and B lymphocytes as well as mature myeloid cells, confirming γc gene-correction of multi-potent progenitor cells (Schmidt et al., 2005). Insertion sites characterised in

differentiated leukocytes were also found in $CD34^+$ cells obtained 8 months later, demonstrating the initial transduction of primitive progenitor cells with self-renewal capacity. These findings are promising with regards to long-term restoration of T cell immunity following retroviral-mediated gene therapy in these patients.

The patient for whom gene therapy was unsuccessful underwent a splenectomy four months post-treatment, due to an enlarged spleen caused by a disseminated Bacille Calmette-Guerain infection (Hacein-Bey-Abina et al., 2002). Analysis of the removed organ revealed it to be likely that transduced cells accumulated in the spleen, thus impairing T cell differentiation in this patient (Fischer et al., 2005). Eight months post-gene therapy the patient was given a bone marrow transplant from an unrelated donor which restored partial T cell immunity.

Overall gene therapy was concluded to be beneficial to most patients, enabling them to live normal lives and cope with environmental pathogens as a result of a restored, functioning immune system. Given the problems related with haploidentical transplantation and the associated failure of B cell immunity post-HSCT, treatment of SCID-X1 patients who lack a suitable genotypically-matched HLA-identical donor by gene therapy presents an effective, alternative treatment with superior immune reconstitution.

1.5.2 British Gene Therapy Trial

The second clinical gene therapy trial for SCID-X1 was initiated at Great Ormond Street Hospital in 2001. Four children with SCID-X1 without an HLA-identical sibling were initially enrolled in the study between July 2001 and December 2002. A similar protocol was used to that in the French trial; however the gammaretroviral vector was pseudotyped with a different envelope, that of the gibbon ape leukaemia virus (GALV) whose receptor is more highly expressed on human HSCs than that for the amphotropic envelope (von et al., 1994), and slightly different culture conditions were used (serum-free media, no protamine sulphate and lower concentrations of IL-3) (Gaspar et al., 2004).

Restoration of cellular and humoral immunity was achieved in all four treated patients; two patients had achieved normal T lymphocyte counts at 29 and 23 months follow-up respectively, with the third patient achieving only partial T cell reconstitution at 24 months post-gene therapy. The fourth patient was significantly older than the other patients enlisted in the trial (33 months old at time of treatment); this patient received the lowest dose of cells per kilogram and showed slower kinetics of lymphocyte recovery than the first three treated patients. Reconstituted T lymphocytes had a polyclonal repertoire and exhibited normal proliferative responses to mitogens. As was observed in the French trial, numbers of reconstituted NK lymphocytes remained low. At time of publication two of the patients had discontinued immunoglobulin replacement therapy; however detection of $CD27^+$ antigen-expressing B cells in all four patients indicated immunological B-cell recovery. Integration site analysis revealed polyclonal contribution to the restored T lymphocyte populations and initial gene transfer into multi-potent $CD34^+$ progenitor cells was indicated by persistence of gene-marked granulocyte populations (Gaspar et al., 2004).

All four patients have returned to normal social environments and the clearance of viral or opportunistic pathogens has demonstrated the therapeutic benefits of the gene therapy procedure (Gaspar et al., 2004).

In January 2003 the clinical gene therapy trial for SCID-X1 at Great Ormond Street Hospital was extended to include a further six patients under the identical criteria. Following gene therapy, all are alive and well and currently living at home (Thrasher, A.J. unpublished data). All patients enrolled in the trial have reached lymphocyte levels at normal ranges as compared to age-matched children and six patients have discontinued prophylaxis. These results suggest improved reconstitution and lower levels of morbidity and mortality than with haploidentical bone marrow transplantation.

1.5.3 Gene Therapy of a SCID-X1 Infant in Australia

In March 2002 an infant with SCID-X1 was treated by gene therapy at The Children's Hospital at Westmead, Sydney (Ginn et al., 2005). The nine month old infant had a less

common variant of SCID-X1 exhibiting an NK^+ phenotype, the result of a splice-site mutation within *IL2RG*, and no HLA-identical sibling donor available.

The patient was treated according to the protocol followed in the French trial and initially underwent partial immunological reconstitution with the appearance of T lymphocytes; however normal levels of T cells were never reached. T cell responsiveness to mitogens remained impaired and the patient continued to require intravenous immunoglobulin. The patient received a relatively low dose of $CD34^+$ cells per kilogram, similar to the French patients who also underwent partial T cell reconstitution. γc remained undetectable on the cell surface of the patient's B cells and only appeared on NK cells at 19 months post-gene therapy. It is hypothesised that the patients NK^+ phenotype may have reduced the selective growth advantage of gene-corrected haematopoietic precursors in the bone marrow (Ginn et al., 2005).

Initially clinical benefit was observed following the appearance of T lymphocytes with the clearance of a chronic rotavirus infection and weight gain. However the patient's clinical condition deteriorated four months after treatment with the development of neurological complications, severe persistent diarrhoea and failure to thrive; a possible cause of which was immune dysregulation. The patient received a bone marrow transplant from a matched-unrelated donor at 26 months post-gene therapy.

1.5.4 Failure of Gene Therapy for SCID-X1 in Older Patients

The results of a clinical trial to treat older SCID-X1 patients with gene therapy were published in 2005 (Thrasher et al., 2005). Two male patients were enrolled in the trial conducted at Great Ormond Street Hospital in London and the Necker Hospital in Paris. The first was a twenty year old who had received an HLA-identical paternal bone marrow transplant at three weeks of age, but whose immune status had deteriorated with time resulting in low T and B cell numbers and an absence of NK cells. The second patient was fifteen years of age at the time of treatment and had an incomplete SCID-X1 phenotype with normal T, B and NK cell counts but severe $CD4^+$

lymphopenia. Both patients were enlisted in the trial under the criterion that no genoidentical or HLA-matched donors were available for HSCT.

Gene therapy was unsuccessful for both patients; whilst transduction rates of $CD34^+$ haematopoietic progenitor cells were comparable to those obtained for the successfully treated younger patients, no significant changes in immunity or clinical status were observed in either patient and patient two died of respiratory failure one and a half years post-gene therapy (Thrasher et al., 2005). These data indicate a potential time-dependency on the capacity to re-initiate thymopoiesis, the absence of which results in disorganisation of the thymic architecture and hypoplasia (Hale et al., 2004). This hypothesis is supported by observations that allogeneic HSCT performed in neonatal SCID patients results in superior thymic output as compared to patients receiving transplants later in life (Myers et al., 2002).

1.5.5 Severe Adverse Events in the French SCID-X1 Gene Therapy Trial

In October 2002, it was reported that one of the patients in the French SCID-X1 gene therapy trial had developed a monoclonal lymphoproliferative disease (AFSSAPS, 2002; Hacein-Bey-Abina et al., 2003c). News of a T cell leukaemia in a second patient from the same trial was made public in January 2003, increasing the likelihood that these severe adverse events were therapy induced. As a consequence, a temporary halt was imposed by the United States Food and Drug Administration (FDA) and French Health Product Safety Agency (AFSSAPS) on gene therapy trials using retroviral vectors to transduce haematopoietic stem cells (AFSSAPS, 2002; Hacein-Bey-Abina et al., 2003a). The French SCID-X1 trial was resumed in May 2004, however in January 2005 it was reported that a third patient in the trial had developed symptoms of a lymphoproliferative disease, leading to a further suspension of the clinical trial by the authorities (AFSSAPS, 2005).

The characteristics of the lymphoproliferative disease affecting the third patient are still under investigation, however in the first two affected patients, an uncontrolled, exponential proliferation of clonal mature T lymphocytes was observed approximately three years after gene therapy. The patients were the two youngest in the trial, at one and three months old at the time of treatment, and had received a high dose of corrected $CD34^+$ cells per kg of body-weight as compared to other patients. It was also noted that T cell development in these patients was especially rapid early after therapy; however prior to the time of lymphoproliferation, both patients' T cell characteristics were indistinguishable from those of age-matched children (Hacein-Bey-Abina et al., 2003b).

In the first patient (patient 4) a monoclonal γδ T cell proliferation was detected thirty months post-gene therapy. Following conventional treatment for T cell acute lymphoblastic leukaemia (T-ALL) the patient received a matched, unrelated bone marrow transplant. The second patient (patient 5) was also treated for T-ALL; the lymphoproliferative disease in this patient was attributed to three different αβ T cell clones and was detected thirty-four months after gene therapy. Unfortunately in October 2004 it was reported that one of the patients who had developed a lymphoproliferation had died (AFSSAPS, 2005).

Analysis of T cell clones from both patients revealed retrovirus vector integration in proximity to the *LMO2* (LIM domain only-2) proto-oncogene promoter, leading to aberrant transcription and translation of the gene product (Hacein-Bey-Abina et al., 2003b). The *LMO2* gene is located on the short arm of chromosome 11; translocations at this locus involving T cell receptor (TCR) genes result in aberrant expression of LMO2 and are implicated in childhood T-ALL (Boehm et al., 1991). LMO2 acts as a bridging molecule in transcription factor complexes (Rabbitts, 1998) and is thought to be necessary early on in haematopoiesis, since a null mutation of the *lmo2* gene in mice leads to failure of yolk sac erythropoeisis and eventual embryonic lethality (Warren et al., 1994). LMO2 is not expressed in mature myeloid or lymphoid cells (Warren et al., 1994) and this down-regulation appears crucial in T lymphocytes, given that transgenic mice constitutively expressing lmo2 in all tissues develop only T cell tumours (Neale et al., 1995).

The leukaemias in the French SCID-X1 gene therapy trial represent the first reports of gene therapy vector-induced insertional mutagenesis in humans. Thus far no other oncogenic events have developed in over forty clinical trials worldwide involving retroviral-mediated gene transfer to HSCs (Kohn et al., 2003). The risk of insertional mutagenesis ultimately leading to malignancy in a gene therapy setting, although recognized, was considered low (Kohn et al., 2003). Until a sole report in 2002, no incidences of insertional mutagenesis in extensive murine studies involving retroviral-mediated gene transfer to HSCs had been observed (Li et al., 2002). In the single study, mice with vector integrations near the proto-oncogene *Evi1* developed acute myeloid leukaemia (AML).

The exact mechanism of leukaemogenesis in the two SCID-X1 patients is unclear. One plausible cause of oncogenesis might have been the presence of replication-competent retrovirus leading to multiple integration events; however none was detected in both cases (Hacein-Bey-Abina et al., 2003b). The long latency preceeding leukaemia development in these patients suggests that insertional activation of *LMO2* alone is insufficient to cause malignancy and that secondary events are required. This hypothesis is consistent with the multi-step model of tumourigenesis; aberrant expression of LMO2 in progenitor cells may have resulted in increased cell expansion and concomitantly the opportunity to acquire further, transforming mutations (Wu and Pandolfi, 2001). At the time of clinical manifestation, the leukaemic cell clones from both patients were found to contain chromosomal abnormalities; in the first patient a t(6;13) translocation had occurred, whilst in the second a SIL-TAL1 fusion transcript and trisomy 10 were detected (Hacein-Bey-Abina et al., 2003b).

It is also possible that the immunodeficient setting in SCID-X1 and/or the γc transgene may have contributed to the onset of leukaemia. Infants with SCID-X1 may accumulate higher numbers of T lymphocyte precursors in the bone marrow due to the block in differentiation resulting from the inability to respond to cytokine signals. LMO2 is expressed in stem and progenitor cells and hence this may increase the likelihood of retroviral vector integrations at this locus. γc is widely expressed in haematopoietic cells and therefore constitutive expression under the control of the viral long terminal repeat (LTR) was postulated to be safe. Moreover, signalling through γc requires the involvement of additional, specific receptor subunits. However, one recent publication

purports that *IL2RG* itself is oncogenic; Woods *et al.* reported the development of thymic lymphomas in five out of fifteen X-SCID mice that had received wild-type or X-SCID bone marrow transduced with lentivirus encoding *IL2RG* (Woods et al., 2006). Conclusions drawn from this study may however be premature; as yet molecular data regarding insertion sites and clonality of the murine tumours have not been published and furthermore, vector sequences were undetectable in one of the lymphomas. Also, insertional mutagenesis as a cause of lymphoma development cannot be excluded, since vector copy number was high and a strong, ubiquitous promoter was incorporated into the lentiviral vector backbone. Investigations into JAK3 activation in these lymphomas would also provide evidence as to the role of γc itself in tumour development in these mice, since γc specifically associates with and signals through this tyrosine kinase (Miyazaki et al., 1994). In the French clinical trial, no over-expression of γc was seen on either of the patients' clones, neither was any constitutive activation of JAK3 observed, indicating that γc was functioning normally (Hacein-Bey-Abina et al., 2003b). Furthermore, pre-clinical studies in sixty-eight mice (X-SCID and wild-type) reconstituted by gammaretroviral or lentiviral *IL2RG* gene transfer resulted in only three cases of lymphoma, one of which was transgene-negative (Thrasher et al., 2006). Trangenic mice expressing the human γc from a CD2 promoter have also been tumour-free for over twelve months (Thrasher et al., 2006). However, a synergistic effect between γc and LMO2 over-expression could in theory have played a role in the lymphoproliferations (Hacein-Bey-Abina et al., 2003b). A retrospective analysis of retroviral integration sites inducing murine leukaemia has provided evidence that LMO2 and γc may cooperate to induce leukaemagenesis (Dave et al., 2004).

Other potential contributing factors are the young age at which the children were treated and the high number of corrected $CD34^+$ cells that these patients received. A potential genetic predisposition to cancer and a possible synergistic influence of a varicella zoster virus (VZV) infection in one of the patients, could also have contributed as secondary events in the emergence of a malignant clone (Hacein-Bey-Abina et al., 2003b). Conversely however, the third patient was treated at an older age, nine months, and does not appear to have an insertion at *LMO2* (Couzin and Kaiser, 2005). Further investigation is needed to discover whether these events are disease-specific, nonetheless they highlight the necessity of further pre-clinical investigation to

evaluate the risks associated with gene therapy; including development of predictive animal models and improvements in vector technology and experimental design.

1.6 Gene Therapy for ADA-SCID and CGD

Efficacy of gene therapy for the treatment of ADA-SCID using a similar methodology as for SCID-X1 has been reported in two clinical trials (Aiuti et al., 2002; Gaspar et al., 2006). In the first trial, Aiuti and colleagues in Milan, Italy, treated two ADA-SCID patients for whom enzyme-replacement therapy, conventionally used to treat this disorder, was unavailable. A gammaretroviral vector derived from the Moloney murine leukaemia virus, whereby expression on the ADA transgene is regulated by the viral LTR, was used to transuce autologous $CD34^+$ cells which were infused back into the patients following a low-intensity, nonmyeloablative conditioning regime used to enhance engraftment of transduced progenitors. In both patients, the sustained engraftment of transduced HSCs and the differentiation of these cells into multiple lineages resulted in increased lymphocyte counts, improved immune function and correction of the metabolic defect of ADA-SCID leading to the reversal of clinical phenotype (Aiuti et al., 2002). Successful reconstitution of immunity and systemic detoxification of ADA metabolites was also observed following treatment of an ADA-SCID patient by gene therapy at Great Ormond Street Hospital, London (Gaspar et al., 2006). A similar protocol to that used by Aiuti and colleagues was performed, however a vector derived from the Spleen focus forming virus (SFFV) and a different conditioning agent (Melphalan as opposed to Busulfan) were used in this trial. To date, no severe adverse events have been observed in either gene therapy trial.

The outcome of a gene therapy trial to treat two adults suffering from CGD, an immunodeficiency caused by a defect in the oxidative antimicrobial activity of phagocytes due to mutations in the gene encoding $gp91^{phox}$, has recently been reported (Ott et al., 2006). As for the successfully treated ADA-SCID children, the CGD patients received non-myeloablative conditioning prior to the infusion of mobilised peripheral blood $CD34^+$ cells transduced with an SFFV-based gammaretroviral vector.

Therapeutic benefit was observed in both patients shortly after the procedure with the clearance of existing bacterial or fungal infections refractory to conventional therapy alone. Four months post-treatment however, an increase in gene-modified myeloid cells was detected with the appearance of multiple dominant clones approximately one month later. Analysis of retroviral vector insertion sites in both patients revealed the dominant clones to result from transactivating insertions within or close to one of three genes – *MDS1-EV1*, *PRDM16* or *SETBP1*. The insertional activation of these genes by the enhancer sequences within the SFFV LTR initially appeared to contribute to therapeutic efficacy as a result of the expansion of gene-corrected myeloid cells; such a benefit was consequently negated however due to transgene silencing resulting from methylation of the SFFV LTR promoter sequences leading to deterioration in the clinical status of both patients and the subsequent death of one patient due to severe sepsis two and a half years post-treatment (M.Grez personal communication). Interestingly and quite unexpectedly, although transgene silencing was associated with methylation of the SFFV promoter, the enhancer component of this viral regulatory element remained unmethylated and functional, resulting in the continual activation of the nearby *MDS1-EV1*, *PRDM16* or *SETBP1* promoters and therefore maintenance of myeloid cell numbers harbouring integration events at these loci (M.Grez personal communication).

1.7 Viral Vectors for Gene Therapy

Viral vectors have been used in 70% of gene therapy clinical trials (Journal of Gene Medicine clinical trials database) and currently represent the most effective means of gene delivery. No single vector is suitable for all applications and the choice of viral vector depends largely on the cell type to be targeted and duration of expression required. Desirable properties of all vectors however are the ability to be reproducibly propagated and purified to high titres, and to mediate transgene delivery and expression to the target cell without substantial toxicity. Many viruses have been exploited to produce gene therapy vectors; some of the most commonly used are derived from

gammaretroviruses, lentiviruses, foamy viruses, adenoviruses, adeno-associated viruses (AAVs) and herpes simplex viruses (HSVs) (see table1. 2).

	Gamma-retrovirus	Lentivirus	Foamy virus	Herpes virus	Adenovirus	AAV	Non-viral
Genetic Material	RNA	RNA	RNA	dsDNA	ssDNA	ssDNA	dsDNA
Packaging Capacity	~9 kb	~10 kb	~12 kb	>30 kb	~30 kb	<5 Kb	Unlimited
Tropism	Broad	Broad	Broad	Strong for neurons	Broad	Broad except for HSCs	Broad
Integration into host genome	+	+	+	-	-	<10%	Rare
Duration of transgene expression	Long	Long	Long	Transient	Transient	Transient	Transient
Transduction of non-dividing cells	-	+	+	+	+	+	+
Inflammatory potential	Low	Low	Low	High	High	Low	Low
Safety Concerns	Insertional mutagenesis	Insertional mutagenesis	Insertional mutagenesis	Inflammatory response	Insertional mutagenesis	Low risk of insertional mutagenesis	-

Table 1.2. The properties of commonly used gene transfer vectors. (Table adapted from Nathwani *et al.*, *Br.J.Haem.Rev.* **128**:3-17 (2004))

1.7.1 Retroviruses

Retroviruses are composed of single-stranded RNA genomes encapsulated in a protein core and packaged into a lipid envelope. Following entry into a target cell, the viral genome is reverse-transcribed into linear double-stranded DNA that is subsequently integrated into the host cellular chromatin. This family of viruses may be grouped into seven genera based on genome sequence and complexity and site of particle assembly; however, in terms of the development of vectors for gene therapy, the focus has almost exclusively been on the following three genera: the *Gammaretroviruses*, the *Lentiviruses* and the *Spumaviruses* (Baum et al., 2006).

All retroviral genomes are flanked by two long terminal repeat (LTR) sequences; at the 5' end these contain the viral promoter and enhancers responsible for initiation of transcription of viral genes, whilst the polyadenylation signal is situated in the 3' LTR. The LTRs and neighbouring sequences act in *cis* during reverse-transcription, integration, viral gene expression and packaging. Framed between the LTR sequences are the *gag*, *pol* and *env* genes required by all retroviruses, these encode the structural proteins, viral replication enzymes and envelope glycoprotein respectively, and the packaging signal (ψ) required for the incorporation of the RNA genomes into viral particles (figure 1.4). Lentiviruses have a more complex genome containing two additional regulatory genes, *tat* and *rev*, required for gene expression, together with a variable set of accessory genes. The spumavirus genome also contains several accessory genes in addition to *gag*, *pol* and *env*, one of which, *bel-1*, is essential for regulation of gene expression (Lee et al., 1993).

1.7.2 Retroviral Vectors

Retroviral vectors are derivatives of viruses that have been engineered to carry a therapeutic gene into target cells. There are several advantageous features of gene therapy vectors derived from this family of viruses; firstly, the proteins required for reverse transcription and integration are carried within the viral particle and thus all

viral genes may be removed from vectors. Secondly, integration of proviral DNA into the host cell genome results in stable transmission of the transgene to subsequent cell generations and therefore offers a potential long-term cure for monogenic disorders. Finally, these vectors have only a low potential of triggering a detrimental inflammatory immune response as compared to vectors derived from other viral species (table 1.2).

To generate a retroviral vector, both coding genes and *cis*-acting sequences are required; however an important safety feature is that these sequences are separated so as to prevent the formation of replication-competent retrovirus (RCR). Consequently the *trans*-acting *gag*, *pol* and *env* genes are removed from the viral genome and instead provided either on heterologous plasmids or within the chromosomes of a packaging cell line. The vector genome, consisting only of the *cis*-acting regions (sequences recognised by viral and cellular proteins during the replication cycle) and the therapeutic gene, can therefore be introduced into packaging cells or co-transfected with packaging plasmids to produce replication-defective vector particles (figure 1.4).

A key factor regarding the potential for insertional mutagenesis is the integration site preferences of retroviral vectors. Completion of the human genome sequence has made possible genome-wide studies of retroviral vector integration, a process initially thought to occur at random. Such studies have identified a bias of MLV-based vector integration within the proximity of transcriptional start sites, whilst lentiviral vectors derived from HIV-1 or SIV target integration within transcriptional units, downstream of transcriptional start sites (Wu et al., 2003; Schroder et al., 2002; Mitchell et al., 2004). These integration site preferences have also been confirmed *in vivo*, following transplantation of HSCs transduced with an MLV or SIV vector into rhesus monkeys (Hematti et al., 2004). On the basis of this data MLV-based vectors appear to harbour the greatest potential to exert enhancer effects on endogenous genes following proviral integration, a propensity demonstrated in one study utilising a promoter-trapping method and in the human SCID-X1 gene therapy trials, whilst lentiviral vectors may pose a risk of gene disruption given their bias for integration within transcriptional units (De et al., 2005; Hacein-Bey-Abina et al., 2003b). Retroviral vectors derived from foamy viruses (FVs) or avian sarcoma-leukosis virus (ASLV) may pose a decreased risk with regards insertional mutagenesis; genome-mapping of FV vector integration

has revealed no bias for integration within active genes as compared to lentiviral vectors, however once integration has occurred into a transcribed region of the genome, FVs exhibit a modest preference for integration near transcription start sites (Trobridge et al., 2006; Nowrouzi et al., 2006). ASLV vectors showed only a weak bias for integration into actively-transcribed genes with no preference for integration near transcription start sites (Mitchell et al., 2004).

1.7.2.1 Gammaretroviral Vectors

Gammaretroviral vectors are most commonly based on the Moloney murine leukaemia virus (MLV) and have been widely used both in experimental studies and clinical trials. As mentioned previously, the main limitation is their inability to cross the nuclear membrane and consequently these vectors are only able to transduce dividing cells, limiting their application in gene therapy to *ex vivo* transduction.

Gammaretroviruses such as MLV have a simple genomic structure containing only the aforementioned three essential viral genes and *cis*-acting sequences such as the packaging signal (ψ), primer binding site (PBS), polypurine tract (PPT), splice donor and splice acceptor sites (figure 1.4A). The PBS and PPT act to position the tRNA primers during reverse transcription, whilst the packaging signal interacts with viral proteins to accomplish specific packaging of viral RNA. The splice donor and splice acceptor sites are located 5' and 3' to the packaging signal respectively; translation of spliced RNA transcripts (such as Env in the wild-type virus), in which the complex secondary structure of the packaging signal has been removed, is thought to proceed more efficiently (Krall et al., 1996). In MLV-based vectors, transgenes are classically placed between the packaging signal and PPT and early generations of these vectors currently used in clinical trials retain full-length LTR sequences and portions of viral *gag-pol* coding sequence incorporating the splice acceptor site (figure 1.4B) (Cavazzana-Calvo et al., 2000; Gaspar et al., 2004). Expression of the transgene in these vectors is therefore regulated by the viral enhancer and promoter sequences in the 5'LTR and the portions of *gag-pol* coding sequence are maintained in these vectors to allow splicing and thus enhanced mRNA stability functions (Krall et al., 1996).

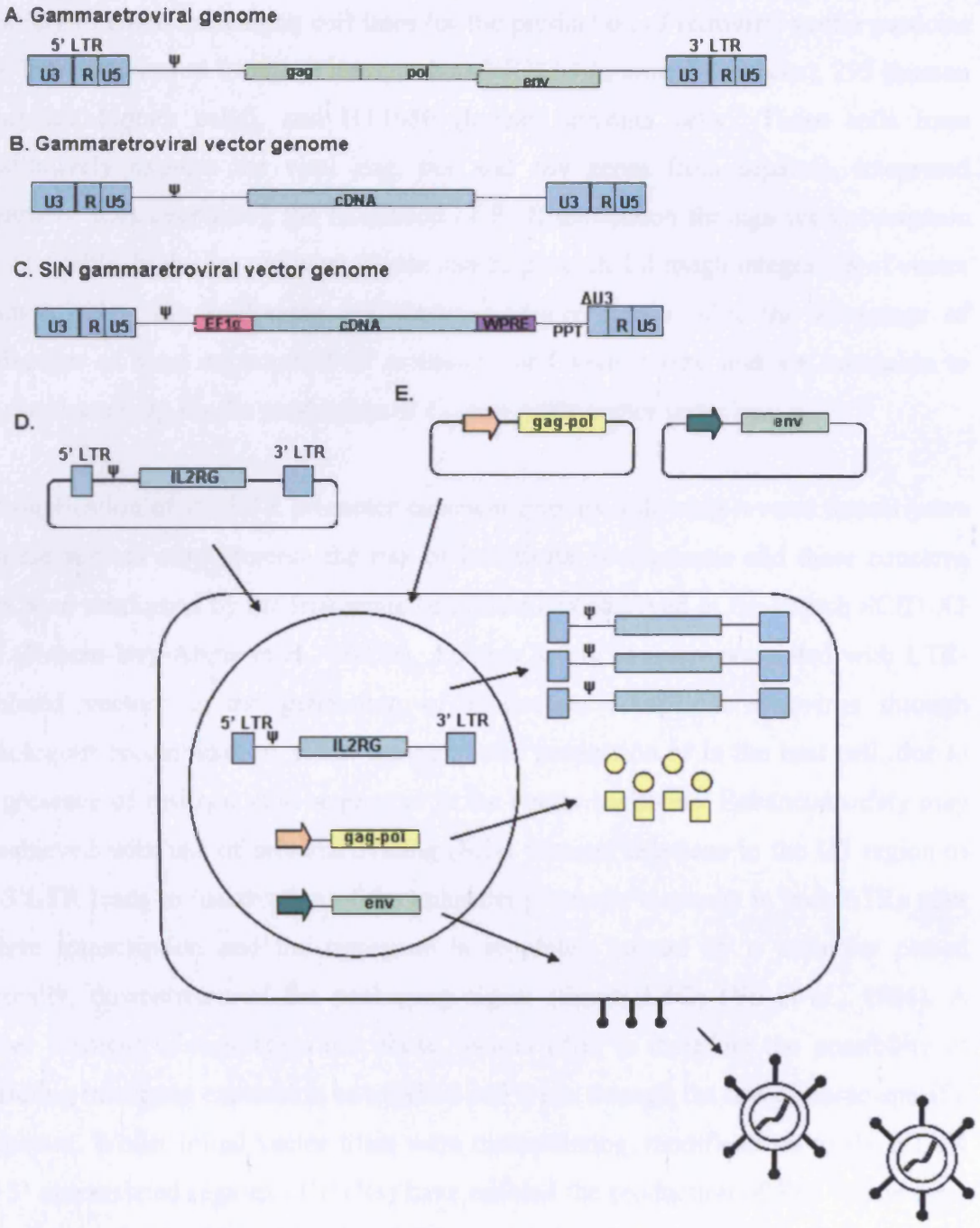

Figure 1.4. Retroviral vector production. (A) Genome of a wild-type gammaretrovirus. (B) Retroviral vector genome in which the viral genes have been removed and replaced with the cDNA of a therapeutic gene (e.g. *IL2RG*). (C) Genome of a self-inactivating (SIN) gammaretroviral vector in which the transgene is regulated by an internal promoter (e.g. EF1α). A Woodchuck hepatititis virus post-transcriptional regulatory element (WPRE) is positioned after the transgene to enhance its expression and viral titre. (D & E) A plasmid encoding the retroviral vector is transfected into a packaging cell line (D) or is co-transfected with packaging plasmids into producer cells (E), resulting in the production of replication-defective retroviral vector particles. (ψ - packaging signal)

A variety of stable packaging cell lines for the production of retroviral vector particles have been engineered from cell lines such as NIH3T3 (murine fibroblasts), 293 (human embryonic kidney cells), and HT1080 (human sarcoma cells). These cells lines constitutively express the viral *gag*, *pol* and *env* genes from separate, integrated constructs, thus decreasing the likelihood of RCR generation through recombinagenic events. Stable, high-titre producer clones can be generated through integration of vector plasmid DNA into packaging cell lines; producer clones offer the advantage of production of viral supernatant of consistent and known titre and are amenable to industrial scale-up for the production of clinical grade vector particles.

The duplication of the LTR promoter-enhancer regions following reverse transcription of these vectors may increase the risk of insertional mutagenesis and these concerns have been reinforced by the leukaemic complications observed in the French SCID-X1 trial (Hacein-Bey-Abina et al., 2003b). Another safety concern associated with LTR-regulated vectors is the generation of replication competent retrovirus through homologous recombination, either during vector production or in the host cell, due to the presence of residual viral sequences in the vector backbone. Enhanced safety may be achieved with use of self-inactivating (SIN) vectors; deletions in the U3 region of the 3'LTR leads to inactivation of the enhancer-promoter elements in both LTRs after reverse transcription and the transgene is regulated instead by a promoter placed internally, downstream of the packaging signal (figure 1.4C) (Yu et al., 1986). A further element of regulation that these vectors offer is therefore the possibility of restricting transgene expression to required cell types through the use of tissue-specific promoters. Whilst initial vector titres were disappointing, modifications to the 5'LTR and 3' untranslated regions (3'UTRs) have enabled the production of SIN vector titres equivalent to those achieved for their LTR-regulated counterparts (Kraunus et al., 2004; Schambach et al., 2006b). These modifications include the incorporation of a post-transcriptional regulatory element (PRE) of the woodchuck hepatitis virus into the 3' UTR to improve RNA export and hence transgene expression and viral titre (Zufferey et al., 1999; Kraunus et al., 2004) and the removal of aberrant translational start codons within the untranslated region 5' of the internal expression cassette (Hildinger et al., 1999; Kraunus et al., 2004).

A major limiting factor for successful gene transfer is efficient binding of viral vector particles to the host cell. The viral envelope glycoprotein dictates the vector host range and substitution of the envelope protein for one from a different virus (a process referred to as pseudotyping) can broaden vector tropism. The majority of clinical gene therapy trials targeting HSCs have used gammaretroviral vectors pseudotyped with either the MLV amphotropic envelope or with the Gibbon ape leukaemia virus envelope (GALV). Amphotrophic retrovirus binds to Pit-2, a sodium-dependent phosphate transporter protein, expressed at relatively low levels on both human and murine HSCs (Orlic et al., 1996). The receptor for the GALV envelope is the closely related phosphate transporter protein Pit-1, that appears to be more highly expressed on human HSCs than the amphotropic receptor resulting in improved transduction efficiency of these cells by GALV pseudotyped vectors (von et al., 1994). Pit-1 is not expressed on murine HSCs, however efficient transduction of these cells may be achieved using the murine ecotropic envelope (Suzuki et al., 2001; Li et al., 2003), which targets a basic amino-acid transporter ubiquitously expressed in murine cells (Wang et al., 1991). Another envelope frequently used for transduction of haemtopoietic cells is that of the Endogenous feline type C virus RD114, an advantage of which is that vectors pseudotyped with this envelope are able to withstand concentration by ultracentrifugation (Kelly et al., 2001).

1.7.2.2 Lentiviral Vectors

Lentiviruses rely on active transport of the preintegration complex through the nuclear membrane and hence vectors derived from these viruses offer the advantage of being able to transduce and stably integrate into the genome of non-dividing cells (Sherman and Greene, 2002). This ability is particularly advantageous in HSC gene therapy since cells may be transduced without prestimulation, thus avoiding prolonged *ex vivo* culture that may result in loss of pluripotency and engraftment ability.

The first lentiviral vectors to be developed were derived from HIV-1 and these vectors have been used extensively in gene transfer studies (figure 1.5) (Vigna and Naldini, 2000). As previously mentioned, HIV-1 is a complex retrovirus that requires two

additional regulatory genes, *tat* and *rev*, and a set of accessory proteins. Initial packaging constructs for these vectors maintained the accessory genes; however these proteins were shown to be dispensible for efficient transduction and integration of lentiviral vectors and were therefore deleted in second generation packaging constructs (Zufferey et al., 1997). The deletion of accessory genes also increased the biosafety of these vectors, since any RCR generated during vector production would lack the essential factors for HIV-1 virulence *in vivo*. A further safety measure was achieved by the production of third generation packaging constructs in which the *rev* gene was placed on a separate plasmid to that of the *gag-pol* genes and the *tat* gene was removed altogether (Dull et al., 1998). The viral tat protein acts as a potent transcriptional transactivator of the HIV-1 LTR and is therefore required for high titre virus production (Cullen, 1998). High vector titre in the absence of *tat* was found to be possible however by replacement of the U3 region in the 5'LTR with a constitutively active heterologous promoter such as that from the Rous sarcoma virus (RSV) (Dull et al., 1998). The viral *rev* gene product functions as a nuclear export factor; the protein binds to an RNA motif, the Rev-response element (RRE), and promotes cytoplasmic export of unspliced and spliced transcripts and hence must be provided during vector production (Cullen, 1998).

Considering the pathogenicity of the parental virus there are several safety concerns surrounding HIV-1 derived lentiviral vectors that must be addressed before clinical application. Among these concerns is the possibility of vector mobilisation in individuals who are or become infected with wild-type virus. The development of self-inactivating lentiviral vectors has significantly increased their biosafety (figure 1.5). These vectors have been demonstrated to retain the *in vitro* and *in vivo* properties of lentiviral vectors retaining wild-type LTRs, whilst they are unable to interact with or be mobilised by wild-type HIV-1 (Zufferey et al., 1998; Bukovsky et al., 1999). Early lentiviral vectors containing intact, wild-type LTR sequences, required transgene expression to be regulated by an internal promoter (as opposed to MLV LTR-regulated vectors) due to the weak basal transcriptional activity of the 5'LTR. Consequently, a further attribute of the self-inactivating design is that promoter interference between the 5'LTR and internal promoter is abolished (Zufferey et al., 1998). Modifications to lentiviral SIN vectors to enhance vector potency and transgene expression include reintroduction of a central polypurine tract (cPPT) and insertion of a WPRE into the

vector backbone (figure 1.5) (Follenzi et al., 2000; Zufferey et al., 1999). Lentiviral vectors have also been derived from non-human lentiviruses (e.g. Equine infectious anaemia virus) on the rationale that they might be more acceptable to clinical application due to their parental viruses not being infectious to humans (Olsen, 1998).

The glycoprotein of the Vesicular stomatitis virus (VSV-G) is most commonly used to pseudotype lentiviral vectors and expands vector tropism since the receptor for VSV-G, although still undetermined, appears to be ubiquitous in all cell types (Carneiro et al., 2006). Furthermore, VSV-G pseudotyped vectors can be efficiently concentrated by ultracentrifugation, enabling the production of serum-free, high-titre vector particles (Akkina et al., 1996). However, VSV-G is associated with cytotoxicity limiting the concentrations of vector which can be used without reducing target cell viability. This toxicity has also hindered the creation of packaging cell lines for production of VSV-G pseudotyped lentivirus, however progress has been reported using inducible expression of the envelope protein (Farson et al., 2001). Efficient pseudotyping of lentivirus has been achieved with RD114, amphotropic MLV envelope and GALV, with particularly high transduction levels of human $CD34^+$ cells achieved using RD114 (Relander et al., 2005). As for gammaretroviral vectors, efficient transduction of murine cells, including HSCs, has been demonstrated using lentivirus pseudotyped with murine ecotropic envelope (Schambach et al., 2006c).

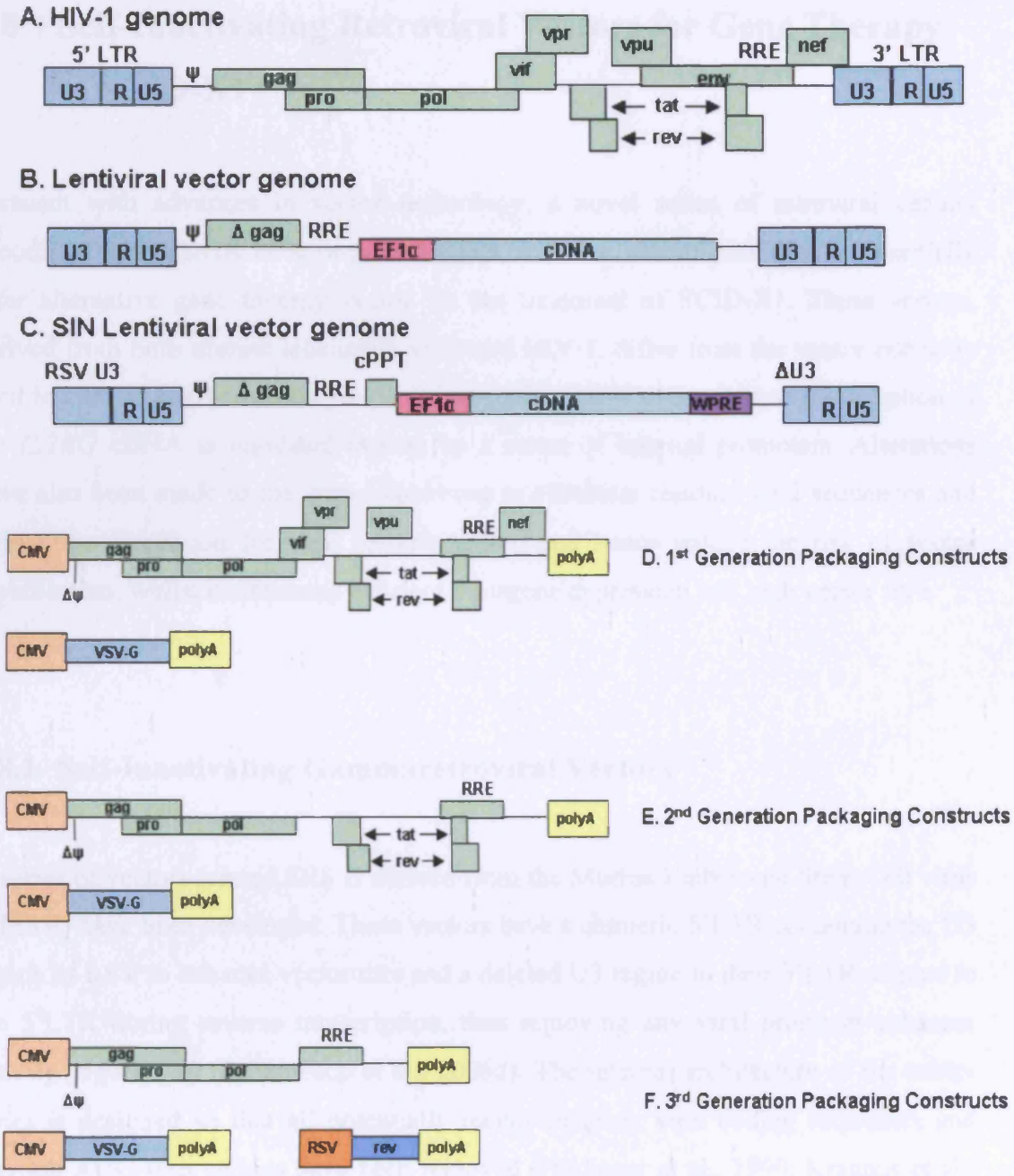

Figure 1.5. Lentiviral vector genomes and packaging constructs. (A) Genome of a wild-type HIV-1. (B) Wild-type LTR lentiviral vector genome in which the cDNA of a therapeutic gene is regulated by an internal promoter (e.g. EF1α). (C) Genome of a self-inactivating (SIN) lentiviral vector containing a modified 5'LTR in which the U3 region has been replaced by the constitutive RSV promoter. The vector contains a cPPT and WPRE to enhance vector potency and transgene expression. (D, E and F) First-, second- and third generation packaging constructs containing sequences from HIV-1 and the VSV-G envelope, used in conjunction with A, B orC. (ψ - packaging signal)

1.8 Self-Inactivating Retroviral Vectors for Gene Therapy of SCID-X1

Pursuant with advances in vector technology, a novel series of retroviral vectors encoding the γc cDNA have been developed, with the aim of providing a potentially safer alternative gene therapy vector for the treatment of SCID-X1. These vectors, derived from both murine leukaemia virus and HIV-1, differ from the vector currently used in clinical trials since they have deleted LTRs (SIN vectors), thus transcription of the *IL2RG* cDNA is regulated instead by a series of internal promoters. Alterations have also been made to the vector backbone to eliminate residual viral sequences and reduce the likelihood for viral recombination, and hence reduce the risk of vector mobilisation, whilst maintaining efficient transgene expression and high vector titre.

1.8.1 Self-Inactivating Gammaretroviral Vectors

A series of vectors termed SRS II derived from the Murine Embryonic Stem Cell virus (MESV) have been developed. These vectors have a chimeric 5'LTR containing the U3 region of RSV to enhance vector titre and a deleted U3 region in their 3'LTR, copied to the 5'LTR during reverse transcription, thus removing any viral promoter-enhancer activity (figure 1.6) (Schambach et al., 2006d). The internal architecture of the vector series is designed so that all potentially recombinagenic viral coding sequences and aberrant AUG start codons have been removed (Hildinger et al., 1999; Kraunus et al., 2004). Regulation of the *IL2RG* cDNA is therefore via an internal promoter placed downstream of this leader region and a modified WPRE, placed 3' to the cDNA, is added to enhance transgene expression (Zufferey et al., 1999). Two different promoters, the U3 region of the Spleen focus forming virus (SFFV promoter) and the short form (intron-deleted) of the human elongation factor 1α promoter (EFS), have been incorporated into the vector backbone to be evaluated for their potential efficacy in a clinical setting (see figure1.7). Both promoters have been demonstrated to regulate high levels of transgene expression in haematopoietic lineages, including human $CD34^+$ cells, and consequently may represent suitable promoters for gene therapy of

haematopoietic disorders (Baum et al., 1995; Demaison et al., 2002; Salmon et al., 2000).

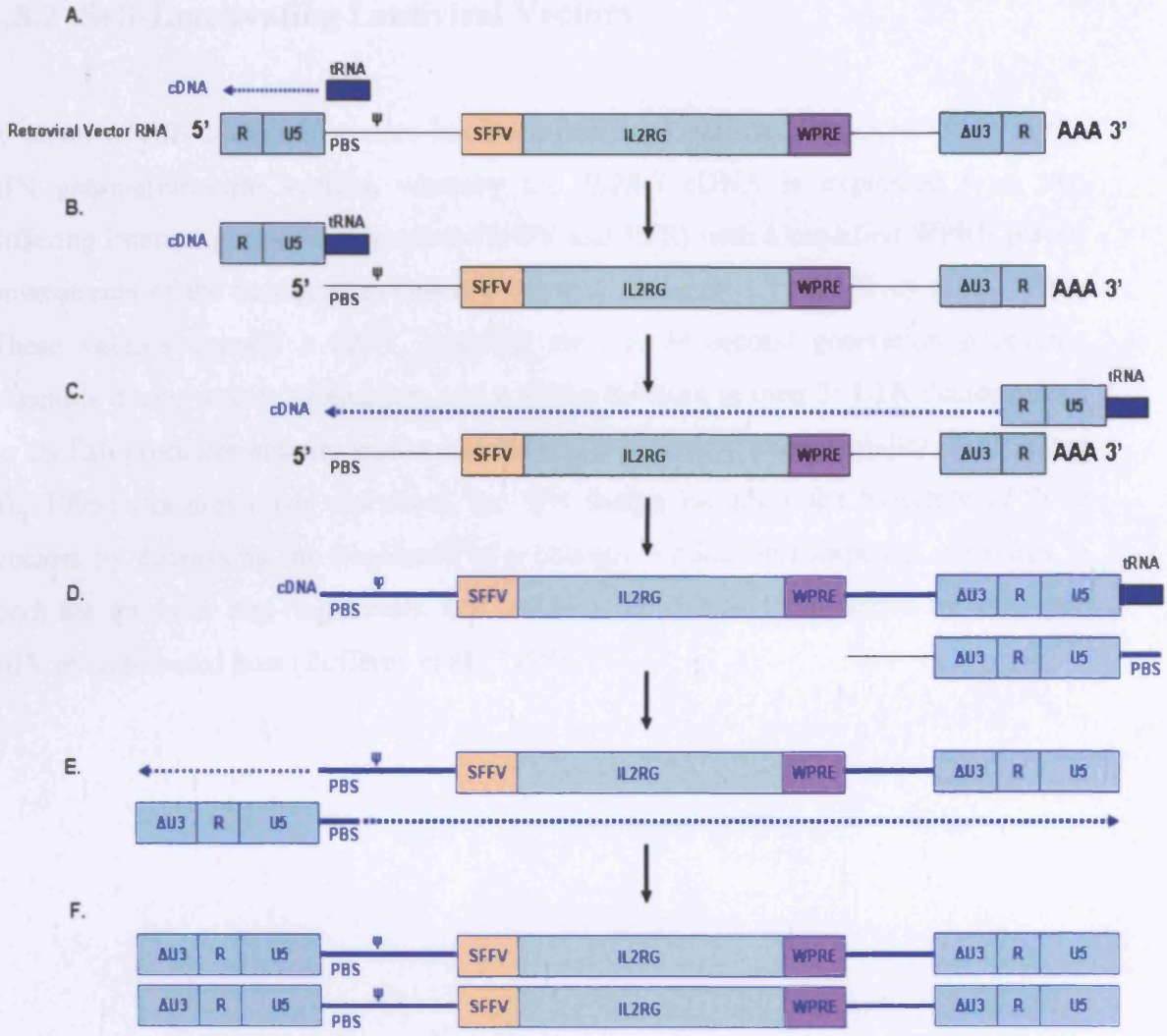

Figure 1.6. Reverse transcription of self-inactivating retroviral vectors. (A) A retrovirus-specific cellular tRNA hybridises with the primer binding site (PBS) on the vector RNA genome (black) and reverse transcriptase makes a DNA copy of the U5 and R regions. (B) The vector U5 and R regions are removed by the RNase H activity of reverse transcriptase. (C) The cDNA (blue) hybridises with the complementary R region at the 3' end of the vector RNA genome and the remainder of the vector genome is copied into cDNA. (D) Most of the remaining vector RNA genome is removed by RNase H. A second cDNA strand is extended from the remaining viral RNA and the deleted U3 region of the 3' LTR copied. (E) The tRNA and remaining vector RNA are removed by RNase H. The copied PBS region in the second DNA strand hybridises with the PBS region on the first strand and both strands are extended. (F) A double-stranded DNA copy of the retroviral vector genome is produced with 'self-inactivated' LTR regions.

1.8.2 Self-Inactivating Lentiviral Vectors

A series of SIN lentiviral vectors has been produced with similar architecture to the SIN gammaretroviral vectors, whereby the *IL2RG* cDNA is expressed from two differing internal promoter sequences (SFFV and EFS) with a modified WPRE placed downstream of the transgene to enhance expression (figure 1.7) (Zufferey et al., 1999). These vectors contain a cPPT, enabling the use of second generation packaging plasmids during vector production, and a 400bp deletion in their 3' LTR demonstrated to abolish promoter activity whilst maintaining high vector titre capability (Zufferey et al., 1998). As previously discussed, the SIN design increases the biosafety of these vectors by decreasing the likelihood of producing replication-competent retrovirus in both the producer and target cells, and reducing the risk of mobilisation by wild-type HIV in an infected host (Zufferey et al., 1998).

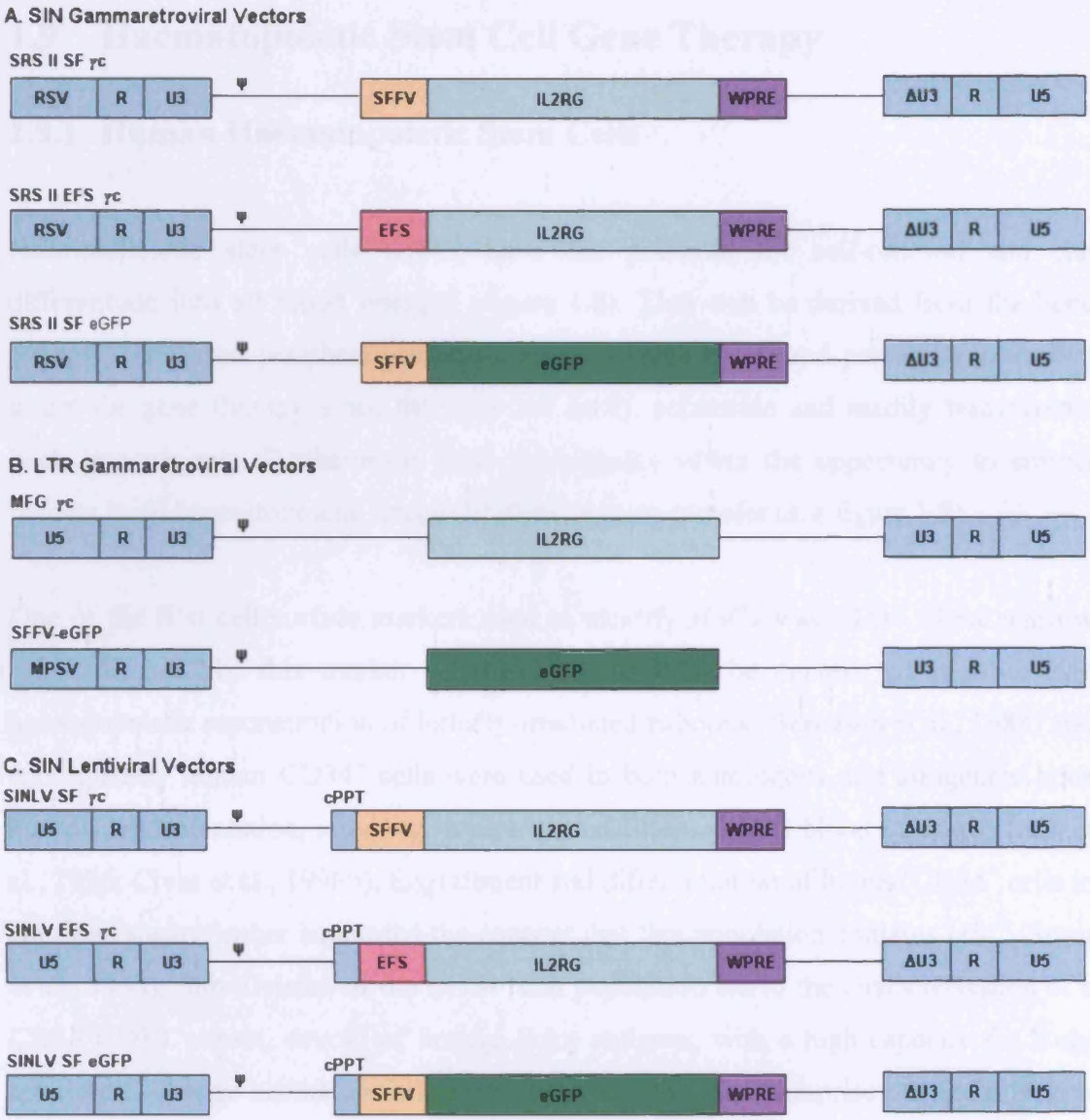

Figure 1.7. The gammaretroviral and lentiviral vectors used in this study. (A) SIN gammaretroviral vectors including SRS II SF γc and SRS II EFS γc, for the treatment of SCID-X1, and SRS II SF eGFP encoding enhanced green fluorescent protein (eGFP) for use as a control vector. (B) LTR-regulated gammaretroviral vectors including MFG γc, currently used in the clinical gene therapy trial for SCID-X1, and SFFV-eGFP (with a chimeric 5'LTR containing the U3 from the Myeloproliferative sarcoma virus (MPSV) to improve vector titre) as a control vector. (C) SIN lentiviral vectors including SINLV SF γc and SINLV EFS γc, for gene therapy of SCID-X1, and SINLV SF eGFP as a control.

1.9 Haematopoietic Stem Cell Gene Therapy

1.9.1 Human Haematopoietic Stem Cells

Haematopoietic stem cells (HSC) have the potential for self-renewal and can differentiate into all blood lineages (figure 1.8). They can be derived from the bone marrow, mobilised peripheral blood or umbilical cord blood and present an attractive target for gene therapy since the cells are easily accessible and readily transplanted back into patients. Furthermore, their pluripotency offers the opportunity to correct defects in all haematopoietic lineages following gene transfer (see figure 1.8).

One of the first cell surface markers used to identify HSCs was CD34. Bone marrow cells enriched for this marker were demonstrated to be capable of multi-lineage haematopoietic reconstitution of lethally-irradiated baboons (Berenson et al., 1988) and consequently human $CD34^+$ cells were used in both autologous and allogeneic bone marrow transplantation, resulting in rapid reconstitution of all blood lineages (Link et al., 1996; Civin et al., 1996b). Engraftment and differentiation of human $CD34^+$ cells in chimeric sheep further supported the concept that this population contains HSC (Srour et al., 1993). Sub-division of the $CD34^+$ cell population led to the characterisation of a $CD34^+CD38^-$ subset, devoid of lineage (Lin) antigens, with a high capacity for long-term multi-lineage haematopoietic engraftment. These cells comprise <0.1% of human bone marrow mononuclear cells and have been demonstrated capable of sustained multi-lineage repopulation of both chimeric sheep and non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) (Civin et al., 1996a; Bhatia et al., 1997).

Recent data suggests that the CD34 antigen is not expressed on all HSCs; human Lin^- $CD34^-CD38^-$ cells from cord blood were demonstrated capable of restoring multi-lineage haematopoiesis in the NOD/SCID mouse model (Bhatia et al., 1998). Long-term engraftment of human bone marrow-derived $CD34^-$ cells in sheep chimeras also confirms that a significant number of HSCs are present in this cell population (Zanjani et al., 2003). Characterisation of alternative cell surface markers for HSCs is therefore underway. One putative marker is AC133 (or CD133) which has been demonstrated to

be expressed on both human $CD34^+$ and a subset of $CD34^-$ cells with high repopulating ability (Gallacher et al., 2000). In the majority of gene therapy clinical trials to date however, cells have been enriched for the $CD34^+$ fraction and clinical-grade devices are available for enrichment of this cell population.

1.9.2 Murine Haematopoietic Stem Cells

Expression of the CD34 antigen on murine HSCs declines with age; $CD34^+$ HSCs are present in foetal and newborn mice, however in adult mice HSCs with long-term repopulating ability are $CD34^-$ (Matsuoka et al., 2001). Consequently cell surface markers other than CD34 are used to define murine HSCs and populations that are $CD34^-Lin^-c\text{-}kit^+Sca\text{-}1^+$ have been demonstrated capable of long-term haematopoietic reconstitution in irradiated mice (Osawa et al., 1996).

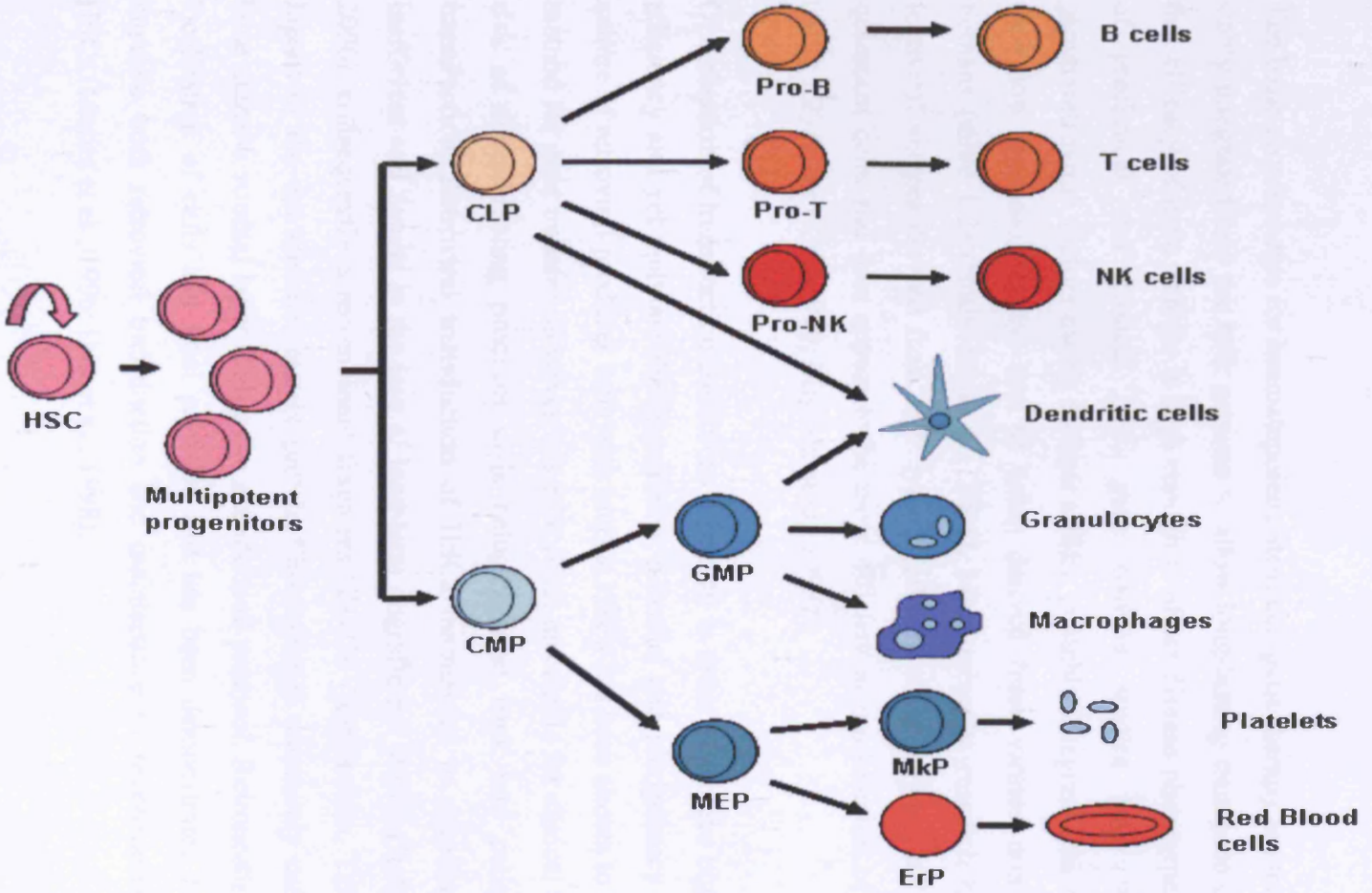

Figure 1.8. Haematopoietic stem cell development. Self-renewing HSCs give rise to multipotent progenitor cells which may develop into common lymphoid progenitor cells (CLPs) and common myeloid progenitors (CMPs). CLPs may differentiate into B, T and NK cells; dendritic cells may be derived from both CLPs and CMPs. CMPs give rise to granulocyte/macrophage precursors (GMPs) and megakaryocyte/erythroid precursors which in turn give rise to all myeloid cell populations. (MkP - megakaryocyte precursor; ErP - erythroid precursor). (Figure adapted from Reya *et al.*, *Nature.* **414**:105-111 (2001))

1.9.3 Requirements for HSC Gene Therapy

The basic requirements for haematopoietic stem cell gene therapy are that the vector is stably integrated into the host genome to allow long-lasting transgene expression and that efficacy of gene transfer is high enough to affect disease phenotype. The majority of preclinical and clinical HSC gene transfer studies have therefore used gammaretroviral vectors owing to their ability to stably integrate into host DNA and their low immunogenicity, due to being derived from viruses non-pathogenic to humans (table 1.2) (Nathwani et al., 2005). More recently, research has focused on lentiviral vectors derived from HIV type 1 (HIV-1) since they are able to transduce quiescent cells and thus appear to be more efficient at transduction of human HSCs (table 1.2) (Case et al., 1999; Miyoshi et al., 1999).

Optimisation of transduction conditions is critical in order to achieve high transduction efficiency and yet maintain the engraftment potential and pluripotency of HSCs. Co-culture of retroviral producer cells with murine HSCs has been shown to be an efficient method for gene transfer; however this protocol is unsuitable for clinical trial due to the risk of contaminating producer cells being infused back into patients following transduction. Retroviral transduction of HSCs maintained in suspension culture is inefficient and results in the loss of long-term engraftment ability (Halene and Kohn, 2000), consequently a recombinant fragment CH-296 (Retronectin, TaKaRa Bio Inc, Japan) of the extracellular matrix protein fibronectin is commonly used to mimic a bone marrow stromal layer during the transduction protocol. Retronectin enhances co-localisation of cells and viral particles and has been demonstrated to significantly improve both retroviral transduction and maintenance of regenerative potential of HSCs (Moritz et al., 1996; Dao et al., 1998).

Improvements in cytokine cocktails have also enhanced retroviral-mediated transduction efficiency of HSCs. As haematopoietic stem cells are mostly quiescent this is particularly important for gammaretroviral vectors, which are only able to transduce cells undergoing active mitosis. Amongst the earliest cytokines demonstrated to have beneficial effects on stem cell survival in culture and retroviral transduction efficiency are interleukins 3 and 6 and stem cell factor (SCF) (Bodine et al., 1989;

Luskey et al., 1992). The addition of Flt-3 ligand, IL-11 and thrombopoietin has also been shown to improve stem cell cycling whilst maintaining self-renewal and repopulating ability of HSCs (Hennemann et al., 1999; Oostendorp et al., 2000). These studies and consequent clinical trials have therefore demonstrated the possiblility to induce self-renewal of HSCs for optimal retroviral-mediated transduction without loss of engraftment potential or pluripotency.

1.10 The Murine Model of SCID-X1

1.10.1 γc-Deficient Mice

The γc-deficient mouse was created to investigate the *in vivo* role of γc in lymphoid development in addition to establishing a murine model of human SCID-X1 (DiSanto et al., 1995; Cao et al., 1995; Ohbo et al., 1996). Mice in which the γc gene has been targeted by homologous recombination display multiple abnormalities of the immune system; however the phenotype, although related, is somewhat different to that of the human disease.

In contrast to SCID-X1 infants, mice deficient in γc develop a small percentage of T lymphocytes, indicating the existence of γc-independent pathways in the murine model that enable T cell development. One candidate pathway is that involving the receptor tyrosine kinase c-kit, since mice lacking both γc and c-kit display an early and severe thymocyte-specific developmental block (Rodewald et al., 1997).

γc-deficient mice exhibit thymic hypoplasia and have almost undetectable levels of circulating lymphoid cells in the peripheral blood. In particular $CD8^+$ T cells are absent from the periphery leading to an increased CD4:CD8 ratio; this is postulated to arise from the requirement of γc-dependent cytokine signals for antigen-induced proliferation of $CD8^+$ T lymphocytes (DiSanto et al., 1996). NK cells are completely absent in this model, as are intra-epithelial lymphocytes (IELs) and TCRγδ T cells, indicating that γc differentially effects the development of lymphoid sub-lineages in mice (DiSanto et al., 1995). Despite the presence of some T cells in the SCID-X1

mouse model, these animals are functionally immunodeficient; the γc-deficient lymphocytes are unable to proliferate in response to mitogens *in vitro* (DiSanto et al., 1995) or clear intracellular pathogens such as *Listeria monocytogenes* and *Toxoplasma gondii in vivo* (Andersson et al., 1998; Scharton-Kersten et al., 1998). Consequently these mice must be maintained in a pathogen-free environment.

Histological analysis of spleens from three week old γc-deficient mice revealed severe lymphocyte hypoplasia, however an age-related increase in $CD4^+$ T cells in the spleen was observed with no concomitant increase in thymic size, resulting in splenomegaly and colitis in these animals (Cao et al., 1995; Sharara et al., 1997). The accumulated $CD4^+$ splenic T cells display an activated/memory phenotype and undergo proliferation and apoptosis at augmented rates than seen for thymocytes (Nakajima et al., 1997). Since a high proportion of γc-deficient animals develop inflammatory bowel disease it is plausible that these $CD4^+$ T cells are activated in response to self-antigens (Nakajima et al., 1997; Andersson et al., 1998). γc-deficient mice also exhibit marked extramedullary haematopoiesis in the spleen thought to result from the increased number of activated cytokine-producing $CD4^+$ T cells in the bone marrow (Sharara et al., 1997). Thus γc-dependent signals appear essential for both T cell development and homeostatic regulatory mechanisms *in vivo*.

SCID-X1 patients have normal numbers of non-functional B cells; however an incomplete block in the development of B cell progenitors in the bone marrow of γc-deficient mice results in only a small number of $B220^+$ IgM^+ mature B lymphocytes developing (DiSanto et al., 1995). The diminished B cell numbers in the SCID-X1 murine model most likely reflects the key role played by IL-7 as a pre-B cell growth factor in mice, but not in humans (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Despite a significant decrease in the numbers of surface IgM^+ B cells in the spleen, bone marrow and periphery of γc-deficient mice, levels of IgM in the sera are normal (DiSanto et al., 1995; Cao et al., 1995; Ohbo et al., 1996). IgE is however undetectable *in vivo* and γc-deficient B cells fail to undergo class-switching *in vitro* demonstrating the effects of defective IL-4 receptor function in these mice (DiSanto et al., 1995; Cao et al., 1995).

The γc-deficient mouse therefore provides a model for human SCID-X1 that recapitulates many features of the disease. This model is invaluable in evaluating the feasibility, efficacy and safety of gene therapy approaches for the treatment of SCID-X1 in an *in vivo* setting.

1.10.2 *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

The susceptibility of γc-deficient mice to develop colitis makes this model unsuitable for long-term engraftment studies; in such cases a second recipient strain, such as *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice, with a more stable phenotype is desirable.

Mice homozygous for a mutation in the *Rag2* gene are viable but fail to produce mature B or T lymphocytes due to the inability to initiate V(D)J recombination (Shinkai et al., 1992). A genetic cross with γc-deficient mice produced alymphoid mice with a stable phenotype, doubly homozygous for the mutant γc and *Rag2* alleles (*Il2rg*$^{-/-}$*Rag2*$^{-}$) (Goldman et al., 1998). These mice were developed as an immunodeficient model for human cell engraftment, useful for the study of therapies for conditions such as GvHD. To further enhance human cell engraftment in this model, a second genetic cross was performed with inbred A/J mice which are naturally deficient in complement (*c5*$^{-/-}$) (Blundell, M.P. personal communication). Thus the *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ triple-knockout mouse, deficient in T, B and NK cells, provide an ideal background with which to examine the efficacy of gene therapy vectors for immune reconstitution of SCID-X1. The alymphoid strain is therefore used as a recipient for gene-corrected γc-deficient haematopoietic stem cells in long-term engraftment studies.

1.11 Project Aims

The severe adverse events recently observed in the French SCID-X1 gene therapy trial underline the necessity of continued research into improving the safety of retroviral vectors for gene therapy. The aims of this project are therefore:

- To evaluate the novel series of self-inactivating gammaretroviral and lentiviral vectors encoding human *IL2RG in vitro*. Viral and endogenous promoters will be assessed for restoration of functional γc expression and compared to the LTR-regulated gammaretroviral vector currently used in the clinical gene therapy trial for SCID-X1.

- To evaluate restoration of lymphopoiesis in the murine model of SCID-X1 following *ex vivo* transduction of HSCs with the retroviral SIN vectors and clinical vector.

- To generate a lentiviral vector in which the eGFP transgene is regulated by the endogenous human common gamma chain promoter (γcP) and assess for promoter tissue-specificity and efficacy both *in vitro* and *in vivo*.

- To generate a lentiviral vector for the treatment of SCID-X1 incorporating the γcP and assess for efficacy in restoration of γc expression both *in vitro* and *in vivo* using the SCID-X1 murine model.

Chapter Two

Materials and Methods

2.1 Materials

Unless otherwise stated, all tissue culture reagents were supplied by Gibco BRL (Invitrogen), all general chemicals were supplied by Sigma and all cytokines were supplied by Peprotech.

General Reagents and Enzymes

1kb Plus DNA Ladder	Invitrogen
Restriction Endonucleases	Promega
Benzonase Nuclease	Novagen
Calf intestinal alkaline phosphatase	New England Biolabs
Proteinase K, PCR-grade	Roche
Agarose	Invitrogen
Agar	MERCK
(methyl-^{3}H) Thymidine 88.0Ci/mmol	Amersham
Retronectin	Takara
Red Cell Lysis Buffer	EBioscience
MethoCult GF M3434	StemCell Technologies
Perm buffer III	Pharmingen
Lyse/Fix buffer	Pharmingen
Ampicillin	Stratagene
ELISA Duo-Set substrate reagent pack	R&D Systems
Formaldehyde (methanol free) 16% Ultrapure	Polysciences, Inc
Ficoll-Paque PLUS	Amersham Biosciences
Cyclosporin A	Sandimmune
Aciclovir	DBL
Sucrose	BDH
Magnesium Chloride	Fisher Scientific

Cell Lines

293T	Human embryonic kidney cell line
HT1080	Human fibrosarcoma cell line (ATCC# CCL-121)
SC-1	Murine fibroblast cell line (ATCC# CRL-1404)
C2C12	Murine myoblast cell line (ATCC# CRL-1772)
U937	Human monocytic cell line (ATCC# CRL-1593.2)
Hep-G2	Human hepatocellular carcinoma cell line (ATCC# HB-8065)
B95-8	Marmoset cell line (ATCC# CRL-1612) that releases high titres of transforming EBV. Supernatant from this cell line was a kind gift from Persis Amrolia (Molecular Immunology Unit, Institute of Child Health).
Phoenix-gp	293-T cells stably transfected with a construct expressing MLV gag-pol proteins for the production of retroviral particles.
SCID-X1 LCL	EBV-transformed peripheral blood mononuclear cells from a SCID-X1 patient at Great Ormond Street Hospital
ED-7R	A kind gift from Professor Ian Alexander at The Children's Hospital, Westmead, Sydney, Australia. This cell line is derived from a γc-deficient T cell line ED40515$^-$ (Ishii et al., 1994) and is stably transduced with a retrovirus encoding the human IL-7Rα receptor cDNA.
ED-7R-γc	These cells were also a gift from Professor Ian Alexander; they are derived from ED-7R cells and stably express human γc.
OP9-eGFP	A kind gift from Dr. Juan Carlos Zúñiga-Pflücker, University of Toronto, Canada. This mouse stromal cell line is stably transduced with a retroviral vector expressing eGFP.
OP9-DL1	Also from Dr. Juan Carlos Zúñiga-Pflücker's laboratory, this mouse stromal cell line is stably transduced with a retroviral vector expressing eGFP and the Delta-Like-1 notch ligand.

Lin$^-$ cell isolation and culture

StemSep mouse progenitor enrichment cocktail	StemCell Technologies
StemSep 0.6" negative selection columns	StemCell Technologies
Stemspan SF expansion medium	StemCell Technologies

Antibodies

Unless otherwise stated below all antibodies were supplied from BD Pharmingen.

IgM-FITC	Jackson Immunoresearch
Goat anti-human *IL2Rγ*	R&D Systems
Anti-goat IgG-HRP	R&D Systems
Monoclonal anti-β-actin	Sigma
Polyclonal goat anti-mouse immunoglobulins-HRP	DakoCytomation
Rabbit anti-mouse IgG	Serotec
Polyclonal rabbit f(ab')$_2$ anti-mouse Ig biotin	DakoCytomation

PCR

Primers	Invitrogen
Taq DNA polymerase, dNTPs, buffer	Promega
DNA polymerase I large (Klenow) fragment	Promega
PfuTurbo DNA polymerase, buffer	Stratagene

Quantitative Real-Time PCR

Primers	Invitrogen
Probes	MWG
Platinum qPCR SuperMix-UDG with ROX	Invitrogen

Kits

Calcium phosphate (CAPHOS) transfection kit	Sigma
Plasmid megaprep/maxiprep kit	Qiagen
QIAquick gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
HIV-1 p24 Antigen ELISA	Beckman Coulter

Western Blotting

M-PER mammalian protein extraction reagent	Pierce
Halt protease inhibitor cocktail	Pierce
Laemmli sample buffer	Sigma
SeeBlue Plus2 pre-stained standard	Invitrogen
NuPAGE NOVEX Bis-Tris gels	Invitrogen
MOPS Buffer	Invitrogen
SDS PAGE running buffer	Invitrogen
ECL Western blotting substrate	Pierce

Bacteria

Escherichia coli strains used:

DH5α Competent Cells — Invitrogen

Used for all plasmid transformations.

Genotype: F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^- m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1

ElectroMAX Stbl4 — Invitrogen

Used for all cloning procedures.

Genotype: *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *rec*A1 *end*A1 *gyr*A96 *gal*$^-$ *thi*-1 *sup*E44 λ^- *rel*A1 Δ(*lac-pro*AB)/F′ *pro*AB$^+$ *lac*Iq *lac*ZΔM15 Tn*10* (Tet^R)

Centrifuges

Microcentrifuge	Heraeus Biofuge Fresco
Tabletop centrifuge	Sorvall Legend RT
Superspeed centrifuge	Sorvall Evolution RC
Ultracentrifuge	Sorvall *Discovery* SE

2.2 Methods

2.2.1 Bacterial Manipulation

2.2.1.1 Growth and Maintenance of E.coli

Escherichia coli (*E.coli*) were grown in liquid LB media at 37°C with agitation at 250 rpm or streaked out on solid LB plates containing 1.5% bacto agar. *E.coli* transformed with plasmids was grown on the same media supplemented with ampicillin (50 μg/ml).

For long-term storage, bacterial cultures were stored in 15% volume for volume (v/v) glycerol at -70°C.

2.2.1.2 Production of Electro-competent E.coli

A 1 L flask of LB media was inoculated 1:1000 from a fresh overnight culture of *E.coli* DH5α or ElectroMAX Stbl4 cells. The culture was grown at 37°C with agitation until the optical density at 550 nm reached between 0.7-0.8. The cells were harvested by centrifugation at 5000 rpm in a superspeed centrifuge for 10 minutes, the supernatant discarded and the cell pellet resuspended in 1 L of cold sterile 10% glycerol. The spin was repeated, the supernatant discarded and the cell pellet was resuspended in a second

1 L of cold sterile 10% glycerol. The cells were centrifuged again for a final time, after which the supernatant was carefully discarded and the cells resuspended in the small remaining volume of glycerol. The cells were transferred to a smaller centrifuge tube and spun at 7000 rpm for 10 minutes. The supernatant was removed and the cell pellet resuspended in 2 ml cold sterile 10% glycerol; 100 µl of cells were transferred to microfuge tubes and snap-frozen in a dry ice-ethanol bath. The electro-competent cells were subsequently stored at -70°C.

2.2.1.3 Bacterial Transformation

E.coli were transformed by electroporation; 50µl of electro-competent cells were thawed slowly on ice and mixed with 1-10 ng of DNA. The cell/DNA mixture was then pipetted into pre-chilled disposable micro-electroporation cuvettes (0.2 cm) and electroporated at 2.5 kV, 200 Ω, 25 µF using a Bio-Rad Gene Pulser. 1 ml of room temperature LB media was added to the cuvette and the media/bacterial mixture was transferred to a 5 ml tube. The cells were shaken at 250 rpm at 37°C for 1 hour, after which they were diluted in LB media and spread on LB agar plates containing ampicillin (50 µg/ml). The plates were incubated overnight at 37°C, after which colonies were picked using sterile 20 µl pipette tips and grown overnight in 5 ml liquid cultures.

2.2.2 Plasmid DNA Preparation

DNA for the SRS II series of retroviral vectors and the SINLV lentiviral vector series, together with the relevant packaging plasmids were kindly provided by Dr A. Schambach, Hannover Medical School, Germany.

2.2.2.1 Small-scale Plasmid DNA Preparation

Plasmid DNA was prepared using Qiagen Mini-Prep kits as per the manufacturer's instructions from 5 ml overnight cultures.

2.2.2.2 Large-Scale Plasmid DNA Preparation

For large-scale plasmid DNA preparation 500 ml LB media containing ampicillin (50 µg/ml) was inoculated with 500 µl of a fresh 5 ml culture and incubated over night at 37°C with agitation (250 rpm). Plasmid DNA was subsequently prepared using Qiagen Mega-Prep kits as per the manufacturer's instructions.

2.2.3 Measurement of DNA Concentration

Plasmid DNA concentration was calculated by measuring the absorbance of light with a wavelength of 260 nm (A_{260}) using a NanoDrop ND-1000 spectrophotometer with a 0.2 mm pathlength; at this wavelength 50 µg/ml of double-stranded DNA has an absorbance of 1.

2.2.4 Cell Culture

2.2.4.1 Propagation of Adherent Cell Lines

All adherent cell lines were maintained in Dulbecco's modified eagle medium (DMEM) containing GlutaMAX supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin (referred to as complete DMEM). Cells were grown in 25 cm^2, 80 cm^2 or 175 cm^2 tissue culture flasks or in 10 cm tissue culture dishes in 37°C incubators in a 5% CO_2 atmosphere. Cells were passaged when 80-90% confluent; the monolayers were first washed with Dulbecco's phosphate buffered saline

(1x) (PBS) and then incubated for 5 minutes at 37°C with trypsin/EDTA. The cells were subsequently diluted 1:10 in fresh complete DMEM and transferred to new tissue culture flasks.

2.2.4.2 Propagation of Non-Adherent Cell Lines

Non-adherent cell lines were maintained in RPMI 1640 media containing GlutaMAX supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin (referred to as complete RPMI). Cells were grown in 25 cm^2, 80 cm^2 or 175 cm^2 tissue culture flasks (standing upright) in 37°C incubators in a 5% CO_2 atmosphere. Cells were passaged following media colour-change; the cells were transferred to 15 ml centrifuge tubes and centrifuged at 1200 rpm in a tabletop centrifuge for 5 minutes, washed with PBS, diluted 1:10 or 1:20 in fresh complete RPMI and transferred to new tissue culture flasks.

2.2.4.3 Long Term Storage of Cell Lines

For long term storage of cell lines a 90% confluent monolayer from a 80 cm^2 tissue culture flask or 2-5 x 10^6 cells were pelleted by centrifugation at 1200 rpm in a tabletop centrifuge for 5 minutes, resuspended in 1 ml freezing medium (90% FCS, 10% dimethylsulfoxide) and transferred to a cryovial. Cells were frozen slowly overnight to -70°C in an isopropanol freezing box and then transferred to liquid nitrogen.

To revive frozen cells - aliquots were thawed rapidly in a 37°C waterbath and slowly transferred to 9 ml cold growth medium. The cells were pelleted at 1200 rpm in a tabletop centrifuge to remove the dimethylsulfoxide and then resuspended in 5 ml growth medium and transferred to a 25 cm^2 tissue culture flask.

2.2.4.4 Generation of SCID-X1 LCLs

10 ml of anticoagulant-treated peripheral blood from a male SCID-X1 infant at Great Ormond Street Hospital was diluted with 10 ml RPMI and layered onto 15 ml Ficoll-Paque PLUS in a 50ml falcon tube. The sample was centrifuged at 2300 rpm for 30 minutes in a table-top centrifuge (with no brake) and the peripheral blood mononuclear cells subsequently aspirated from the ficoll-plasma interface and transferred to a clean falcon tube. The cells were washed twice with RPMI and then counted. 5 x 10^6 cells were centrifuged at 1200 rpm and resuspended in 200 µl Epstein Barr Virus (EBV) supernatant (produced from a B95-8 cell line) by gently flicking the base of the falcon tube. 1.8 ml complete RPMI supplemented with cyclosporin A (1 µg/ml) was added and 200 µl of the resuspended cells (5 x 10^5 cells) was aliquoted into five wells of a 96-well flat bottom tissue culture plate. A further 1 ml of complete RPMI containing cyclosporin A was added to the remainder of the cells which were then aliquoted (2.5 x 10^5 cells – 200 µl/well) into 10 wells of the 96-well plate. The outer wells of the plate were filled with sterile distilled water and the cells were incubated at 37°C/5% CO_2. 1 week later 100 µl of media was removed from each well and replaced with 100 µl fresh complete RPMI. After 2 weeks of incubation the cells were beginning to expand and one of the 5 x 10^5 cell wells was combined with two of the 2.5 x 10^5 cell wells into a single well of a 24-well tissue culture plate. After one week of incubation at 37°C/5% CO_2 the expanded cells were transferred to a 25 cm^2 vented tissue culture flask in 5 ml complete RPMI. The cells were treated with acyclovir for two weeks to remove any remaining EBV.

2.2.4.5 OP9 Stromal Layers

Early passage OP9-eGFP and OP9-DL1 stromal cells were grown to confluency in 10 cm tissue culture dishes in Minimum essential media (MEM) alpha supplemented with 20% (v/v) foetal calf serum and 5% (v/v) penicillin/streptomycin. 2 x 10^4 stromal cells per well were seeded in 24-well tissue culture plates and left to adhere overnight. The following day the media was removed from the plates and 6,500-10,000 lin^- cells (transduced or untransduced) were seeded on top of the stromal layers in 2 ml of co-

culture media (see below). Every four days 1 ml of media was removed from the top of the plates so as not to disturb the differentiating lin$^-$ cells and 1 ml of fresh co-culture media added. On days six and thirteen the cells were removed from the plates by vigorous pipetting, strained through a 70 μm nylon cell strainer to remove the OP9 stromal layers, stained with antibodies to lymphocyte cell surface markers and analysed by flow cytometry for lymphoid differentiation.

OP9-eGFP co-culture media

420 ml MEM alpha
60ml foetal calf serum
250 μl murine IL-7 (10 ng/ml)
250 μl murine Flt-3 (5 ng/ml)
10.5 ml PG2 solution
10.5 ml HSG solution

PG2 Solution

5 ml Penecillin/Streptomycin
5 ml Sodium Pyruvate (100 mM)
0.5 ml 2-Mercaptoethanol (50 mM)

OP9-DL1 co-culture media

420 ml MEM alpha
60 ml foetal calf serum
25 μl murine IL-7 (10 ng/ml)
250 μl murine Flt-3 (5 ng/ml)
10.5 ml PG2 solution
10.5 ml HSG solution

HSG Solution

5 ml HEPES (100 mM)
5 ml Sodium Pyruvate (100 mM)
0.5 ml gentamicin (50 mg/ml)

2.2.4.6 Isolation and Culture of Murine Lin$^-$ Cells

Bone marrow cells were harvested from the femurs and tibias of *Il2rg*$^{-/-}$ or C57/Bl6 mice, pelleted by centrifugation at 1200 rpm in a table-top centrifuge and resuspended in PBS 1% BSA within the range of 2-8 x 10^7 cells/ml. The lin$^-$ cells were subsequently isolated using a StemSep mouse progenitor enrichment cocktail and negative selection columns as per the manufacturer's instructions (StemCell Technologies). Murine lin$^-$ cells were cultured in StemSpan SFEM serum-free medium supplemented with 1% (v/v) penicillin/streptomycin and cytokines (100 ng/ml mSCF, 100 ng/ml mFlt-3, 100 ng/ml hIL-11, 20 ng/ml mIL-3).

2.2.4.7 Methylcellulose Colony Forming Unit Assay

1 x 10^4 transduced or untransduced murine lin$^-$ cells were washed in Dulbecco's phosphate buffered saline (PBS), pelleted by centrifugation at 1200 rpm in a table-top centrifuge and resuspended in 450 µl RPMI. 50 µl of 10x benzonase buffer (100 mM $MgCl_2$) and 25 units (1µl) of benzonase nuclease was added and the cells were incubated at 37°C for 20 minutes to remove any contaminating plasmid DNA. The cells were then pelleted by centrifugation at 1200 rpm and resuspended in 300 µl RPMI. 3 ml of methocult was mixed with the cells and 1.5 ml seeded into 10 mm tissue culture dishes and incubated at 37°C for 14-21 days. Approximately 28 colonies per condition were picked using a P20 Gilson pipette, lysed immediately in 20 µl DNA lysis buffer (10 mM Tris 1 mM EDTA pH 8.0, 0.5% NP40, 0.5% Tween 20, 1.25 mg/ml Proteinase K) and stored at -20°C. Genomic DNA was subsequently isolated following the protocol outlined in 2.2.14.

2.2.5 Flow Cytometry

All samples were analysed using a CyAn ADP flow cytometry analyser and Summit version 4.1 software (DakoCytomation).

2.2.5.1 Staining of Primary Murine Cells

For analysis by flow cytometry, all primary murine cells (except thymocytes and purified lin$^-$ cells) were first treated with red cell lysis buffer for 5 minutes at room temperature with occasional shaking – 1 ml of lysis buffer was added per 100 µl of peripheral blood, whilst pelleted splenocytes or bone marrow cells were resuspended in 5 ml lysis buffer. The lysis reaction was stopped by dilution with 10 ml PBS and cells were pelleted by centrifugation at 1200 rpm for 5 minutes in a table-top centrifuge and resuspended in the appropriate volume of FACS buffer (PBS, 0.5% (w/v) BSA, 0.05% (v/v) NaN_3). All primary cells were treated with mouse Fc Block (anti-mouse

CD16/CD32 (Fcγ III/II receptor)) to prevent high background staining; 2 µl Fc block (0.5 mg/ml) was added per 1 x 10^6 cells in 100 µl FACS buffer and cells were incubated on ice for 10 minutes. Cells were pelleted by centrifugation at 1200 rpm for 5 minutes, resuspended in 100 µl FACS buffer containing a 1:100 (FITC-conjugated) or 1:200 (APC- or PE-conjugated) dilution of the appropriate antibody and incubated on ice for 30 minutes in the dark. For analysis of T lymphocyte reconstitution, cells were stained with the following anti-mouse antibodies: CD3e-FITC, CD8a-APC, and CD4-FITC. For analysis of B lymphocyte reconstitution cells were stained with the following anti-mouse antibodies: CD45R/B220-APC and IgM-FITC. For analysis of NK and myeloid populations, cells were stained with the following anti-mouse antibodies: NK1.1-APC and CD11b-PE. Cells cultured on OP9 stromal layers were stained with anti-mouse CD25-PE and CD44-APC for analysis of T cell differentiation or with anti-mouse CD45R/B220-APC and CD-19 FITC for analysis of B cell differentiation. Following staining cells were washed once with 1 ml FACS buffer, pelleted by centrifugation and resuspended in 300 µl FACS buffer and transferred to FACS tubes. Cells were stored in the dark at 4°C and analysed by flow cytometry within 24 hours of staining.

2.2.5.2 Staining of Transduced Cell Lines for Human γc Expression

Transduced cells were removed from tissue culture plates and washed once with FACS buffer to remove any traces of media. For analysis of surface human γc expression, 1 x 10^6 cells were stained with 0.5 µl anti-human CD132-PE antibody in 100 µl FACS buffer on ice, in the dark for 30 minutes. Cells were subsequently washed with 1 ml FACS buffer, spun at 1200 rpm for 5 minutes and then resuspended in 300 µl fresh FACS buffer. Cells were stored in the dark at 4°C and analysed by flow cytometry within 24 hours of staining.

2.2.6 Western Blotting

5 x 10^5 primary or cultured cells were washed in PBS and pelleted in 1.5 ml microfuge tubes by centrifugation at 1200 rpm for 5 minutes in a table-top centrifuge. Protein was harvested from cell pellets by resuspension in 100 µl M-PER mammalian protein extraction reagent supplemented with 1 µl HALT protease inhibitor cocktail and incubation on ice for 10 minutes with occasional shaking. The cell lysates were spun at 13,000 rpm in a microfuge at 4°C for 15 minutes and the supernatants containing the nuclear and cytoplasmic protein transferred to fresh eppendorfs. The samples were diluted 1:1 with 100 µl 2 x Laemmli sample buffer and heated to 95°C for 5 minutes. 30µl of protein samples were loaded onto 4-12% Bis-Tris Novex pre-cast gels with 10 µl of SeeBlue Plus2 pre-stained standard loaded into the outer-most well. Protein samples were separated by polyacrylamide gel electrophoresis at 200 V for 50 minutes in an XCell *SureLock* Mini-Cell (Invitrogen) in 1 x NuPAGE MOPS SDS running buffer. The separated proteins were subsequently immunoblotted onto PVDF membrane using the XCell II blot module (Invitrogen) at 30 V for 1 hour. The PVDF membrane was washed with methanol for 30 seconds, rinsed in distilled water and then washed in 1 x NuPAGE transfer buffer prior to immunoblotting.

Following immunoblotting, PVDF membranes were incubated overnight with blocking buffer (PBS, 0.1% (v/v) Tween 20, 5% (w/v) milk powder) to prevent non-specific antibody staining. Membranes were subsequently washed five times in PBS-T (PBS, 0.1% (v/v) Tween 20) and incubated with primary antibody in 3 ml blocking buffer for 2 hours at room temperature with agitation. The membranes were washed a further five times in PBS-T and then incubated with an HRP-conjugated secondary antibody in 3 ml blocking buffer for 45 minutes at room temperature with agitation. The membranes were washed for a final five times after which 1 ml of ECL Western blotting substrate was added directly to the membrane and incubated for 1 minute at room temperature. Protein bands were visualised using an UVIchemi (UVItec) chemiluminescence documentation system and UVIsoft (UVItec) software.

2.2.7 pSTAT5 Assay

$1x10^5$ transduced or mock-transduced ED-7R cells and untransduced ED-7R-γc control cells were incubated overnight in complete RPMI media. The following day the cells were washed once in PBS, pelleted by centrifugation in a table-top centrifuge at 1200 rpm and resuspended in 200 µl of RPMI only. The cells were split between two capped FACS tubes (100 µl in each) and 6 µl human IL-2 (100 ng/µl) was added to one of the tubes. The cells were incubated at 37°C/5% CO_2 for 10 minutes, at which time 2 ml of pre-warmed (to 37°C) FACs lyse/fix buffer (1:5 dilution) was added to each tube. The cells were then incubated for a further 10 minutes at 37°C/5% CO_2. The cells were pelleted by centrifugation, after which the supernatant was decanted and the cell pellets resuspended in the small volume of remaining media at the bottom of the tubes. 1 ml of cold Perm Buffer III was then added to each tube and the cells incubated on ice for 30 minutes. The cells were pelleted and washed once with 1 ml FACs buffer. The cells were then stained on ice for 30 minutes in the dark with 5 ul anti-pSTAT5-PE in 100 µl FACs buffer. The cells were subsequently washed twice with 1 ml FACs buffer and finally resuspended in FACS buffer supplemented with 1% (v/v) paraformaldehyde solution and analysed by flow cytometry.

2.2.8 Gammaretrovirus Preparation and Transductions

2.2.8.1 Gammaretrovirus Production

Phoenix-gp packaging cells were grown in complete DMEM; the day before transfection, 5 x 10^6 cells were seeded in 10 cm tissue culture dishes. Retroviral vector DNA (8 µg) and packaging plasmids were transfected using the calcium phosphate precipitation method. To generate ecotropic retroviral particles the packaging plasmids used were as follows, 10 µg M57 (gag/pol expression plasmid) and 2 µg K73 or 5 µg K37 (Ecotropic or RD114 envelope expression plasmids repectively). The DNA was added to sterile distilled water to make a total volume of 450 µl to which 50 µl $CaCl_2$ (2.5 M) was added. 15 ml sterile falcon tubes were pre-loaded with 500 µl 2 x HEPES

buffered saline (HBS) and the solution was bubbled using an electronic pipettor and sterile 1 ml pipette whilst the DNA/$CaCl_2$ solution was added drop by drop. The mixture was incubated for 20 minutes at room temperature during which time the complete DMEM was removed from the plates containing the phoenix-gp cells and replaced with 9 ml of transfection medium (complete DMEM with 20 mM HEPES) supplemented with 25 μM chloroquine (to inhibit DNA degradation by lysosomes). The 1 ml transfection reaction was then added to the plates slowly whilst gently swirling the media to evenly distribute the calcium phosphate/DNA precipitate. After 6 hours incubation at 37°C/5% CO_2 the media was replaced with fresh transfection media without chloroquine. Supernatants containing the viral particles were collected at 24, 36, 48 and 60 hours post-transfection, pooled and filtered through a 0.22 μm filter. Viral supernatant was concentrated by centrifugation at 18,500 g, overnight at 4°C for 14-16 hours. Retroviral pellets were resuspended in DMEM supplemented with 20 mM HEPES and stored in aliquots at -70°C.

2.2.8.2 Titration of Gammaretroviral Supernatants

Ecotropic and RD114 pseudotyped gammaretroviral preparations were titrated on murine SC-1 fibroblast cells and human HT1080 fibrosarcoma cells respectively.
5 x 10^4 cells/well were seeded in 12-well tissue culture plates in complete DMEM and left to adhere overnight. The following day the media was removed and serial dilutions of concentrated gammaretroviral supernatant were added directly to the cells and topped up with 500 μl complete DMEM supplemented with protamine sulphate (4 μg/ml). The plates were spun at 950 g for 1 hr in a table-top centrifuge and then incubated at 37°C/5% CO_2 for 6 hours, after which the cells were topped up with 1.5 ml complete DMEM. The plates were incubated for two days at 37°C/5%CO_2, at which time the cells were harvested and analysed by flow cytometry. Viral titre (transducing units per ml of supernatant) was determined by multiplication of the number of transgene-positive cells by the dilution.

2.2.8.3 Transduction of SC-1 cells with Ecotropic Gammaretrovirus

5 x 10^4 SC-1 cells per well were seeded in 12-well plates in complete DMEM and left to adhere for three hours or overnight. The media was subsequently aspirated and the virus added directly to the cells, 500 µl complete DMEM supplemented with protamine sulphate (4 µg/ml) was added to each well and the plates were spun at 950 g for 1 hour in a tabletop centrifuge. The cells were then incubated at 37°C/5% CO_2 for 6 hours after which time the cells were topped up with 1.5 ml complete DMEM. 48 hours later the cells were removed from the plates for analysis.

2.2.8.4 Transduction of SCID-X1 LCLs and ED-7R cells with RD114 Pseudotyped Gammaretrovirus

24-well non-tissue culture treated plates were coated with retronectin (48 µg/ml) overnight at 4°C or for 2 hours at ambient temperature. The plates were washed twice with PBS 1% BSA and then once with PBS only. RD114 pseudotyped gammaretrovirus was then added to each well, together with 500 µl RPMI and plates were spun at 950 g in a table-top centrifuge at 4°C for 30 minutes. The media and gammaretroviral supernatant was then removed and the SCID-X1 LCLs or ED-7R cells were added to the virally pre-loaded wells in complete RPMI. The cells were removed for analysis 48 hours later.

2.2.8.5 Gammaretroviral Lin$^-$ Cell Transduction

Lin$^-$ cells were pre-stimulated for 48 hours in StemSpan SFEM serum-free medium supplemented with 1% (v/v) Penecillin/Streptomycin, mSCF (100 ng/ml), mFlt3 (100 ng/ml), mIL-3 (20 ng/ml) and hIL-11 (100 ng/ml) at a density of 0.5-1 x 10^6 cells/ml. Non tissue-culture 6-well plates were coated with 2 ml retronectin (50 µg/ml) and incubated overnight at 4°C. The retronectin-coated plates were washed twice with PBS 1% BSA and then once with PBS only and subsequently preloaded with gammaretroviral supernatant by centrifugation for 30 minutes at 950 g at 4°C.

Following centrifugation the gammaretroviral supernatant was removed and the pre-stimulated lin$^-$ cells transferred to the virally pre-loaded plates and incubated overnight at 37°C, 5% CO_2. On day four gammaretrovirus was again pre-loaded onto retronectin-coated plates, the lin- cells were removed from the intial plates using enzyme-free cell dissociation buffer and transferred to the new plate for a second of transduction. The transduced cells were harvested on day five for intravenous injection into irradiated mice or for co-culture on OP9 stromal layers.

2.2.9 Lentivirus Preparation and Transductions

2.2.9.1 Lentivirus Production

1.2 x 10^7 293T cells grown in complete DMEM were seeded in 175 cm^2 tissue culture flasks the day before transfection. Lentiviral vector DNA (40 μg) and packaging plasmids pMDG.2 (VSV-G) (10 μg) and pCMVΔ8.74 (gag-pol) (30 μg) were added to 5 ml OPTI-MEM, filtered through a 0.22μm filter and combined with 5 ml filtered OPTI-MEM supplemented with 1 μl polyethylenimine (PEI) (10 mM) transfection reagent. The transfection reaction was incubated at room temperature for 20 minutes during which time the 293T cells were washed once with OPTI-MEM media. The 10 ml DNA/PEI complexes were subsequently added to the cells and they were then incubated at 37°C/5%CO_2 for 4 hours, after which the media was replaced with 14 ml complete DMEM. Viral supernatant was harvested 48 and 72 hours post-transfection, filtered through a 0.22 μm filter and concentrated by ultracentrifugation at 98,000 g for 2 hours. Retroviral pellets were resuspended in serum-free media (DMEM or StemSpan SFEM) and stored in aliquots at -70°C.

2.2.9.2 Titration of Lentiviral Supernatants - Infectious Titre

Viral titre was determined by the transduction of SC-1, HT1080 or ED-7R cells (dependent upon the experiment to be performed with the specific lentiviral preparation). 5 x 10^4 cells/well were seeded in 12-well tissue culture plates and left to

adhere for 3 hours (SC-1 and HT1080s only). The media was subsequently aspirated from the cells and serially diluted lentiviral supernatant added. Infectious viral titre was determined by the analysis of transgene-positive cells by flow cytometry 72 hours post-transduction.

2.2.9.3 Titration of Lentiviral Supernatants - p24 Titre

The quantity of p24 antigen in lentiviral supernatants was measured using a Coulter HIV-1 p24 antigen assay as per the manufacturer's instructions. Supernatants were diluted 1:1 x 10^6, 1:5 x 10^6 and 1:1 x 10^7 and added to p24 antibody-coated wells together with diluted calibration standards. Samples were lysed, washed and then incubated with biotinylated anti-HIV-1. The samples were washed again and subsequently incubated with streptavidin-HRPO followed by the addition of tetramethybenzidine (TMB) and Coulter stop reagent (4N H_2SO_4). The absorbance of each plate was then read at 450 nm and the quantity of p24 reagent calculated from a standard curve generated from the diluted calibration standards.

2.2.9.4 Lentiviral Cell Line Transduction

Cells were seeded at a density of 5 x 10^4 cells/well in 12-well tissue culture plates in the appropriate growth medium and left to adhere (adherent cell lines only) for three hours or overnight at 37°C/5% CO_2. Concentrated lentiviral supernatant was added directly to the growth media and the cells were incubated at 37°C/5% CO_2 for 48 hours and subsequently analysed for transgene expression by flow cytometry.

2.2.9.5 Lentiviral Lin$^-$ Cell Transduction

Lin- cells were seeded at a density of 0.5-1 x 10^6 cells per ml in StemSpan SFEM serum-free media supplemented with 1% (v/v) penicillin/streptomycin and cytokines (mSCF (100 ng/ml), mFlt3 (100 ng/ml), mIL-3 (20 ng/ml), hIL-11 (100 ng/ml)). Concentrated lentiviral supernatant was added directly to the growth media and the

cells were transduced overnight at 37°C/5% CO_2. The following day the transduced cells were harvested for intravenous injection into irradiated mice or for co-culture on OP9 stromal layers.

2.2.10 Murine Reconstitution Experiments

Between 3-10 x 10^5 transduced lin^- cells, resuspended in 300 µl RPMI, were intravenously injected into the tail veins of sublethally irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice using a 27 G bevel needle attached to a tuberculin syringe. Recipients were irradiated with a total of 6 Gy in a split dose over two days.

2.2.11 Murine Secondary Transplantations

1 x 10^8 whole bone marrow cells isolated from the femurs and tibias of primary transplant recipients were resuspended in 300 µl RPMI and intravenously injected into the tail veins of lethally irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice using a 27 G bevel needle attached to a tuberculin syringe. Recipients were irradiated with a total of 10 Gy in a split dose over two days.

2.2.12 Splenocyte Proliferation Assay

Splenocytes were plated at a density of 2 x 10^5 cells/well, in triplicate, in 96-well plates in complete RPMI media. The cells were stimulated for 48 hours at 37°C/5% CO_2 with and without 1 mg/ml Concanavalin A and/or IL-2 (20 ng/ml). The cells were then pulsed with 1 µCi/well of methyl-^{3}H-thymidine for 12 hours and uptake determined using a beta-counter (MicroBeta TAILUX, Wallac).

2.2.13 Immunoglobulin ELISAs

Approximately 100 µl of peripheral blood was collected from the cardiac chambers of transplanted mice immediately following sacrifice and stored at room temperature for approximately one hour to allow coagulation. Samples were then centrifuged at 13,000 rpm in a micro-centrifuge for 10 minutes and the serum transferred to fresh eppendorfs and stored at -20°C.

Maxisorp 96-well NUNC-Immuno ELISA plates (NUNC) were coated overnight with 100 µl/well capture antibody (see table 2. - diluted to the appropriate concentration in PBS), wrapped in cling film and stored overnight at 4°C. The coated plates were washed three times with wash buffer (100 µl PBS/0.05% (v/v) Tween 20) and blocked for 1 hour at room temperature in the appropriate blocking buffer (see table 2.1). The plates were washed a further three times and 100 µl of diluted serum added in triplicate. Plates were incubated at room temperature for 1 or 2 hours (see table 2.1), at which time they were washed three times. 100 µl/well of detection antibody (see table 2.1) was added to each well and plates were incubated at room temperature for 1 hour (for the IgG1 and IgG2a ELISAs in which the detection antibody is HRP conjugated this incubation was performed in the dark). The plates were subsequently washed three times; for the IgG ELISA, 100 µl/well of Streptavidin-HRPO was added and the plates were incubated for 30 minutes at room temperature in the dark before a further three washes were performed. Substrates A and B from the ELISA Duo-set substrate reagent pack were mixed in equal quantities, 100 µl of the mixed solutions was added to each well and the plates were incubated at room temperature in the dark for approximately 10 minutes to allow colour change, at which time the reaction was stopped by adding 50 µl stop solution/well (2N H_2SO_4). The absorbance of each plate was then read at 405 nm using a FLUOstar Optima plate reader (BMG LABTECH).

ELISA	Capture antibody	Blocking Buffer	Serum dilutions	Sample incubation time	Detection Antibody	Tertiary antibody
IgG	Rabbit anti-mouse IgG (1:500)	PBS/ 1%BSA/ 5% Sucrose	1:4000 - 1:128000	1 hour	Polyclonal rabbit F(ab')2 anti-mouse Ig-biotin (1:5000)	Strep-HRPO (1:1000)
IgG1	Rabbit anti-mouse IgG1 (2µg/ml)	PBS/ 1%BSA	1:750 - 1:6000	1 hour	HRP-rat anti-mouse IgG1 (2µg/ml)	-
IgG2a	Rabbit anti-mouse IgG2a (2µg/ml)	PBS/ 1%BSA	1:750 - 1:6000	1 hour	HRP-rat anti-mouse IgG2a (2µg/ml)	-

Table 2.1. The antibodies and conditions used for immunoglobulin ELISAs.

2.2.14 Genomic DNA Isolation

5 x 10^4 cells were pelleted by centrifugation at 13,000 rpm for 10 minutes in a micro-centrifuge, resuspended in 20 µl DNA lysis buffer (10 mM Tris, 1 mM EDTA pH 8.0, 0.5% NP40, 0.5% Tween 20, 1.25 mg/ml Proteinase K) and incubated at 56°C for 2 hours. Lysed cell pellets were pulsed at 13,000 rpm, incubated at 95°C for 15 minutes and then pulsed again at 13,000 rpm. 180µl of distilled DNase-free water was added, followed by centrifugation at 13,000 rpm for 5 minutes. The supernatant containing the genomic DNA was then transferred to sterile eppendorfs and kept at 4°C for short-term storage and transferred to -20°C for long-term storage.

2.2.15 PCR

PCR reactions were performed in a total volume of 25 µl containing 100 ng template DNA, forward and reverse primers at 0.5 µM, dNTPs, each dNTP at 200 µM and 2.5 U of *Taq* DNA polymerase in the appropriate buffer (Promega). The concentration of $MgCl_2$ to be used was determined for each specific reaction (typically 1.5 mM

however). Typically cycling conditions were as follows: 30 cycles of 96°C for 30 seconds, 58°C for 1 minute, 72°C for 1 minute.

2.2.15.1 Calculation of Lin$^-$ Cell Transduction Efficiency by PCR from Methylcellulose Colonies

Retroviral transduction efficiency was calculated by PCR from DNA isolated from methylcellulose colonies using gammac-F and gammac-R primers specific for the *IL2Rg* cDNA. The reaction was performed at an annealing temperature of 60°C and amplified a 459 bp DNA product from cells transduced with gammaretroviral or lentiviral vectors encoding the human *IL2RG* transgene.

Forward primer – gammac-F (exon 3): 5' CTGGCTGTCAGTTGCAAAAA 3'
Reverse primer – gammac-R (exon 8): 5' GAGATAACCACGGCTTCCAA 3'

To confirm the presence of genomic DNA from each picked colony and to determine transduction efficiency, a PCR was performed using HPRT-F and HPRT-R primers to exon 9 of the murine housekeeping gene, *Hprt*. The reaction was performed at an annealing temperature of 58°C and amplified a 324 bp product.

Forward primer – HPRT-F: 5' TCCCCAGACTTTTGATTTGC 3'
Reverse primer – HPRT-R: 5' GGAAAATACAGCCAACACTGC 3'

2.2.15.2 PCR of the IL2RG Promoter

The 1.24 kb promoter was amplified from human genomic DNA using a high fidelity *PfuTurbo* DNA polymerase and primers hIL2RG prom-XhoF and hIL2RG prom-BamR or hIL2RG prom-EcoF and hIL2RG prom-BamR incorporating restriction endonuclease recognition sequences at their 5' ends. The PCR reactions were performed at an annealing temperature of 61°C.

Forward primers:
hIL2RG prom-XhoF: 5' GTCCTCGAGCTATCCCCCTCTCCATCTT 3'
hIL2RG prom-EcoF: 5' GCGAATTCCTATCCCCCTCTCCATCTTG 3'

Reverse primer:
hIL2RG prom-BamR: 5' ATGGATCCGGCGCTTGCTCTTCATTC 3'

2.2.15.3 Purification of PCR Products

PCR products to be used for cloning procedures were purified using a QIAquick PCR purification kit as per the manufacturer's instructions.

2.2.16 Quantitative Real-Time PCR for the Determination of Proviral Copy Number

Quantitative real-time PCR was used to determine proviral copy number in transduced murine lin$^-$ cells or primary cells from reconstituted mice. Genomic DNA was isolated as described in 2.2.14 and approximately 100 ng of DNA used as a template for each PCR. Reactions were performed in triplicate in a final volume of 50 µl containing forward and reverse primers at 0.9 µM, a fluorescently-labelled probe at 0.2 µM and a pre-mixed mastermix (Platinum qPCR SuperMix-UDG with ROX) diluted to 1 x with sterile distilled water. DNA samples were analysed using primers and probe to the human γc cDNA (a gift from Elke Grassman, Cincinatti Children's Hospital Medical Center, Cincinatti, USA) for detection of integrated proviral copies and using primers and probe to exon 5 of the murine titin gene (*Ttn*) (a gift from A Galy, Genethon, Paris, France) to enable quantitation of the number of cells in each particular sample. Standard curves of diluted plasmids harbouring either the γc cDNA or murine *titin* gene (gifts from Dr Steven Howe and Dr Mike Blundell, Molecular Immunology Unit, ICH) were used to quantitate integrated viral copies and the number of copies of the murine *titin* gene which was then normalised for cell number. Reactions were performed using

an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the default cycling parameters – 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Primers and probe for the detection on integrated provirus (γc cDNA):
Forward primer – gamma chain US F1: 5' TGCTAAAACTGCAGAATCTGGT 3'
Reverse primer – gamma chain US R1: 5' AGCTGGGATTCACTCAGTTTG 3'
Probe – gamma chain US: 5' FAM-CCTGGGCTCCAGAGAACCTAACA-TAMRA 3'

Primers and probe for the detection of the murine *Ttn* gene:
Forward primer – Titin M ex5 F: 5' AAAACGAGCAGTGACGTGAGC 3'
Reverse primer – Titin M ex5 R: 5' TTCAGTCATGCTGCTAGCGC 3'
Probe – Titin M ex5: 5' FAM-TGCACGGAAGCGTCTCGTCTCAGTC-TAMRA 3'

2.2.17 DNA manipulation

2.2.17.1 Restriction Enzyme Digestion

Plasmid DNA (typically 0.5-5 μg) was digested in a final volume of 25-50 μl of 10 x buffer (supplied by the manufacturer and diluted to 1 x with distilled water) and bovine serum albumin (0.1 mg/ml). 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 and DNA digestion was verified by agarose gel electrophoresis. Double or triple digestions were performed either in compatible buffers or sequentially, following clean-up of DNA by ethanol precipitation.

2.2.17.2 Ethanol Precipitation of DNA

The volume of the DNA solution was estimated and one-tenth of the volume of 3 M sodium acetate (pH 5.2) and two times the volume of 100% ethanol were added. The solution was mixed by inversion and placed at -20°C for 1 hour. The precipitated DNA was subsequently pelleted by centrifugation at 13,000 rpm in a microfuge for 30 minutes at 4°C. The supernatant was discarded and the pellet washed in 1 ml 70% ethanol, followed by centrifugation for 10 min under the same conditons as previously. The supernatant was again discarded and the DNA pellet briefly air dried and resuspended in an appropriate volume of 1 x TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or distilled water.

2.2.17.3 Agarose Gel Electrophoresis

DNA fragments were resolved by electrophoresis through 1% agarose gels in 1 x TAE buffer (40 mM Tris-acetate, 5 mM EDTA). To prepare the gels, agarose was dissolved in 1 x TAE buffer by boiling in a microwave, after cooling ethidium bromide was added at 0.5 μg/ml for visualisation of DNA. DNA samples were mixed with Orange G loading buffer (10 mM Tris pH 7.5, 50 mM EDTA, 10% Ficol 400, 0.4% Orange G) before loading onto agarose gels. A 1 kb plus DNA ladder was loaded onto each gel to enable size determination of DNA fragments. Gels were electrophoresed using a voltage of 50-100 V (up to 150 mA) and the separated DNA fragments subsequently visualised by exposure to ultra-violet light using an UVIdoc gel documentation system.

2.2.17.4 Gel Purification of DNA

Following electrophoresis DNA fragments were excised from agarose gels using a clean scalpel blade under ultra-violet light. The DNA was then extracted from the agarose using a QIAquick gel extraction kit as per the manufacturer's instructions.

2.2.17.5 Dephosphorylation of Digested Plasmid DNA Ends

In cases where digested vector DNA ends had compatible termini, the digested plasmid DNA was treated with calf intestinal alkaline phosphatase (CIP) to dephosphorylate the DNA ends prior to ligation. Dephosphorylation reactions were perfomed directly in restriction endonuclease buffers; immediately following digestion, 10 units of CIP enzyme was added to the reaction mixture and incubated at 37°C in a waterbath for 1 hour. Dephosphorylated plasmid DNA was subsequently purified by ethanol precipitation.

2.2.17.6 Filling of 5'-Protruding Ends by Treatment with Klenow

DNA Polymerase I Large (Klenow) fragment was used to fill in the 5' overhang created by digestion of the vector with this restriction endonuclease and thus create a blunt end. Immediately following digestion with *Xho*I, five units of Klenow enzyme were added directly to the reaction mixture together with 40 μM of each dNTP and the reaction was incubated at room temperature for 10 minutes. The reaction was then stopped by heating to 75°C for 10 minutes. The blunt-ended DNA was subsequently purified by ethanol precipitation prior to ligation.

2.2.17.7 Ligation

Ligations were performed using vector to insert ratios of 1:3 or 1:5 (100 ng vector DNA) in a final volume of 10 μl of 10 x T4 DNA ligase buffer (diluted to 1 x with distilled water) containing 1 unit of T4 DNA ligase. Ligation reactions were incubated overnight (16 hours) at 15°C. The ligation reactions were either immediately transformed into electro-competent *E.coli* (2 μl) or stored at -20°C for later use.

2.2.18 Neonatal Injections

This procedure was performed by Dr Simon Waddington. 1-day old neonatal mice were placed on ice slush until they no longer moved when touched. The heads were subsequently illuminated and 40 µl of concentrated lentiviral supernatant injected into the superficial temporal vein (a prominent vessel located on either side of the head, visible just below the eye) using a 33 G Hamilton needle. Following the procedure, the mice were allowed to return to normal temperature on a warming mat before being returned to their mothers.

2.2.19 Statistical Analysis

Where applicable data was analysed using a two-tailed, unpaired t-test.

2.2.20 Bioinformatics

2.2.20.1 Human and Murine Genomic Sequences

Human and murine genomic sequenes were obtained from the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/) or from the Ensembl Genome Browser (http://www.ensembl.org/index.html).

2.2.20.1 Alignment of Human and Murine Sequences

Sequences were aligned using a multiple sequence local alignment and visualisation tool (Mulan, http://mulan.dcode.org).

2.2.20.2 Analysis of Transcription Factor Binding Sites

Sequences were analysed for transcription factor binding sites using the online tool TFSEARCH tool (www.cbrc.jp). For analysis of transcription factor binding sites conserved in both the human and murine genomes, the MultiTF tool (http://multitf.dcode.org) was used.

2.2.20.3 Analysis of Putative CpG Islands

Sequences were analysed for the presence of putative CpG islands using CpG plot (http://www.ebi.ac.uk/emboss/cpgplot/).

2.2.20.4 Analysis of Alu Elements

Sequences were analysed for the presence of Alu Ements using the online RepeatMasker program (www.repeatmasker.org).

Chapter Three

Self-Inactivating Gammaretroviral Vectors for SCID-X1

3.0 Aims

- To assess the performance of the SRS II series of SIN gammaretroviral vectors *in vitro*
- To compare the SRS II series of vectors to MFG γc for the ability to restore the lymphoid differentiation potential of *Il2rg*$^{-/-}$ lin^{-} cells *in vitro*
- To assess the ability of the SRS II vector series to restore immunity in the SCID-X1 murine model following *ex vivo* gene therapy

3.1 Introduction

The intact 5' and 3' Moloney murine leukaemia virus (MLV) LTRs within MFG γc contain potent, duplicated enhancer sequences and a promoter region that regulate high levels of transcription. The enhancer sequences consist of two, approximately 75 bp, direct repeats within which several transcription factor binding sites are located (figure 3.1A). To reduce the potential for insertional mutagenesis, the SRS II series of self-inactivating gammaretroviral vectors have been developed that incorporate only a single internal regulatory element as opposed to the two LTR sequences present in MFG γc (see figure 1.7). The internal SFFV and EFS promoters were selected due to their ability to regulate high transgene expression in haematopoietic lineages.

The potent promoter activity of the U3 region of the SFFV LTR has been shown to efficiently express transgene in both haematopoietic progenitor cells and their multi-lineage progeny *in vivo*, suggesting its potential as a promoter element in gene therapy vectors for haematological disorders (Demaison et al., 2002; Baum et al., 1995; Tumas et al., 1996). As for MLV, the U3 region of the SFFV LTR contains duplicated enhancer regions consisting of several transcription factor binding sites, including the conserved LVb recognition site for the Ets family of transcription factors (figure 3.1B). In addition, a binding site for the ubiquitous Sp1 transcription factor is also present within the SFFV U3, located 5' to the 42 bp enhancer repeat sequences (Baum et al., 1997). The Ets family of transcription factors control expression of a large number of genes in haematopoietic cells, in particular during lymphoid development

(Anderson et al., 1999), and consensus sequences for Sp1 and Ets factors are observed in several genes specifically expressed in haematopoietic lineages (as reviewd in (Baum et al., 1997).

The EFS promoter is derived from the regulatory sequences 5' to the human elongation factor 1α gene, a housekeeping gene that encodes an enzyme which catalyses the binding of aminoacyl-tRNA to ribosomes (Uetsuki et al., 1989). The promoter is described as the short form of the EF1α promoter (EFS) due to the deletion of the first intron, demonstrated to also contain promoter activity (Wakabayashi-Ito and Nagata, 1994), removed so as to avoid any problems associated with splicing. EF1α is abundantly expressed in most eukaryotic cells and the CpG rich promoter has been demonstrated to induce high levels of trangene expression in human $CD34^+$ cells, primary T cells and *in vitro* differentiated blood lineages, whilst remaining unaffected by the SIN design (Salmon et al., 2000; Dardalhon et al., 2001). Analysis of putative transcription factor binding sites located within this 242 bp promoter using the TFSEARCH tool (www.cbrc.jp) revealed the presence of several consensus sequences for haematopoietic-specific transcription factors, including the lymphoid restricted Ik-2, the myeloid zinc finger protein MZF1 and GATA-1, a transcription factor important in erythroid development (figure 3.1C). This promoter therefore presents an attractive alternative to viral regulatory sequences for incorporation into viral gene therapy vectors.

To assess vector performance in terms of transgene expression, the SIN gammaretroviral vectors encoding human γc were analysed both *in vitro* and *in vivo* and compared directly to the LTR-regulated vector, MFG γc.

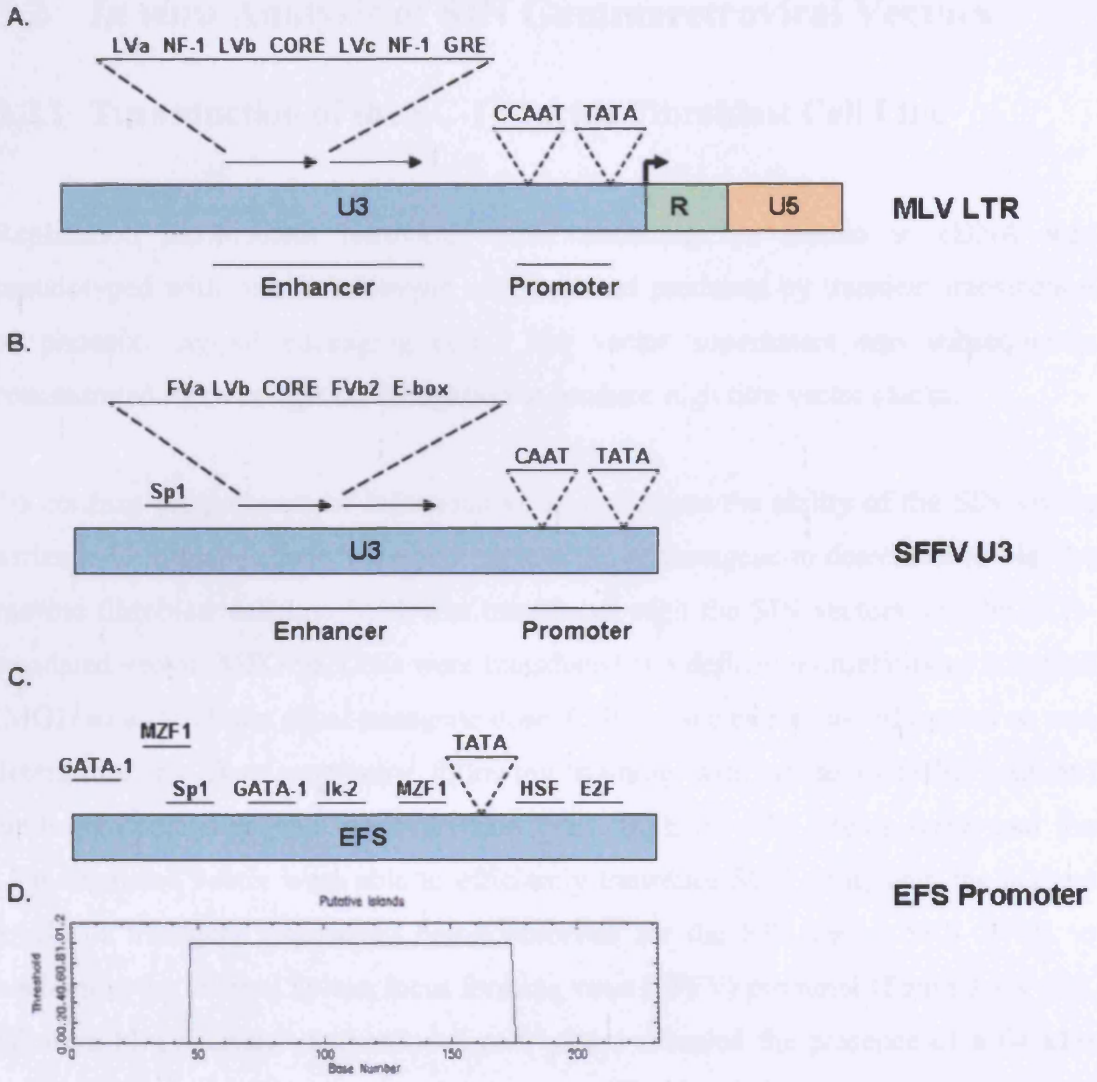

Figure 3.1. Comparison of the promoter/enhancer sequences within the MLV LTR, the SFFV U3 region and the EFS promoter. (A) The MLV LTRs contain duplicated enhancer regions containing several transcription factor binding sites and a promoter region containing CAAT and TATA motifs. (B) The SFFV U3 contains a Sp1 binding site upstream of the two enhancer direct repeat sequences and a promoter region also containing CAAT and TATA motifs. (C) The 242 bp EFS promoter contains several putative haematopoietic-specific transcription factor binding sites and a TATA box (analysed using the TFSEARCH tool). (D) A putative CpG island of 128 bp within the EFS promoter is identified following analysis with CpG plot (www.ebi.ac.uk/emboss/cpgplot/).

3.2 *In vitro* Analysis of SIN Gammaretroviral Vectors

3.2.1 Transduction of the SC-1 Murine Fibroblast Cell Line

Replication incompetent retroviral vector encoding the human γc cDNA was pseudotyped with murine ecotropic envelope and produced by transient transfection of phoenix gag-pol packaging cells. The vector supernatant was subsequently concentrated by overnight centrifugation to produce high titre vector stocks.

To confirm the presence of infectious virus and assess the ability of the SIN vector series to transduce cells *in vitro* and express the γc transgene to detectable levels, the murine fibroblast cell line SC-1 was transduced with the SIN vectors and the LTR-regulated vector, MFG γc. Cells were transduced at a defined multiplicity of infection (MOI) so as to obtain equal transgene dose. Cell surface expression of human γc was determined by flow cytometry following staining with an anti-CD132 (anti-γc) antibody conjugated with phycoerythrin (PE). Both the SIN vector series and the LTR-regulated vector were able to efficiently transduce SC-1 cells, with the highest levels of transgene expression being observed for the SIN vector SRS II SF γc containing the internal Spleen focus forming virus (SFFV) promoter (figure 3.2A - B). Western blot analysis of transduced cell lysates revealed the presence of a 64 kDa band representing human γc and two smaller bands that possibly represent underglycosylated or degraded forms of the protein (figure 3.2C); similar isoforms have also been observed in γc-retrovirally transduced SCID-X1 B cell lines and following Western blot analysis of a γc-expressing cell line (data not shown) (Kleiman et al., 1998).

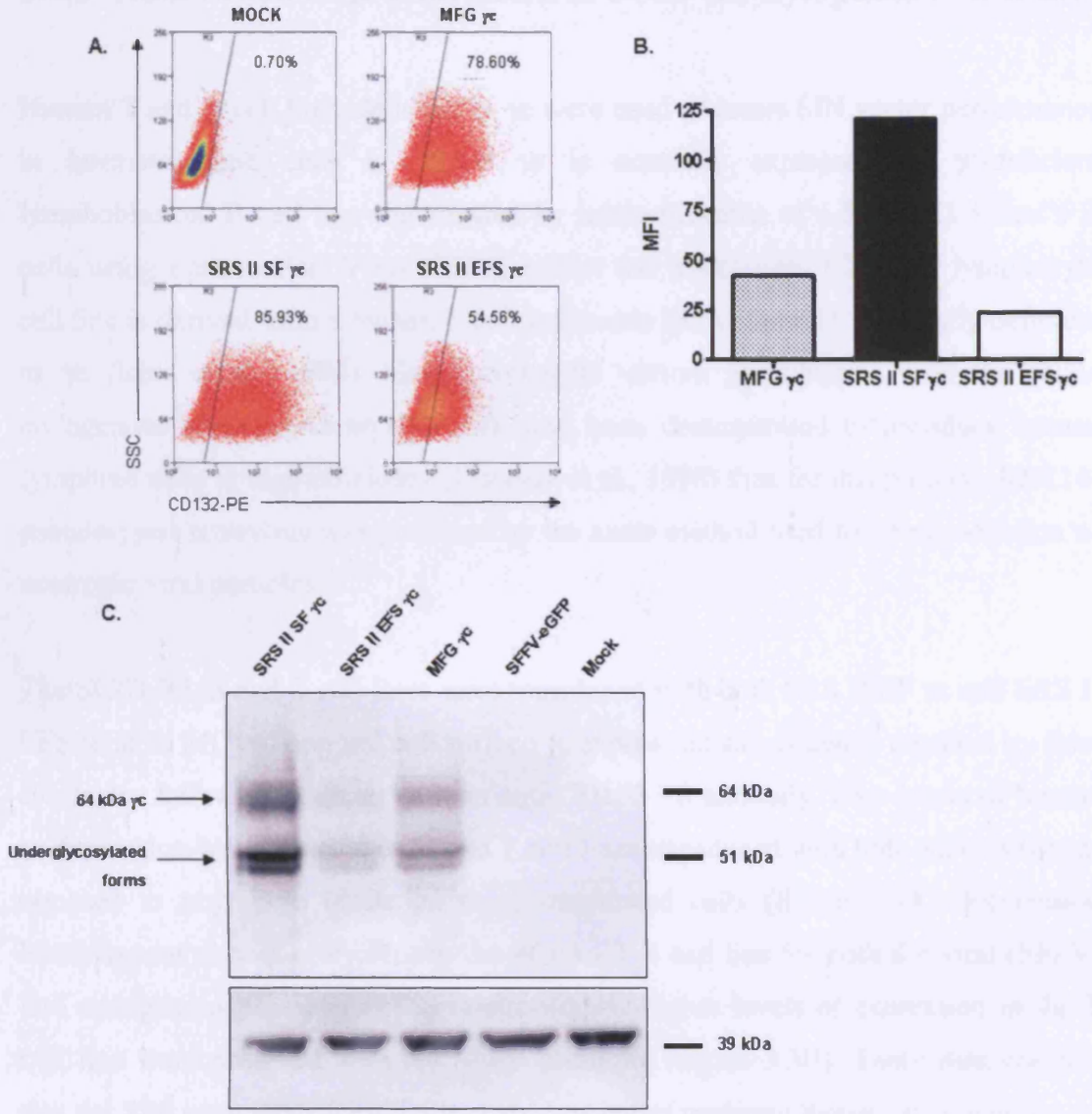

Figure 3.2. Human γc expression in transduced SC-1 cells. (A) Flow cytometric analysis of SC-1 cells transduced with the SIN vector series and MFG γc at an MOI of 1. Plots are from a representative single transduction. (B) The mean fluorescent intensity (MFI) was compared for the transduced cells revealing that the highest expression levels of human γc were observed for SRS II SF γc (n = 1). (C) Western blot analysis of transduced SC-1 cell lysates. To confirm antibody specificity and as a vector control, SC-1 cells were also transduced with an LTR-regulated vector expressing enhanced green fluorescent protein (SFFV-eGFP). The blot was also probed with an antibody to the 41 kDa β-actin protein (lower lane) to demonstrate equivalent loading in each lane.

3.2.2 Restoration of γc Expression in SCID-X1 Lymphoid Cell Lines

Human T and B cell lines deficient in γc were used to assess SIN vector performance in haematopoietic cells in which γc is normally expressed. A γc-deficient lymphoblastoid B cell line was created by immortilisation of a SCID-X1 infant's B cells using Epstein Barr Virus (EBV), whilst the γc-deficient ED-7R T lymphocyte cell line is derived from a human T cell leukaemia line (ED40515$^-$) naturally deficient in γc (Ishii et al., 1994). Gammaretroviral vectors pseudotyped with the feline endogenous virus envelope (RD114) have been demonstrated to transduce human lymphoid cells at high efficiency (Onodera et al., 1998) thus for this purpose, RD114-pseudotyped retrovirus was produced by the same method used for the production of ecotropic viral particles.

The SCID-X1 B and T cell lines were transduced with both SRS II SF γc and SRS II EFS γc at an MOI of one and cell surface γc expression subsequently detected by flow cytometry following staining with an anti-CD132-PE antibody. Low levels of human γc expression were detected on B and T cell lines transduced with both SIN vectors as opposed to negligible levels on mock-transduced cells (figure 3.3A). Expression levels appear almost equivalent in the SCID-X1 B cell line for both the viral (SFFV) and endogenous promoter (EFS) whilst slightly higher levels of expression in the T cell line were observed with the SFFV promoter (figure 3.3B). These data confirm that the SIN gammaretroviral vectors are capable of restoring human γc expression on the cell surface of SCID-X1 lymphoid cell lines.

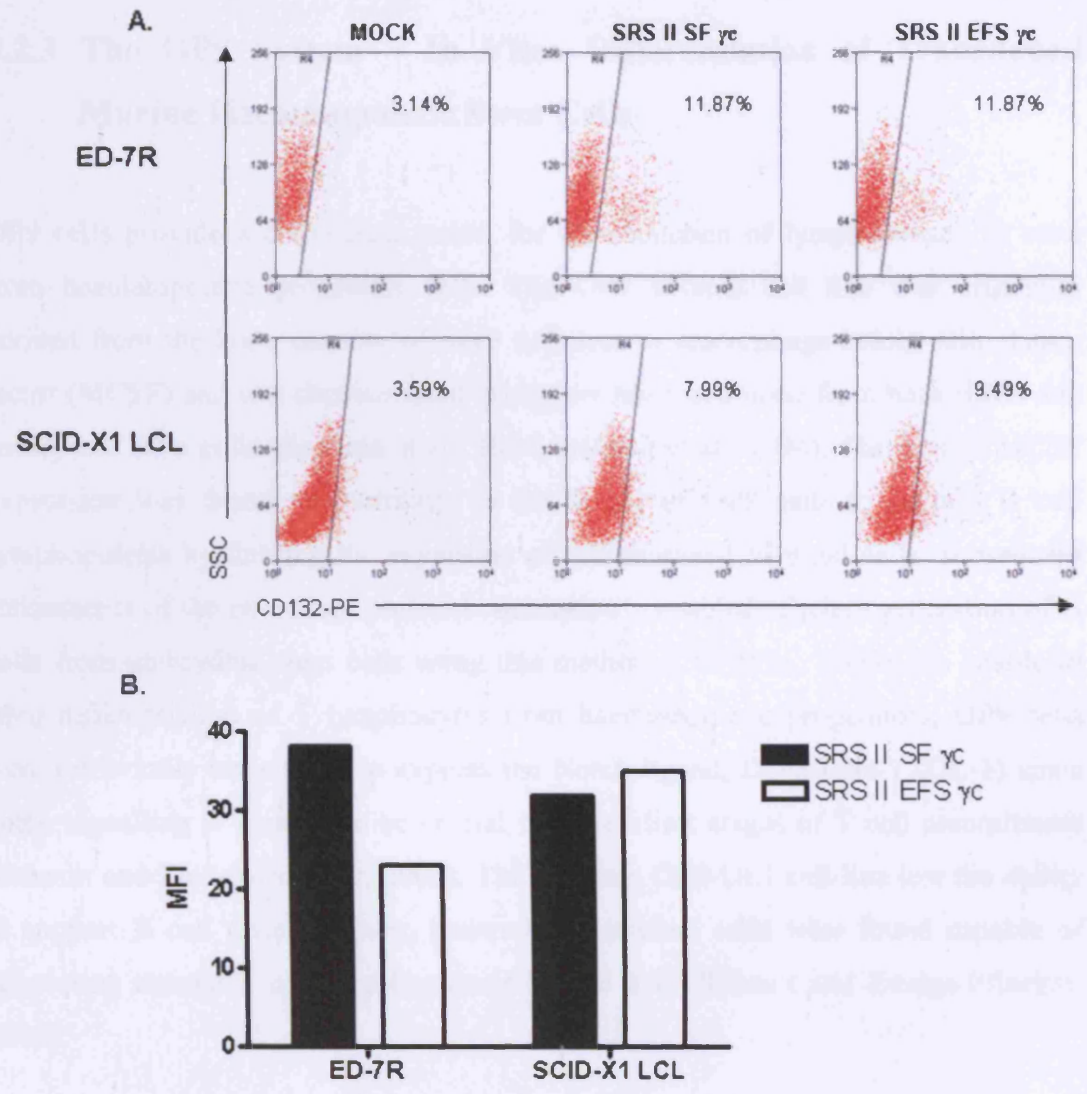

Figure 3.3. Human γc expression in transduced SCID-X1 lymphoid cells.
(A) Flow cytometric analysis of SCID-X1 T (ED-7R) and B (SCID-X1 LCL) cell lines transduced with SRS II SF γc and SRS II EFS γc at an MOI of 1. (B) The mean fluorescent intensity (MFI) of transduced SCID-X1 B cells was similar for both promoters, whilst the SFFV promoter appeared to be more potent in ED-7R cells (n = 1).

3.2.3 The OP9 System – In Vitro Differentiation of Transduced Murine Haematopoietic Stem Cells

OP9 cells provide a convenient model for the induction of lymphopoiesis *in vitro* from haematopoietic progenitor cells. The OP9 stromal cell line was originally derived from the bone marrow of mice deficient in macrophage colony-stimulating factor (MCSF) and was demonstrated to support haematopoiesis from both HSCs and embryonic stem cells (Kodama et al., 1994; Nakano et al., 1994). The lack of MCSF expression was found to contribute to the ability of OP9 cells to support B cell lymphopoiesis by limiting the expansion of differentiated myeloid cells; subsequent refinements of the co-culture protocol consequently enabled efficient generation of B cells from embryonic stem cells using this method (Cho et al., 1999). To enable *in vitro* differentiation of T lymphocytes from haematopoietic progenitors, OP9 cells were retrovirally transduced to express the Notch ligand, Delta-Like-1 (DL-1) since notch signalling is thought to be crucial in the earliest stages of T cell commitment (Schmitt and Zuniga-Pflucker, 2002). The resultant OP9-DL1 cell line lost the ability to support B cell differentiation, however the stromal cells were found capable of supporting normal T cell lymphopoiesis (figure 3.4) (Schmitt and Zuniga-Pflucker, 2002).

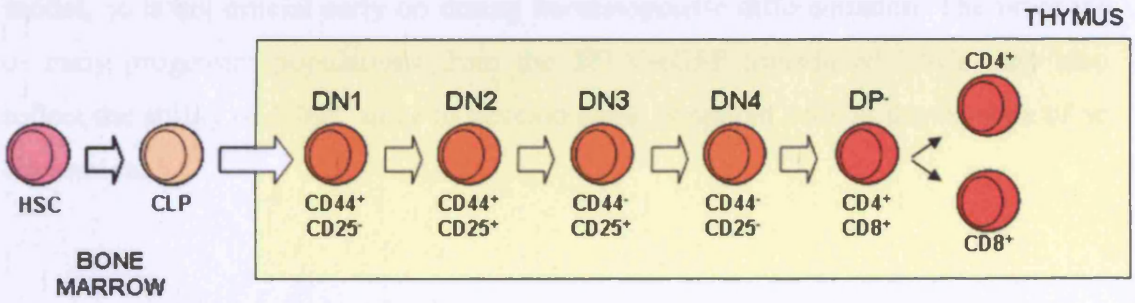

Figure 3.4. The different stages of T cell development in the murine thymus.

OP9-eGFP (OP9 cells transduced with a retroviral vector expressing eGFP only) and OP9-DL1 stromal cell lines were therefore used to investigate the potential of *Il2rg*$^{-/-}$ murine HSCs to undergo lymphoid differentiation *in vitro* following transduction with the SRS II gammaretroviral vector series. Murine HSCs were isolated using lineage negative selection of whole bone marrow cells from *Il2rg*$^{-/-}$ or C57/Bl6 mice (as a positive control). The *Il2rg*$^{-/-}$ lin^{-} cells were prestimulated in serum-free media with cytokines for forty-eight hours and subsequently underwent two rounds of retroviral transduction with either MFG γc, the SIN vectors expressing human γc or with the control vector, SFFV-eGFP, over two days, before seeding on OP9 stromal layers (figure 3.5A). The C57/Bl6 control lin^{-} cells were maintained in serum-free media with cytokines for four days prior to seeding on the OP9 stromal layers. Cells were co-cultured with the OP9 stromal cell lines for thirteen days in the presence of IL-7 and Flt-3 to support lymphoid differentiation and analysed by light microscopy and flow cytometry.

Following five days of co-culture on either the OP9-eGFP or OP9-DL1 stromal cell lines, densely-packed adherent 'cobblestone colonies' were visible for all cells, indicating early progenitor cell populations. Round, healthy-looking haematopoietic cells were also identifiable sitting on top of the stromal cell layer (figure 3.5B). No differences were observed at this stage between the control C57/Bl6 HSCs and the *Il2rg*$^{-/-}$ HSCs that had been transduced with either the SIN vectors expressing γc or with the control vector SFFV-eGFP; thus it appears that in the SCID-X1 murine model, γc is not crucial early on during haematopoietic differentiation. The presence of early progenitor populations from the SFFV-eGFP transduced HSCs may also reflect the ability of *Il2rg*$^{-/-}$ mice to develop some lymphoid cells in the absence of γc expression.

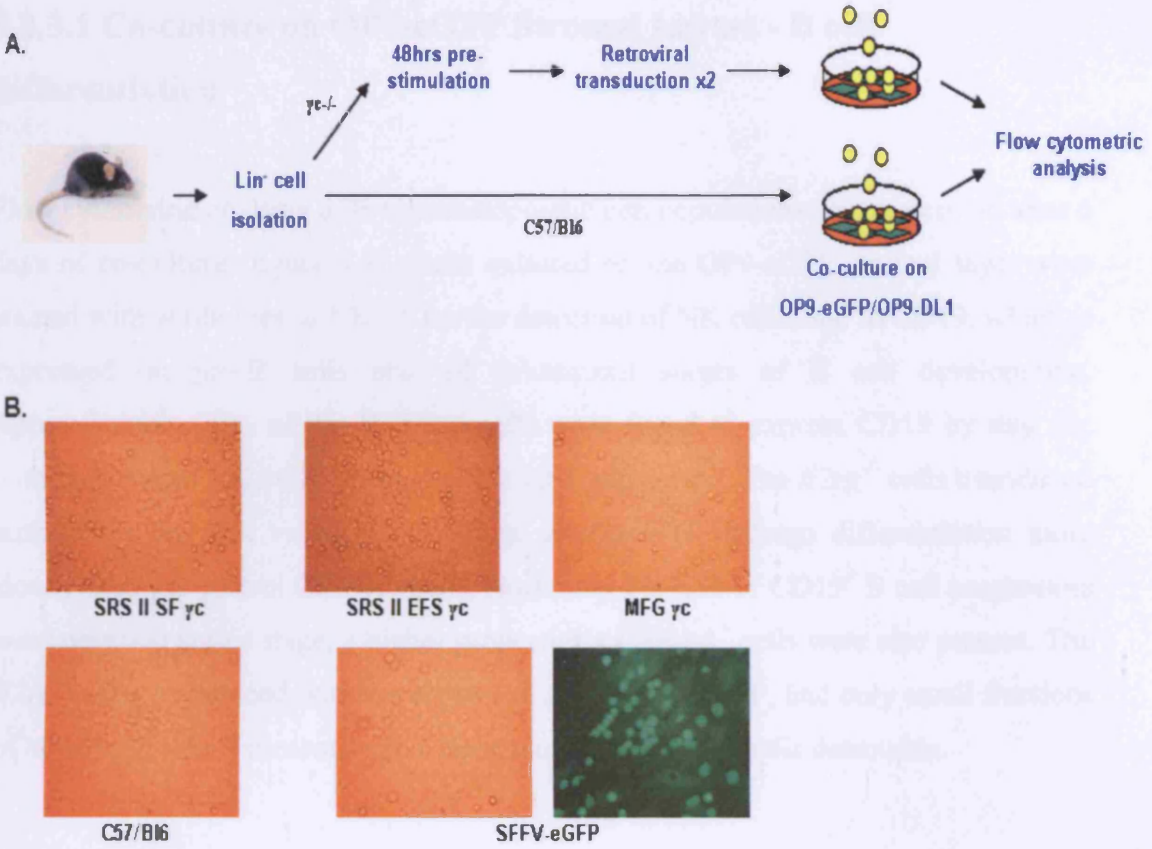

Figure 3.5. Cobblestone colony formation following incubation of γc-retrovirally transduced *Il2rg*$^{-/-}$ lin^{-} cells on OP9 stromal layers. (A) An outline of the experimental protocol used. (B) Cobblestone colonies representing early progenitor cell populations are visible after five days co-culture of transduced HSCs on the OP9-GFP stromal cell line. For the control vector SFFV-eGFP these colonies appear bright green, indicating that they are formed from transduced *Il2rg*$^{-/-}$ donor cells. The fainter, eGFP-expressing OP9 stromal cells are also observed under the fluorescence microscope. Round, differentiated haematopoietic cells resting on top of the stromal layer are also detected at this stage.

3.2.3.1 Co-culture on OP9-eGFP Stromal Layers - B cell differentiation

Flow cytometric analysis of the haematopoietic cell populations was performed after 6 days of co-culture (figure 3.6). Cells cultured on the OP9-eGFP stromal layer were stained with antibodies to NK1.1 for the detection of NK cells, and to CD19, which is expressed on pro-B cells and all subsequent stages of B cell development. Approximately 70% of the C57/Bl6 cells were found to express CD19 by day six, with only 4% of NK cells detected in the total population. The *Il2rg*$^{-/-}$ cells transduced with either the SIN vectors or MFG γc appeared to undergo differentiation more slowly than the control C57/Bl6 cells, since only 10-15% of CD19^{+} B cell progenitors were detected at this stage, a higher proportion of NK1.1^{+} cells were also present. The *Il2rg*$^{-/-}$ cells transduced with the control vector, SFFV-eGFP, had only small fractions of both populations present, with a large number of eGFP^{+} cells detectable.

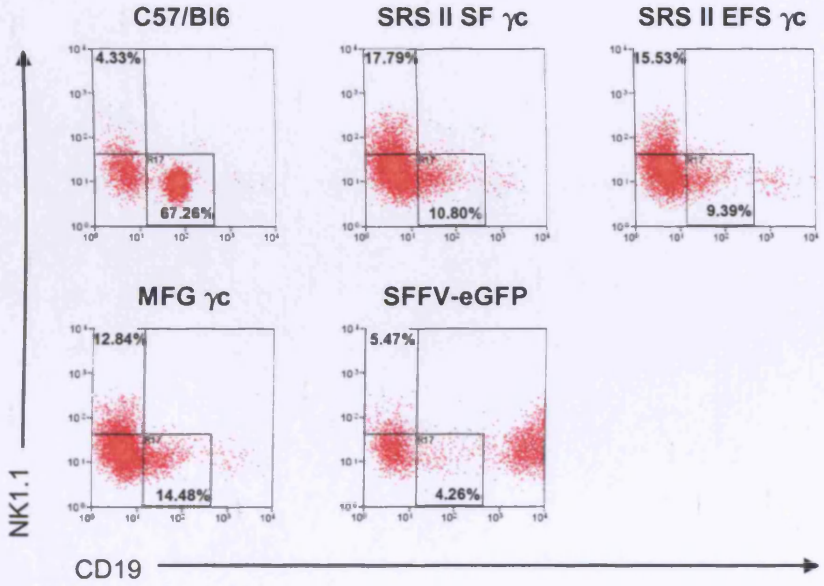

Figure 3.6. Day six analysis of OP9-eGFP co-cultures. Cells were stained with antibodies to NK cell marker NK1.1 and B cell marker CD19 and analysed by flow cytometry. A large population of CD19^{+} early B lymphocytes are observed for C57/Bl6 control cells, with smaller CD19^{+} populations present for the *Il2rg*$^{-/-}$ cells transduced with the SIN vectors or MFG γc. A large eGFP^{+} population is present for the *Il2rg*$^{-/-}$ cells transduced with SFFV-eGFP (ungated population on the far right of the plot) with a small fraction of eGFP^{-}CD19^{+} cells detected.

After ten days co-culture, large numbers of round, differentiated haematopoietic cells were visible resting on top of the OP9-eGFP stromal layers (figure 3.7A). Surprisingly, large numbers of these cells were also present for the *Il2rg*$^{-/-}$ HSCs transduced with SFFV-eGFP, however by day twelve almost all of these cells were found to be dead, indicating the requirement of γc for B cell differentiation and survival. The C57/Bl6 and γc-retrovirally transduced *Il2rg*$^{-/-}$ cells were analysed by flow cytometry on day thirteen following antibody staining for B cell markers CD19 and B220. Almost 100% of cells were characterised as CD19^{+}B220^{+} B lymphocytes, with no differences observed between the wild-type C57/Bl6 cells and the retrovirally-transduced *Il2rg*$^{-/-}$ cells (figure 3.7B). These results indicate that the SIN gammaretroviral vectors are capable of efficiently transducing primitive *Il2rg*$^{-/-}$ murine HSCs under the culture conditions used and expressing functional human γc on the cell surface at levels that enable B cell differentiation *in vitro*. Furthermore, B cell differentiation of the transduced cells was identical to that seen for wild-type γc^{+} HSCs or for *Il2rg*$^{-/-}$ HSCs transduced with the LTR-regulated vector MFG γc.

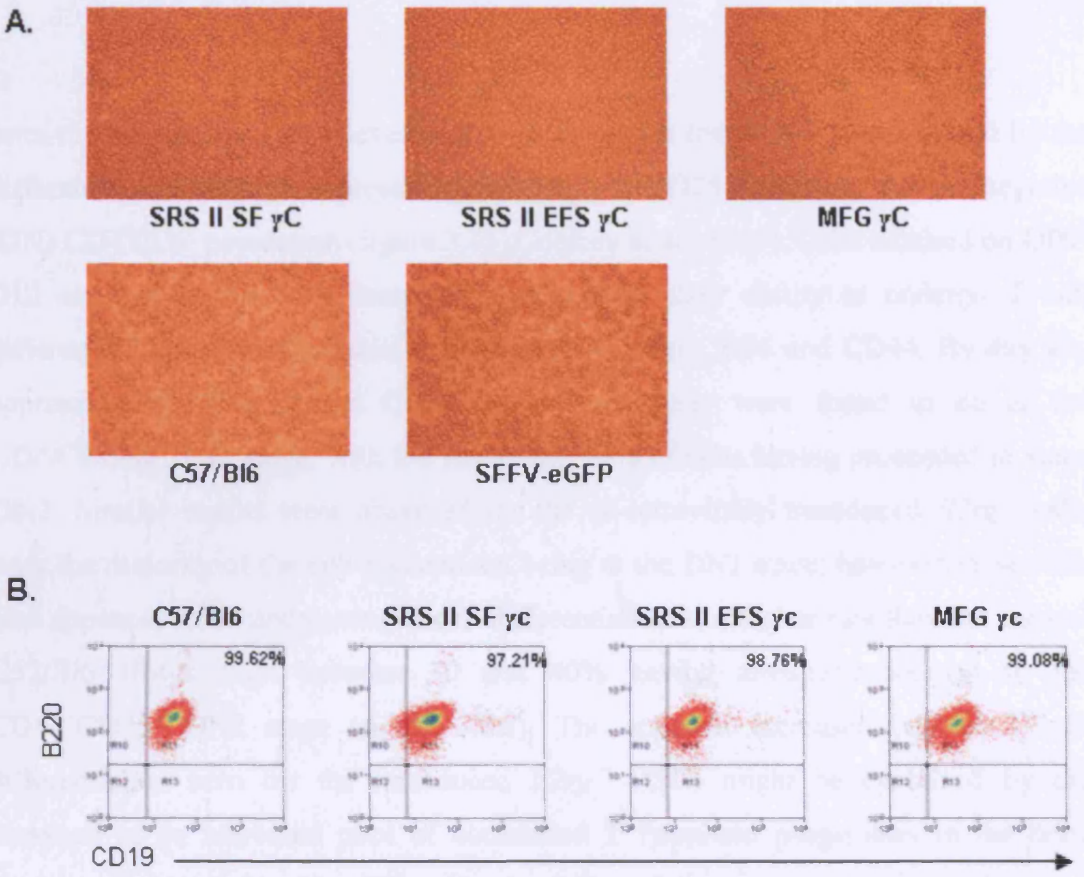

Figure 3.7. Analysis of OP9-eGFP co-cultures on days ten and thirteen. (A) After ten days co-culture large numbers of round haematopoietic cells are observed resting on top of the OP9 stromal layers. (B.) Cells were analysed by flow cytometry after thirteen days of co-culture following staining for B cell surface markers CD19 and B220. After thirteen days of co-culture $CD19^{+}B220^{+}$ B lymphocytes are detected for both wild-type and retrovirally transduced *Il2rg*$^{-/-}$ HSCs.

3.2.3.2 Co-culture on OP9-DL1 Stromal Layers - T cell differentiation

Intra-thymic murine T cell development proceeds via four stages characterised by the differential cell surface expression of CD44 and CD25 within the double negative (DN) $CD4^-CD8^-$ population (figure 3.4) (Godfrey et al., 1993). Cells cultured on OP9-DL1 stromal layers were therefore analysed for their ability to undergo T cell differentiation *in vitro* by staining with antibodies to CD25 and CD44. By day six, approximately 90% of the C57/Bl6 wild-type cells were found to be at the $CD44^+CD25^-$ DN1 stage, with the remaining 10% of cells having proceeded to stage DN2. Similar results were observed for the γc-retrovirally transduced *Il2rg*$^{-/-}$ cells with the majority of the cell populations being at the DN1 stage; however these cells also appeared to be undergoing T cell differentiation at a higher rate than the control C57/Bl6 HSCs, with between 30 and 40% having already proceeded to the $CD44^+CD25^+$ DN2 stage (figure 3.8B). The apparent increased rate of T cell differentiation seen for the transduced *Il2rg*$^{-/-}$ HSCs might be explained by the presence of an increased pool of committed T lymphoid progenitors in the bone marrow of these mice. No differences in differentiation were observed at this time point between *Il2rg*$^{-/-}$ cells transduced with the SIN vectors and those transduced with the LTR-regulated vector MFG γc. SFFV-eGFP transduced *Il2rg*$^{-/-}$ cells appeared arrested at the DN1 stage of development with both $CD44^+eGFP^+$ and $CD44^+eGFP^-$ populations observed, but no $CD44^+CD25^+$ DN2 stage cells detected. This block is most likely due to the inability of the *Il2rg*$^{-/-}$ cells to signal through the IL-7 receptor, since this cytokine plays an important role during early lymphocyte development (von Freeden-Jeffry et al., 1995; Puel et al., 1998). Furthermore, thymocyte development in mice treated with antibodies to IL-7 or in IL-7Rα-deficient mice is unable to proceed efficiently beyond the DN1 stage (Bhatia et al., 1995; He et al., 1997). However, *Il2rg*$^{-/-}$ mice are able to develop some T cells in the absence of γc, consequently it is likely that factors absent in this *in vitro* system are required for the γc-independent development of T cells. C-kit, the cytokine receptor for SCF, has been postulated to partially compensate for the γc-deficiency, since thymocyte development is completely abrogated in mice doubly-deficient for both receptors (Rodewald et al., 1997); OP9 cells have been demonstrated to produce SCF when cultured under

similar conditions and hence it appears that this pathway is unable to rescue T lymphopoiesis beyond the DN1 stage *in vitro* (Cho et al., 1999).

As was observed for the cells cultured on the OP9-eGFP stromal layers, after ten days of co-culture large numbers of round, differentiated haematopoietic cells were visible (data not shown). These cells were again analysed by flow cytometry on day thirteen following staining with antibodies to CD25 and CD44. For both wild-type C57/Bl6 cells and the γc-retrovirally transduced *Il2rg*$^{-/-}$ cells, the majority of the population was found to be at stage DN2, with fewer cells remaining at the DN1 pro-T cell stage (figure 3.8C). Cells were also detected at the later CD44^{-}CD25^{+} DN3 and CD44^{-} CD25^{-} DN4 stages, indicating the occurrence of ongoing T cell differentiation *in vitro*. No viable cells remained in the co-culture of *Il2rg*$^{-/-}$ cells transduced with the SFFV-eGFP control vector by this stage of the experiment. The SRS II series of SIN gammaretroviral vectors are therefore able to efficiently transduce murine lin^{-} cells and express the γc transgene at functional levels that permit T cell differentiation *in vitro*.

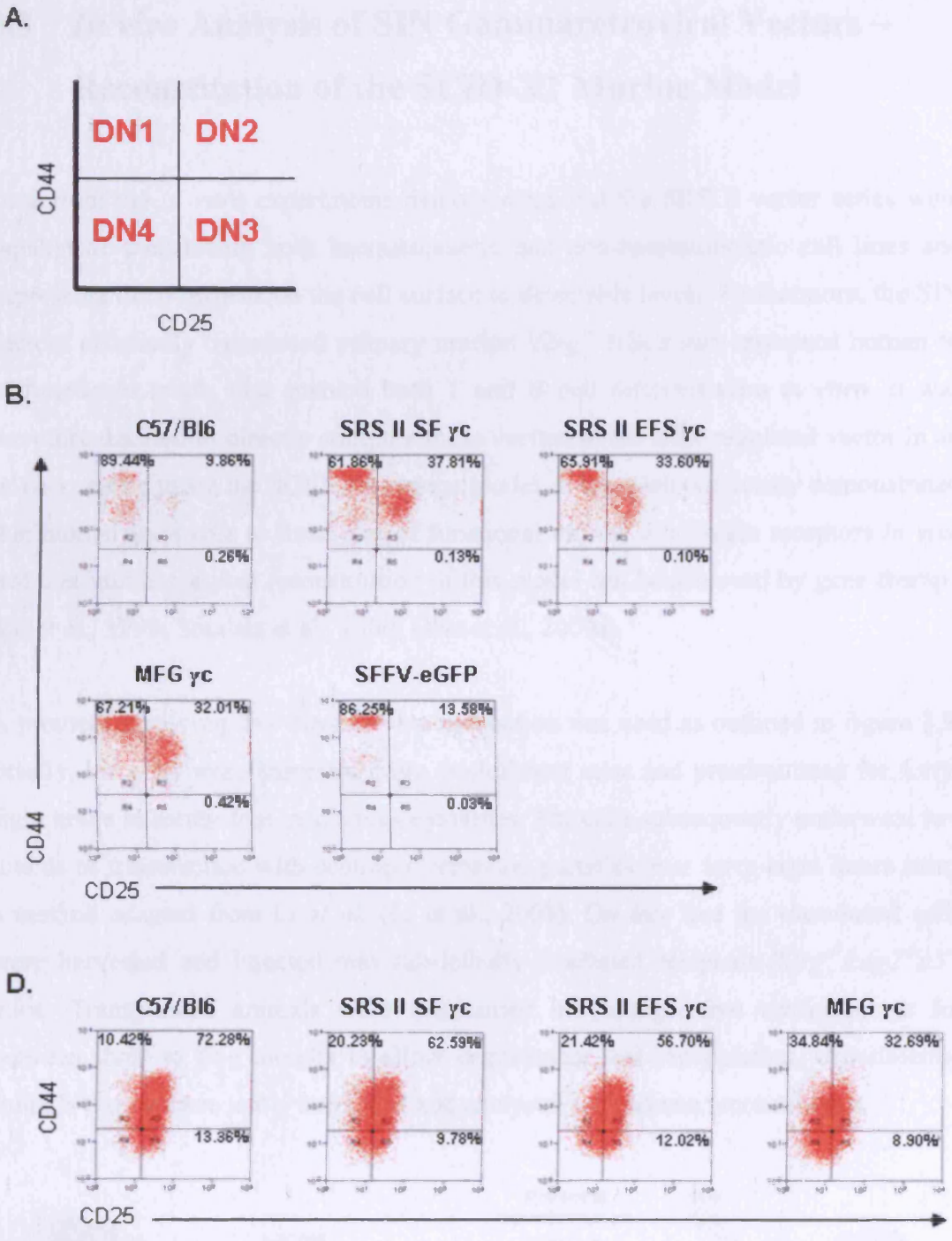

Figure 3.8. Analysis of OP9-DL1 co-cultures. (A) Illustration of the four stages of T cell development as characterised by flow cytometry following staining with antibodies to CD25 and CD44. (B) Cells were stained with antibodies to early T cell markers, CD44 and CD25, after six days co-culture on OP9-DL1 stromal layers and analysed by flow cytometry. Both the C57/Bl6 and γc-retrovirally transduced cells appeared to be differentiating from the DN1 to the DN2 stage of T cell development. Control SFFV-eGFP transduced cells appeared arrested at DN1. (C) After thirteen days of co-culture the SFFV-eGFP transduced cells had all died whilst the wild-type C57/Bl6 and γc-retrovirally transduced cells appeared to be differentiating further towards DN4.

3.3 *In vivo* Analysis of SIN Gammaretroviral Vectors – Reconstitution of the SCID-X1 Murine Model

Data from the *in vitro* experiments demonstrated that the SRS II vector series were capable of transducing both haematopoietic and non-haematopoietic cell lines and expressing the transgene on the cell surface to detectable levels. Furthermore, the SIN vectors efficiently transduced primary murine *Il2rg*$^{-/-}$ HSCs and expressed human γc to functional levels that enabled both T and B cell differentiation *in vitro*. It was therefore decided to directly compare these vectors to the LTR-regulated vector in an *in vivo* setting using the SCID-X1 murine model. It has been previously demonstrated that human γc is able to form part of functional murine interleukin receptors *in vivo* and that immunological reconstitution of this model can be achieved by gene therapy (Lo et al., 1999; Soudais et al., 2000; Otsu et al., 2000a).

A protocol involving five days *ex vivo* incubation was used as outlined in figure 3.9. Briefly, lin$^-$ cells were harvested from γc-deficient mice and prestimulated for forty-eight hours in serum-free media plus cytokines. The cells subsequently underwent two rounds of transduction with ecotropic retroviral particles over forty-eight hours using a method adapted from Li *et al.* (Li et al., 2003). On day five the transduced cells were harvested and injected into sub-lethally irradiated recipient *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. Transplanted animals were maintained in pathogen-free environments for between three to five months to allow engraftment and repopulation, experimental animals were subsequently sacrificed and analysed for immune reconstitution.

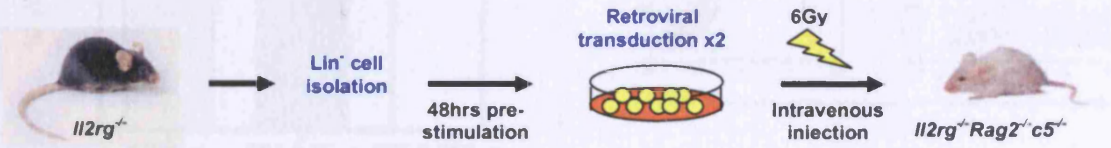

Figure 3.9. *Ex vivo* lin$^-$ transduction protocol. A schematic of the *ex vivo* transduction protocol. Lin$^-$ cells isolated from *Il2rg*$^{-/-}$ mice were prestimulated over forty-eight hours before undergoing two rounds of transduction with ecotropic retrovirus over two days using an MOI of between 1 and 2. The transduced cells were intravenously injected into sub-lethally irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice on day five.

3.3.1 Lin$^-$ Transduction levels

To assess pre-transplant lin$^-$ transduction levels, on day five a proportion of transduced cells were seeded in semi-solid media supplemented with murine cytokines and incubated at 37°C for two weeks. During this time progenitors or colony-forming units (CFUs) proliferated to form discrete colonies of cells which were subsequently isolated and lysed for genomic DNA extraction. Colonies were then analysed by polymerase chain reaction (PCR) for the presence of integrated vector using primers specific for the γc transgene, enabling calculation of the proportion of transduced progenitor cells that were injected into the recipient mice. For SFFV-eGFP transduced cells, transduction efficiency was assayed by flow cytometry to measure the proportion of eGFP$^+$ cells in the population. It was not possible to assay the γc-retrovirally transduced cells by this method as the anti-CD132-PE antibody appears not to recognise human γc in primary murine cells (personal observations). This might be due to the presence of altered glycosylation patterns in murine cells or that the antibody is unable to recognise human γc when forming part of heterologous murine interleukin receptors. Analysis of CFUs by PCR confirmed that high transduction levels were achieved for both the SIN vectors and the LTR-regulated vector, MFG γc (figure 3.10).

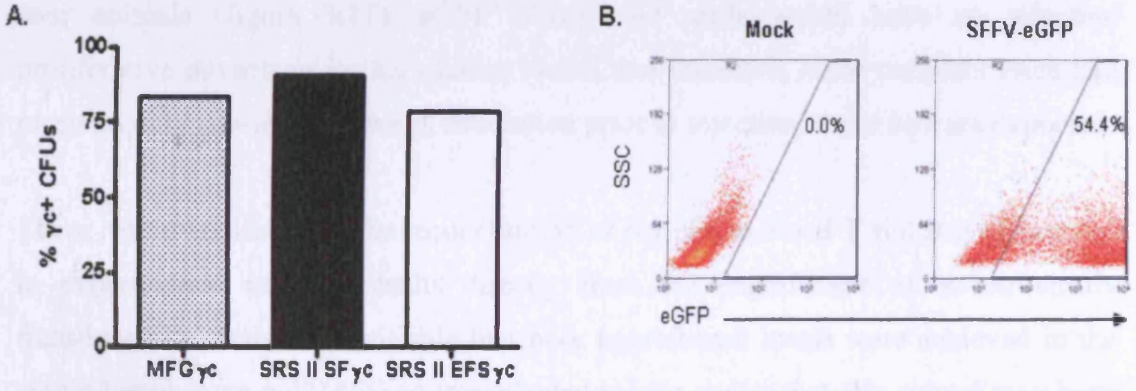

Figure 3.10. Lin$^-$ transduction efficiency. (A) Pre-transplant CFU transduction efficiencies of a representative lin$^-$ cell transduction. (B) Transduction efficiency for SFFV-eGFP transduced lin$^-$ cells was assayed by flow cytometric analysis.

3.3.2 Detection of Circulating Lymphoid Cells in the Peripheral Blood of Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Fifteen to eighteen weeks post-transplant, samples of peripheral blood from repopulated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice were analysed for the presence of circulating lymphoid cells by staining with antibodies to B (B220) and T (CD3) cell markers and analysis by flow cytometry. Peripheral blood from mice that had received *Il2rg*$^{-/-}$ lin^{-} cells transduced with the control vector, SFFV-eGFP, were also analysed for eGFP expression.

Circulating T and B lymphocytes were detected in the peripheral blood of eight out of eight mice that had received *Il2rg*$^{-/-}$ lin^{-} cells transduced with SRS II SF γc and in all six SRS II EFS γc mice (figure 3.11). Six out of seven mice repopulated with cells transduced with MFG γc also had CD3^{+} and B220^{+} cells present in the periphery. Peripheral blood mononuclear cells from all animals were also stained for cell surface human γc expression (data not shown) however none was detected by flow cytometry, further confirming previous observations that the anti-CD132 antibody does not function in the murine setting. Recipient mice that had been injected with *Il2rg*$^{-/-}$ lin^{-} cells transduced with the control SFFV-eGFP vector had no detectable circulating B or T lymphocytes; eGFP^{+} cells were however detected in the periphery of two out of four animals (figure 3.11). eGFP^{+} *Il2rg*$^{-/-}$ lin^{-} cells would have no selective proliferative advantage in this murine model and therefore, since recipient mice had received only sub-lethal doses of irradiation prior to injection, these data are expected.

These results indicate that the reconstitution of peripheral blood T and B lymphocytes in experimental animals results directly from the engraftment of γc-retrovirally transduced lin^{-} cells. It is possible that poor engraftment levels were achieved in the single lymphocyte-null MFG γc transplanted mouse and/or that this animal may have received lower doses of corrected cells as a result of unsuccessful intravenous injection. These preliminary results also suggest that the novel self-inactivating gammaretroviral vectors are able restore lymphopoiesis in a SCID-X1 animal model at similar levels to the older LTR-driven retroviral vector currently proven effective in human gene therapy clinical trials.

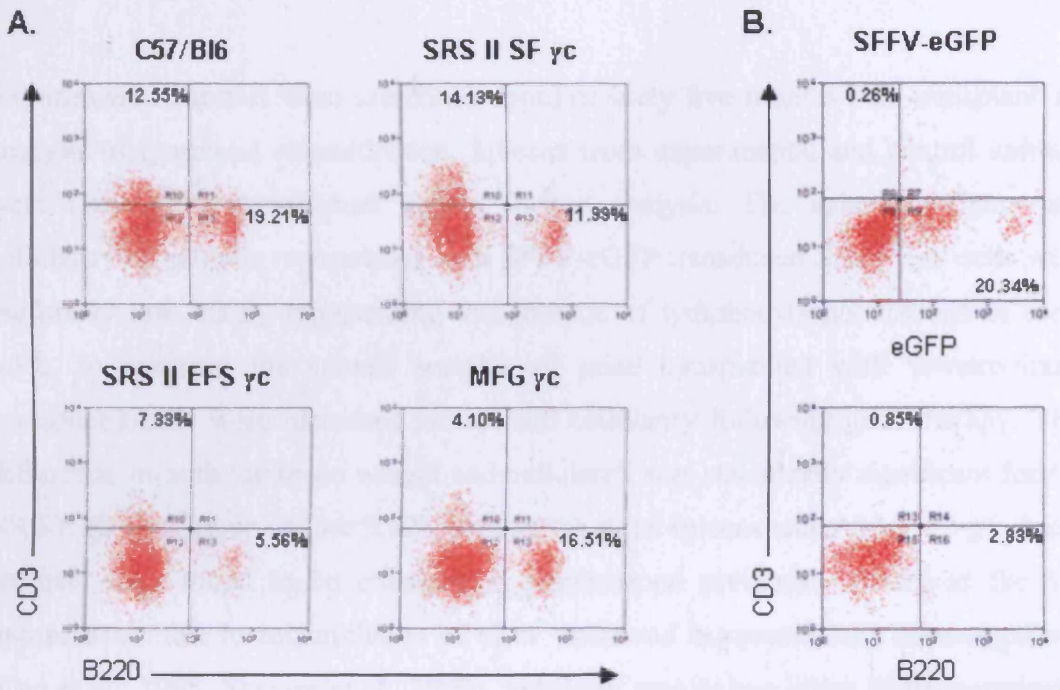

Figure 3.11. Reconstitution of circulating T and B lymphocytes in engrafted animals. (A) Restoration of circulating T and B lymphocytes in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with γc-retrovirally transduced *Il2rg*$^{-/-}$ lin^{-} cells. Peripheral blood mononuclear cells were stained with antibodies to CD3 (T cell marker) and B220 (B cell marker) and analysed by flow cytometry. Plots from one representative animal from each group are shown. The C57/Bl6 cells are from an untransplanted control animal. (B) No B or T cells were detected in mice that had received cells transduced with control vector SFFV-eGFP, however eGFP^{+} cells were detected in the periphery of two out of four animals.

3.3.3 Splenic Weights and Cellularity of Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ *c5*$^{-/-}$ Mice

Experimental animals were sacrificed approximately five months post-transplant for analysis of lymphoid reconstitution. Spleens from experimental and control animals were removed and weighed before further analysis. The splenic weights and cellularity of animals repopulated with SFFV-eGFP transduced *Il2rg*$^{-/-}$ lin^{-} cells were uniformly low, likely representing the absence of lymphocyte populations in these mice. In contrast, the splenic weights of mice transplanted with γc-retrovirally transduced cells were increased in size and cellularity following gene therapy. This difference in both the mean weight and cellularity was statistically significant for the SRS II EFS γc group (figure 3.12). Two of the three spleens taken from *Il2rg*$^{-/-}$ donor animals were found to be enlarged, a phenomenon previously described for this mouse strain due to accumulation of CD4^{+} cells and extramedullary haematopoiesis (Cao et al., 1995; Sharara et al., 1997). Similarly, one or two cases of splenomegally were observed in each of the groups of mice that had received γc-retrovirally transduced cells.

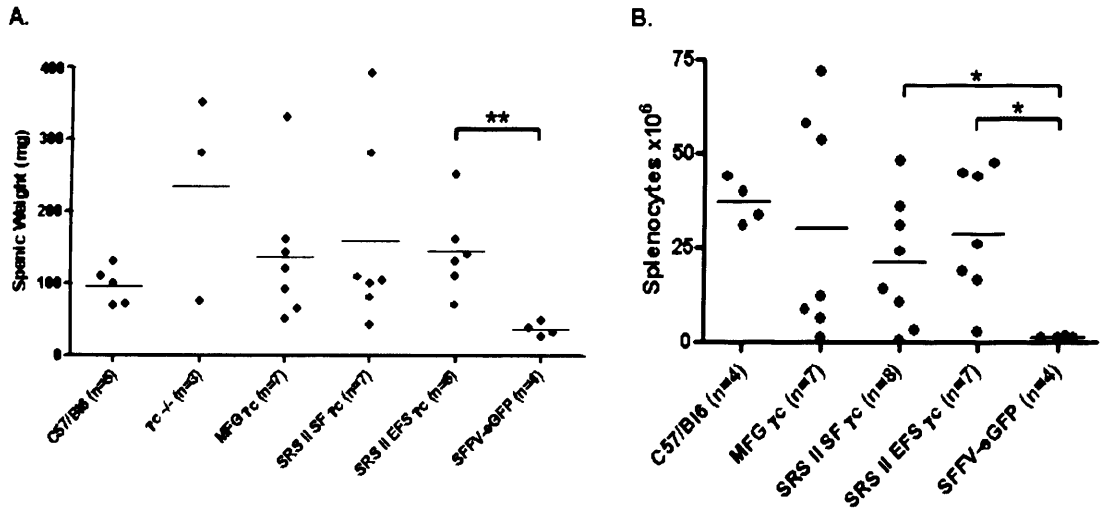

Figure 3.12. Splenic weights and cellularity of transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. (A) Splenic weights and (B) cellularity are increased for mice repopulated with γc-retrovirally transduced cells as opposed to those that received SFFV-eGFP transduced cells, most likely due to lymphoid reconstitution. Bars represent the mean values for each group. (*, $P<0.05$; **, $P<0.01$)

3.3.4 Flow Cytometric Analysis of Lymphoid Populations in the Spleen and Bone Marrow

Cells harvested from the spleens and bone marrow of transplanted mice were analysed for restoration of lymphopoiesis by staining for a variety of lymphocyte cell surface markers and analysis by flow cytometry.

Four to five months post-transplant, T, B and NK cell populations were detected in the spleens and bone marrow of all SRS II EFS γc and SRS II SF γc treated mice, with similar levels of lymphoid reconstitution also detected in six out of seven MFG γc transplanted mice (table 3.1 and figure 3.13A). The percentages of recovered $CD4^+$ and $CD8^+$ T cells in the spleens of SIN vector transplanted animals were found to be within similar ranges as those detected in mice transplanted with MFG γc-transduced cells (table 3.1) and comparable to the levels detected in wild-type C57/Bl6 mice. Furthermore, splenic T lymphocyte CD4/CD8 ratios in the SIN vector transplant recipients were comparable to those in wild-type mice, and considerably lower than for *Il2rg*$^{-/-}$ donor mice which have increased $CD4^+$ T cell numbers (data not shown) (DiSanto et al., 1995; Cao et al., 1995).

The presence of mature $B220^+IgM^+$ B cells in the spleens and bone marrow of treated mice, in particular indicates lymphocyte reconstitution as a direct result of engraftment of retrovirally-transduced lin^- cells, since the γc-deficient donor strain is almost completely devoid of mature B lymphocytes. Furthermore, the small percentage of NK cells detected indicates restoration of lymphopoiesis due to functional γc-mediated signalling in the reconstituted animals, this lineage being entirely absent in *Il2rg*$^{-/-}$ mice. B cell reconstitution in the SIN vector treated mice was found to be as efficient as for those mice that had received cells transduced with the LTR-regulated gammaretroviral vector (table 3.1).

No aberrant increases in lymphocyte or myeloid populations were detected by flow cytometry in mice that were noted to have enlarged spleens.

In accordance with peripheral blood analysis, one of the MFG γc transplanted mice (MFG γc 3) failed to reconstitute lymphocytes of any lineage (data not shown). As previously discussed, poor reconstitution in this animal might be attributable to low levels of engraftment following transplantation or to a low dose of γc-corrected lin$^-$ cells as a result of unsuccessful intravenous injection.

Animals that had received SFFV-eGFP transduced *Il2rg*$^{-/-}$ lin$^-$ cells failed to develop CD4$^+$ or CD8$^+$ T lymphocytes or NK cells populations in either the spleen or bone marrow; however some B220$^+$ B cells were detected. In two of the four transplanted mice, a small percentage of eGFP$^+$ cells were detected in both these tissues, a proportion of which appeared potentially to co-stain with CD3 or B220 lymphoid markers (figure 3.13B), although no CD3$^+$ T cells were detected in the two mice that failed to develop eGFP$^+$ cell populations. The B lymphocytes detected in these mice appeared mostly to be immature B220lo cell populations with only a small percentage in the spleen expressing the more mature B220hi cell surface marker (figure 3.13). These cells represent engraftment of both transduced and untransduced *Il2rg*$^{-/-}$ donor lin$^-$ cells and highlights the ability of early B lineage development in γc-deficient mice, even in the absence of γc signalling.

	CD4 %	CD8 %	B220 IgM%	NK1.1 %
C57/Bl6 (n = 3)	15.75 ± 0.96	10.17 ± 0.27	58.53 ± 0.46	2.49 ± 0.84
MFG (n = 6)	18.55 ± 2.69	5.47 ± 0.58	16.72 ± 3.28	0.36 ± 0.09
SRS II SF γc (n = 7)*	26.9 ± 5.92	9.66 ± 3.27	18.13 ± 4.01	0.65 ± 0.17
SRS II EFS γc (n = 7)+	16.83 ± 1.87	6.92 ± 1.19	20.31 ± 3.31	1.36 ± 1.12

Results are presented as the mean % ± SEM

* n = 6 for NK1.1

+ n = 6 for B220 IgM

Table 3.1. Lymphoid reconstitution in the spleens of transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ *c5*$^{-/-}$ mice.

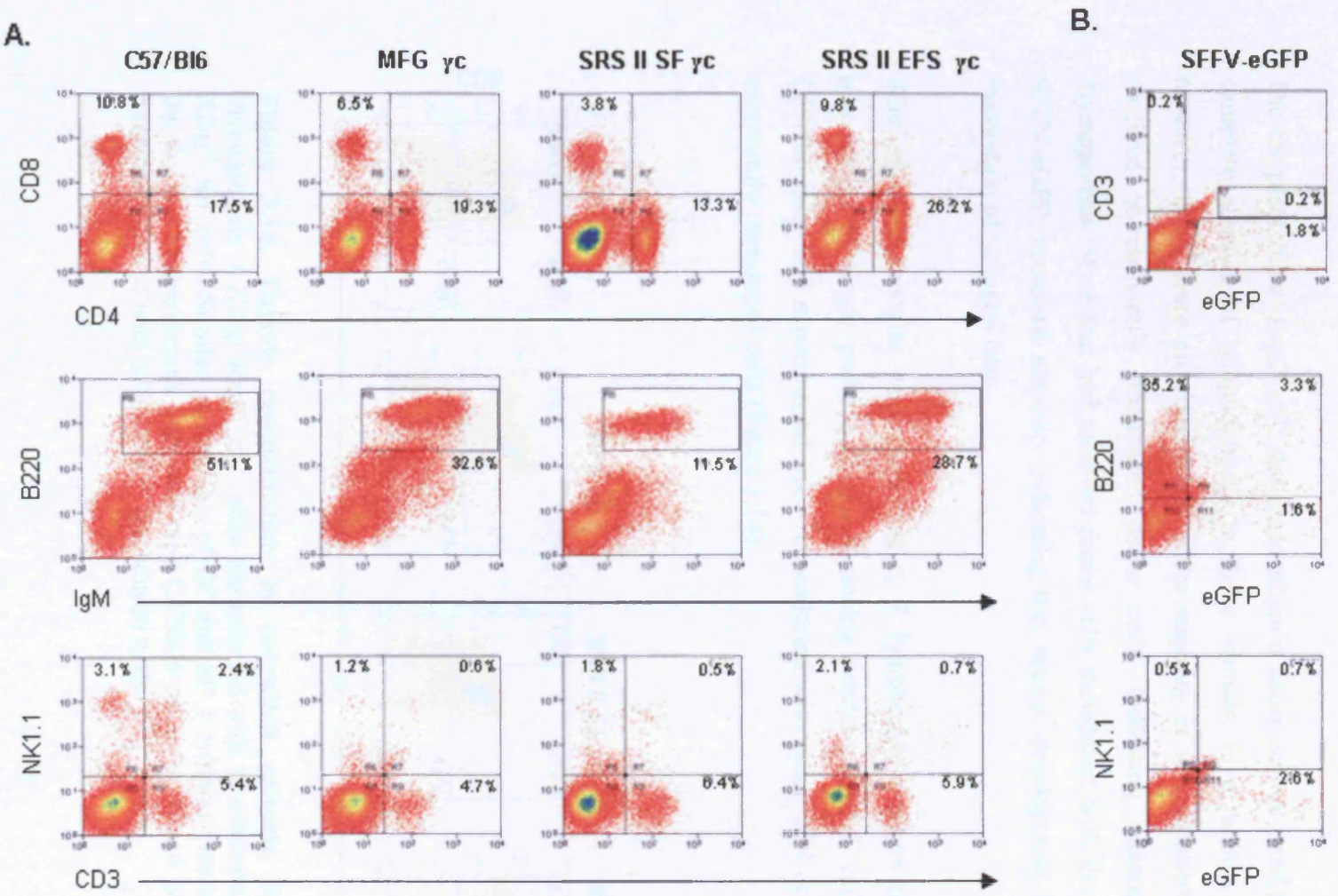

Figure 3.13. Reconstitution of T, B and NK cell populations in the spleens and bone marrow of engrafted animals.
(A) Representative flow cytometry plots from the analysis of lymphocyte restoration in the spleen and bone marrow of *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with γc-retrovirally transduced *Il2rg*$^{-/-}$ lin^{-} cells. CD4^{+} and CD8^{+} T cells and mature B220^{+}IgM^{+} B cells present in the spleens of transplanted mice (first two rows). CD3^{+} T lymphocytes and NK1.1^{+} NK cells detected in the bone marrow of transplanted animals (bottom row). (B) Mice transplanted with SFFV-eGFP transduced cells failed to develop any T, NK or mature B cells. A small percentage eGFP^{+} cells were however detected in the spleens and bone marrow of two out of four mice.

3.3.5 Restoration of Thymopoiesis in Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

The recipient *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ strain are athymic under normal conditions due to the complete absence of lymphopoiesis in these animals. Five months post-transplant however, thymi were clearly present in the majority of reconstituted mice that had received γc-retrovirally transduced donor cells, indicating restoration of active thymopoiesis. Mice that had received donor cells transduced with the control vector, SFFV-eGFP, remained athymic, indicating that thymic development is likely due to restoration of γc-signalling.

Staining of thymocytes with antibodies to T lymphocyte markers revealed similar percentages of single positive (SP) and double positive (DP) T cells in wild-type C57/Bl6 mice and experimental mice reconstituted with either SIN or LTR-regulated retrovirally transduced cells (figure 3.14).

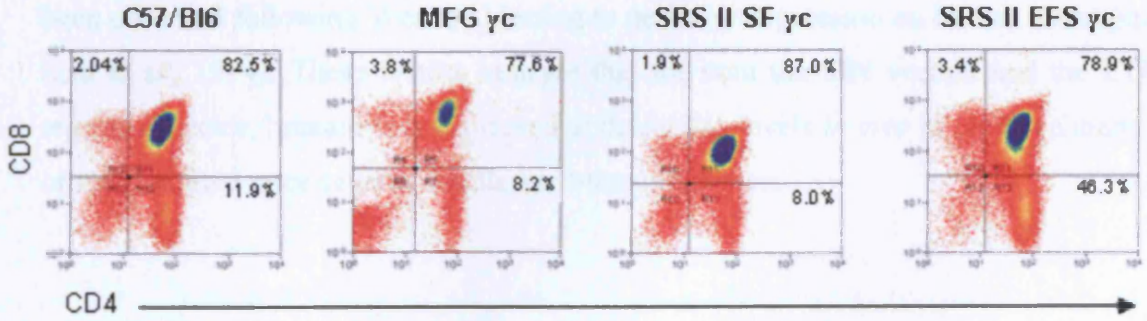

Figure 3.14. Thymic reconstitution in engrafted animals. Restoration of thymopoiesis in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with γc-retrovirally transduced *Il2rg*$^{-/-}$ lin^{-} cells. Similar percentages of DP and SP T lymphocytes are detected in the thymi of experimental and wild-type C57/Bl6 mice. No thymi were present in mice transplanted with SFFV-eGFP transduced cells.

3.3.5 Detection of Human γc Expression in the Bone Marrow of Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

To confirm expression of the human γc transgene in reconstituted mice, Western blot analysis was performed on bone marrow cell lysates. Proteins were separated by SDS-PAGE, immunoblotted and probed with an anti-CD132 antibody. Immunoblots were later re-probed with an antibody to β-actin to confirm the presence of protein in all sample lanes. As shown in figure 3.15, protein bands detected by the anti-CD132 antibody were present in cell lysates from *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with γc-retrovirally transduced lin^{-} cells but not for animals that received SFFV-eGFP transduced cells; it must be noted however that the SFFV-eGFP protein sample was grossly underloaded, although a positive band is detected for the SRS II SF γc sample containing an equivalent low amount of protein. A dominant band of approximately 68 kDa was detected in all reconstituted mice, slightly larger than the 64 kDa isoform of γc previously detected *in vitro* (figure 3.2) possibly representing altered murine glycosylation patterns *in vivo*. Smaller bands were also detected by the anti-CD132 antibody, likely representing under-glycosylated precursor forms of the protein, as has been observed following Western blotting to detect γc expression on human neutrophils (Liu et al., 1994). These results indicate that for both the SIN vectors and the LTR-regulated vector, human γc is expressed at detectable levels *in vivo* in the bone marrow of reconstituted mice several months post-transplantation.

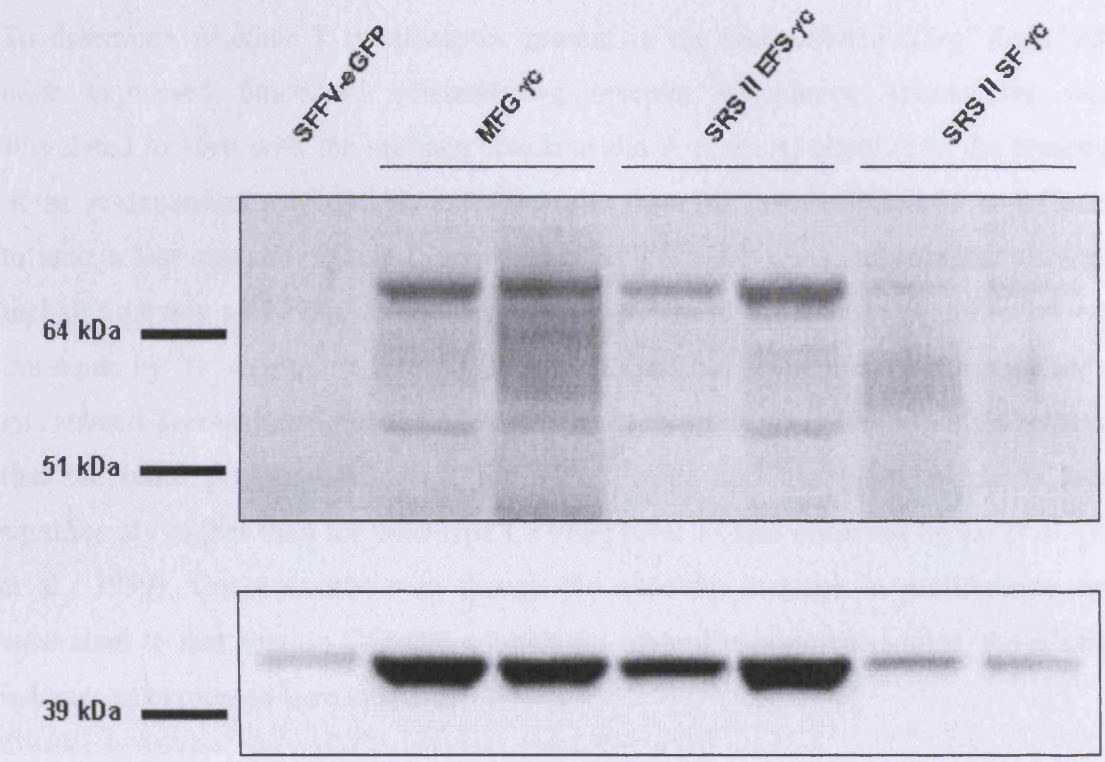

Figure 3.15. Expression of human γc in the bone marrow of transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. Cell lysates from the bone marrow of transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice were separated by SDS-PAGE, immunoblotted and probed with an anti-CD132 antibody. Bands probably representing glycosylated and under-glycosylated forms of human γc are detected in the bone marrow of animals that received γc-retrovirally transduced cells. Control animals transplanted with SFFV-eGFP transduced cells had no detectable γc-positive bands indicating antibody specificity. The blot was also probed with an antibody to the 41 kDa β-actin protein (lower lane) to demonstrate protein loading in each lane. Results are shown for two representive mice for each of the MFG γc, SRS II EFS γc and SRS II SF γc groups.

3.3.6 *In Vitro* Proliferation of Splenocytes from Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

To determine whether T lymphocytes present in the reconstituted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice expressed functional γc-containing receptor complexes, splenocytes were stimulated *in vitro* with the mitogen concanavalin A (Con A) alone or in the presence of the γc-dependent cytokine, IL-2. Splenocytes from MFG γc 3, SRS II SF γc 8 (found to have a low splenocyte count) and control SFFV-eGFP transplanted mice were not included in this assay due to insufficient cell numbers. Splenocyte proliferation was measured by ^{3}H –thymidine incorporation and expressed as the mean stimulation index (stimulated average/unstimulated average) for each group. It is worth noting however that the basal proliferation levels for *Il2rg*$^{-/-}$ mice and transplant recipients were significantly higher than for wild-type C57/Bl6 mice as also observed by Lo *et al.* (Lo et al., 1999). Consequently even though the absolute increase in proliferation was equivalent to that seen in C57/Bl6 animals for several experimental mice, the relative increase as expressed here is lower.

In all reconstituted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice, T cells were capable of proliferation in response to Con A at levels higher than those observed for splenocytes from *Il2rg*$^{-/-}$ donor mice (figure 3.16). These observations correlate with published data by Disanto *et al.* that thymocytes from *Il2rg*$^{-/-}$ mice fail to respond to Con A *in vitro* with or without IL-2 (DiSanto et al., 1995). In all but two of the transplanted mice, IL-2 further stimulated the Con A mitogenic response, with significantly increased levels of proliferation observed for animals reconstituted with lin^{-} cells transduced with either SIN vector (figure 3.16). These data demonstrate that SIN gammaretroviral human γc gene transfer in this model is able to restore expression of functional IL-2 receptor complexes on murine T cells.

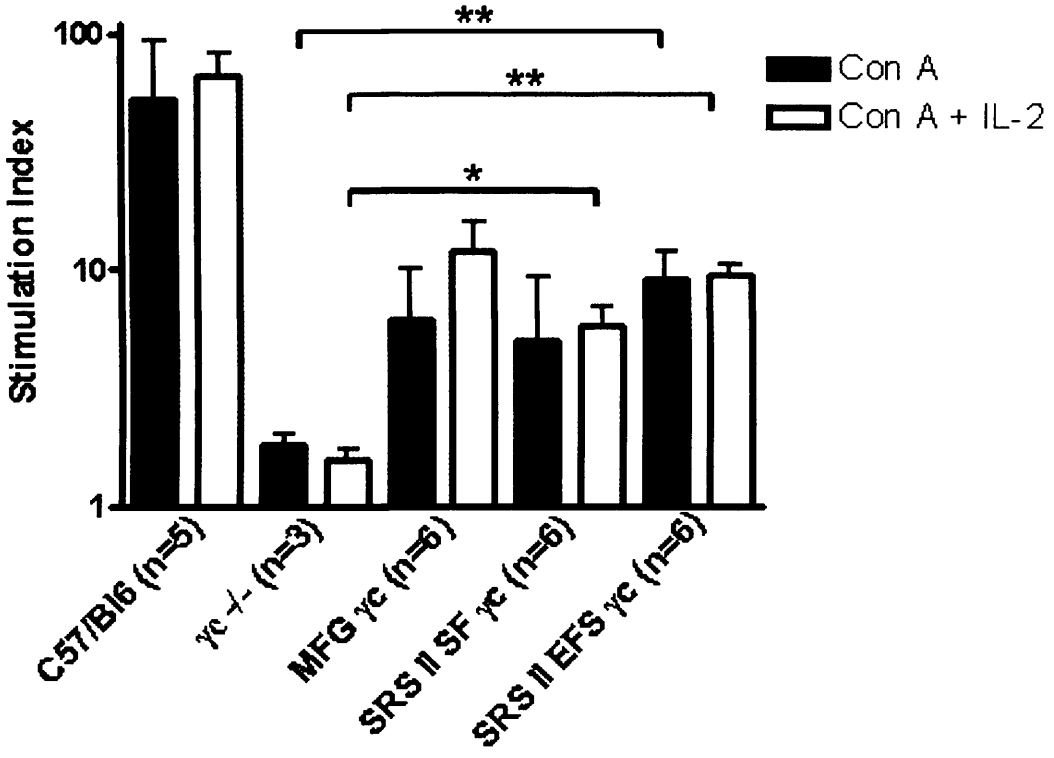

Figure 3.16. Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice exhibit mitogen-induced lymphocyte proliferation *in vitro*. Splenocytes from *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ transplant recipients and C57/Bl6 mice are able to proliferate in response to the mitogen, Con A. In all but two experimental mice, the proliferative response was increased in the presence of IL-2, indicating the reconstitution of functional γc-containing interleukin receptors in these animals. The mean (+/-SD) stimulation indices for each experimental group are shown. (SD, standard deviation; *, $P<0.05$; **, $P<0.01$)

3.3.7 Immunoglobulin levels in Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

To assess humoral immunity in reconstituted MFG γc and SRS II EFS γc mice (serum from reconstituted SRS II SF γc animals was unavailable for analysis) circulating plasma immunoglobulin levels were analysed by enzyme-linked immunosorbent assay (ELISA). Serum from *Il2rg*$^{-/-}$ donor mice was used as a control since the *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ *c5*$^{-/-}$ recipient strain is alymphoid and hence unable to produce immunoglobulin. γc-deficient mice exhibit abnormal serum immunoglobulin levels reflecting the defective B cell development in these animals (Cao et al., 1995); in particular levels of IgG1 and IgG2a are diminished in this model (figure 3.17). Following γc gene transfer however, levels of total IgG immunoglobulin (IgG) in the plasma of mice reconstituted with MFG γc or SRS II EFS γc retrovirally-transduced cells had increased as compared to levels in *Il2rg*$^{-/-}$ donor mice (figure 3.17A). Furthermore, IgG1 in the sera of these experimental mice were increased to levels comparable to those detected for wild-type C57/Bl6 animals (figure 3.17B). IgG1 class-switching has been demonstrated to be dependent on IL-4 secreted by T_H2 $CD4^+$ T helper cells, hence these data in particular indicate restoration of both T lymphocyte function and γc-containing cytokine receptors in the reconstituted mice (Kuhn et al., 1991). Reconstitution of serum IgG2a appeared more efficient in the SRS II EFS γc mice, with IgG2a detectable in only one of the two MFG γc mice analysed (figure 3.17C). In this assay comparison to IgG2a levels in wild-type mice was not possible due to the antibody being unable to recognise the IgG2a allele present in C57/Bl6 animals.

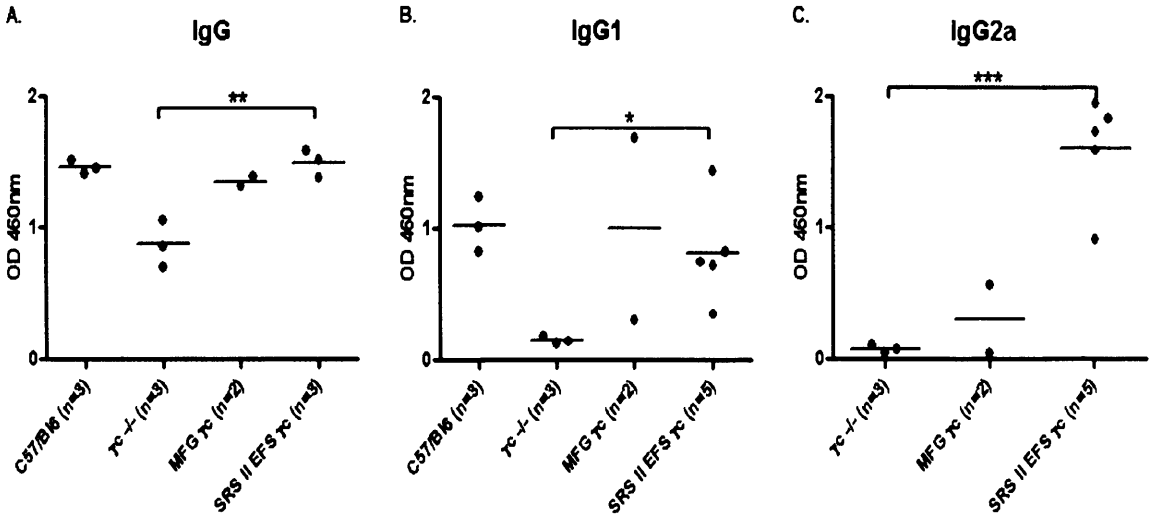

Figure 3.17. Serum immunoglobulin levels in transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. (A) Total IgG, (B) IgG1 or (C) IgG2a levels in the sera of reconstituted mice was assayed by ELISA. Levels of IgG and IgG1 in reconstituted MFG γc and SRS II EFS γc animals were found to be similar to those observed in normal C57/Bl6 mice. IgG2a was only detected in the sera of SRS II EFS γc reconstituted mice. Bars represent the mean values for each group. (*, P<0.05; **, P<0.01; ***, P<0.001)

3.3.8 Proviral Copy Number in Sorted Populations from Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Lymphoid and myeloid populations from the spleen and thymus of a representative *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mouse transplanted with either MFG γc or SRS II SF γc transduced cells were sorted according to their cell surface markers. Genomic DNA was subsequently extracted and proviral copy number determined by quantitative real-time PCR using primers and Taqman probe specific for the human γc cDNA. Dilutions of the gammaretroviral plasmid, MFG γc, and a plasmid containing the murine *titin* gene were used to produce standard curves within the assay against which copies of integrated provirus and cell number could be calculated respectively.

Provirus was detected in sorted splenic CD3$^+$ T cells and IgM$^+$ mature B cells from mice reconstituted with either MFG γc or SRS II SF γc transduced cells at low levels of less than one copy per cell, indicating the likelihood of untransduced *Il2rg*$^{-/-}$ donor lin$^-$ cells having also engrafted in these animals (figure 3.18A). Due to poor genomic DNA yield, proviral copy number in sorted myeloid CD11b populations in the spleen were only determined for the SRS II SF γc reconstituted mice; proviral copy number was found to be extremely low in these myeloid populations representing the lack of selective engraftment potential of this lineage in the SCID-X1 setting. Provirus was also detected in sorted CD3$^+$ and CD4$^+$ T lymphocytes from the thymi of SRS II SF γc reconstituted mice (figure 3.18B).

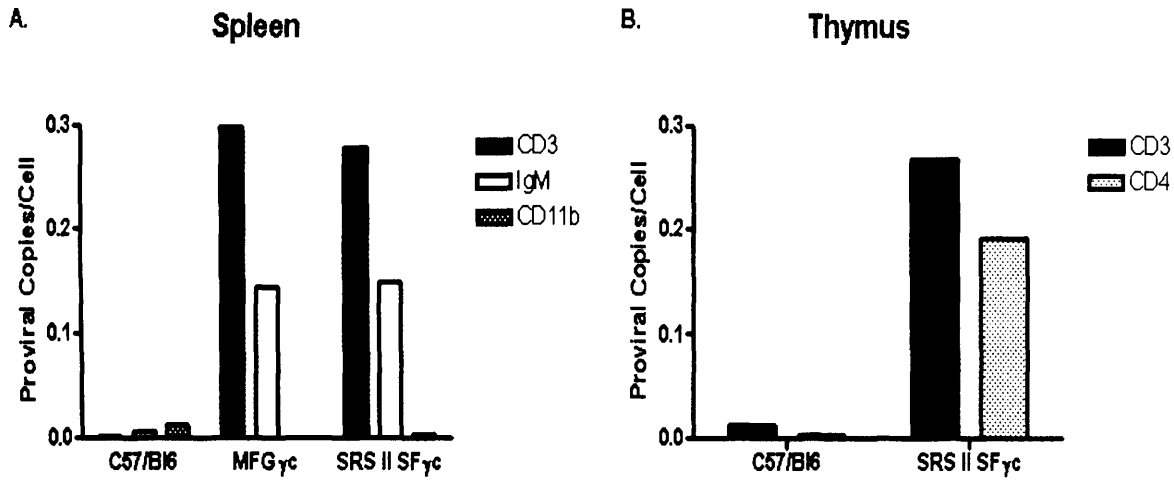

Figure 3.18. Proviral copy number in sorted lymphoid and myeloid populations from transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. (A) Provirus was detected in sorted lymphoid populations from the spleens of reconstituted mice. (B) Proviral copy number in sorted T lymphocyte populations from the thymus of an SRS II SF γc reconstituted mouse. Data shown represents one animal from each group.

3.3.9 Proviral Copy Number in the Bone Marrow and Spleens of Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Proviral copy number was also determined for unsorted cell populations from the spleens and bone marrow of experimental mice. Proviral copy number in the bone marrow was relatively low, less than 0.2 copies per cell for all experimental animals, indicating a high degree of chimerism between transduced and untransduced cells, likely as a result of sub-lethal irradiation of recipient mice and the low multiplicity of infection used (figure 3.19A). No large differences in proviral copy number were observed for mice that had received cells transduced with either the LTR-regulated vector or the SIN gammaretroviral vectors, although two mice in the SRS II SF γc group had the highest copy numbers recorded. Proviral copy number appeared to correlate with efficacy of reconstitution, with the lowest copy number determined for the recipient MFG γc 3 (0.0001) which failed to reconstitute any lymphocytes in either tissue. Negligible γc-proviral copies were obtained for the bone marrow of control SFFV-eGFP mice.

Proviral copy number was determined to be slightly higher in the spleens of reconstituted mice for each group, with a range of 0.1-1.2 recorded for all animals that had restored lymphocyte populations (figure 3.19B). One experimental mouse from each SIN vector group was found to have a copy number greater than one, indicating the likelihood of some cells in the spleens of these mice having more than one vector integration per cell. The higher copy numbers observed in the spleens of experimental animals is likely due to the higher proportions of reconstituted lymphoid cells in this organ, interestingly the SRS II SF γc mouse with the highest copy number was found to have an enlarged spleen.

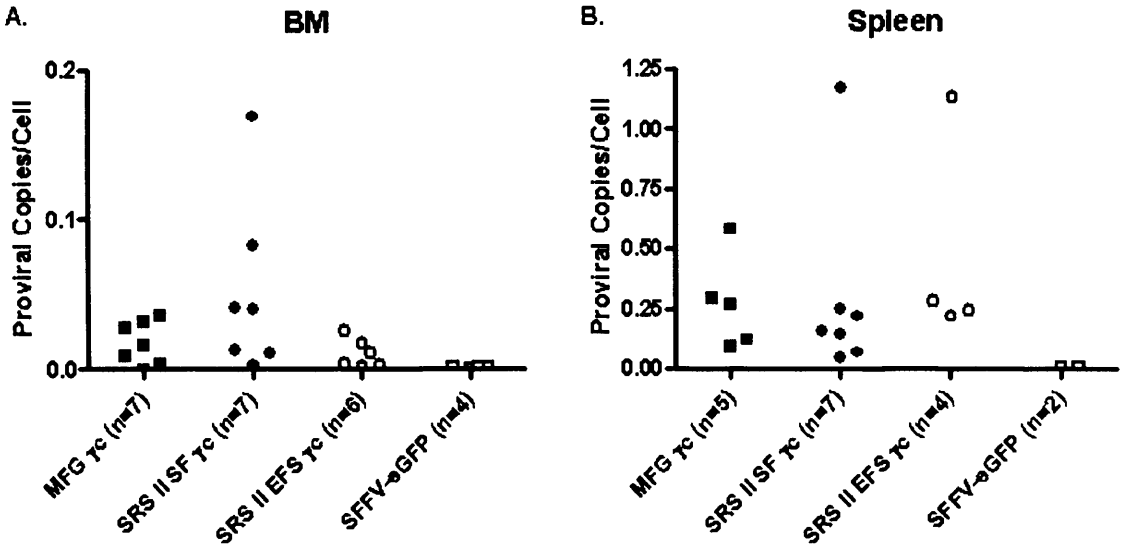

Figure 3.19. Proviral copy number in the bone marrow and spleens of transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. Low proviral copy numbers were detected in the (A) bone marrow and (B) spleens of reconstituted mice. Negligible γc-provirus was detected for control SFFV-eGFP transplanted animals

3.3.10 Restoration of Lymphopoiesis in Secondary Transplant Recipients

To confirm that self-renewing haematopoietic progenitors were targeted during the original gene transfer protocol and hence demonstrate the longevity of such a procedure, secondary transplants were performed. Ten million cells isolated from the bone marrow of reconstituted mice and wild-type C57/Bl6 mice were therefore transplanted into lethally irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ recipients of six to ten weeks of age. A single secondary transplant was performed from the SRS II SF γc, SRS II EFS γc and MFG γc primary ransplanted animals. Mice that had received secondary transplants were sacrificed for analysis approximately seven to eight months after transplantation; the recipient of SRS II EFS γc reconstituted bone marrow was sacrificed only eight weeks post-transplant due to the onset of wasting and colitis-like symptoms. Spleens, thymi and bone marrow were harvested from each animal for analysis by flow cytometry and proviral copy number.

3.3.10.1 Flow Cytometric Analysis of the Spleens and Bone Marrow from Secondary Transplant Recipients

As for the initial reconstituted animals, splenocytes and bone marrow cells from the secondary transplant recipients were stained with antibodies for a variety of lymphocyte and myeloid cell surface markers and analysed by flow cytometry. In all secondary transplant recipients, except those that received bone marrow from SFFV-eGFP control mice, CD4$^+$ and CD8$^+$ T lymphocyte populations were restored in the spleen and bone marrow (figure 3.20). Reconstitution of the B cell compartment in these mice appeared less efficient, with mature B220$^+$IgM$^+$ B cells detectable only for SRS II SF γc and SRS II EFS γc secondary transplant recipients. The population of immature B220$^+$ B lymphocytes detected in the MFG γc secondary transplant recipient was comparable to the B220$^+$ populations observed in mice that received SFFV-eGFP bone marrow, thus it is likely that the origin of these cells are untransduced donor *Il2rg*$^{-/-}$ precursors. As with B lymphocyte reconstitution, restoration of NK cells was most

efficient in the SRS II EFS γc secondary transplant recipient, with $NK1.1^+$ populations detectable in both the bone marrow and spleen. In all secondary transplant recipients the percentage of splenic myeloid $CD11b^+$ cells was noted to be increased as compared to mice transplanted with C57/Bl6 bone marrow. This trend was also observed in SFFV-eGFP secondary transplants however, thus it unlikely that these populations result from γc-transduced cells. No $eGFP^+$ cells were detected in SFFV-eGFP transplant recipients, likely due to the lack of selective engraftment potential of transduced cells in the primary transplanted mice being further reinforced in the secondary transplant recipients (figure 3.20B).

These results demonstrate the successful transduction and engraftment of haematopoietic progenitor cells with pluripotent potential by the SIN gammaretroviral vectors in the original transplant recipients, since bone marrow from these mice was able to restore T, B and NK cell populations in alymphoid secondary transplant recipients. It is possible that only transduced committed lymphoid precursors were originally transplanted in the MFG γc mouse from which the secondary transplant was performed as B lymphopoiesis was not restored.

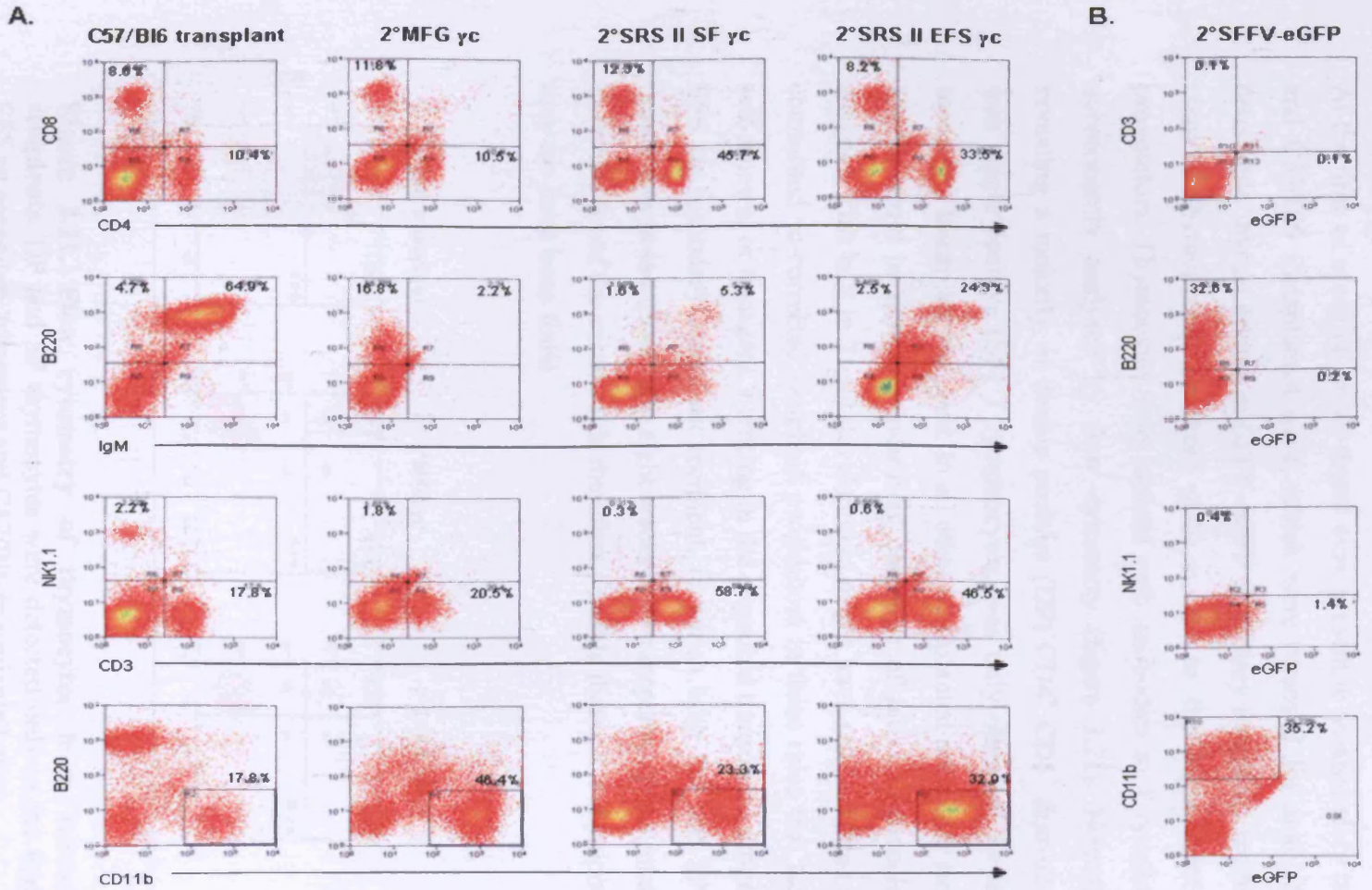

Figure 3.20. Reconstitution of lymphoid populations in the spleens of secondary transplant recipients. (A) Representative flow cytometry plots from the analysis of lymphocyte restoration in the spleens of secondary transplant recipients. Bone marrow from a wild-type C57/Bl6 mouse was transplanted into *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ recipients to serve as controls. CD4^{+} and CD8^{+} T cells were detected in the spleens of all secondary transplant recipients, whilst mature B220^{+}IgM^{+} B cells were only present in the spleens of mice that had received SRS II SF γc and SRS II EFS γc bone marrow (first two rows). NK cell reconstitution was most efficient in the SRS II EFS γc secondary transplant recipient (third row). An increase in the CD11b^{+} myeloid fraction was observed in the spleens of all secondary transplant recipients. (bottom row) (B) Mice transplanted with SFFV-eGFP bone marrow failed to develop any T, NK or mature B cells.

3.3.10.2 Flow Cytometric Analysis of Thymocytes from Secondary Transplant Recipients

At the time of sacrifice, small thymi were present in γc-secondary transplant recipients and C57/Bl6 transplanted mice, which were removed for analysis. The absence of detectable thymic organs in SFFV-eGFP secondary transplant recipients indicates that restored thymopoiesis in these mice is due to the engraftment of γc-transduced progenitors. Thymocytes were stained with antibodies to T lymphocyte markers and subsequently analysed by flow cytometry (figure 3.21). Normal thymic staining, revealing a majority of double positive (DP) $CD4^+$ $CD8^+$ thymocytes differentiating into single positive (SP) T lymphocytes, was only observed for the SRS II EFS γc secondary transplant recipient. In all other transplanted mice only single $CD4^+$ or $CD8^+$ T cells could be detected, indicating cessation of active thymopoiesis at the time of analysis. This halt in T cell development might have resulted from the engraftment of committed γc-corrected lymphoid progenitors in these mice that lack the capacity for self-renewal or transgene silencing in the engrafted transduced progenitors. The SRS II EFS γc secondary transplant recipient, in which both DP and SP thymocytes were detected, was sacrificed only eight weeks post-transplant, approximately 5 months prior to remainder of the cohort. It is therefore possible that active thympoiesis in this animal may too have been finite.

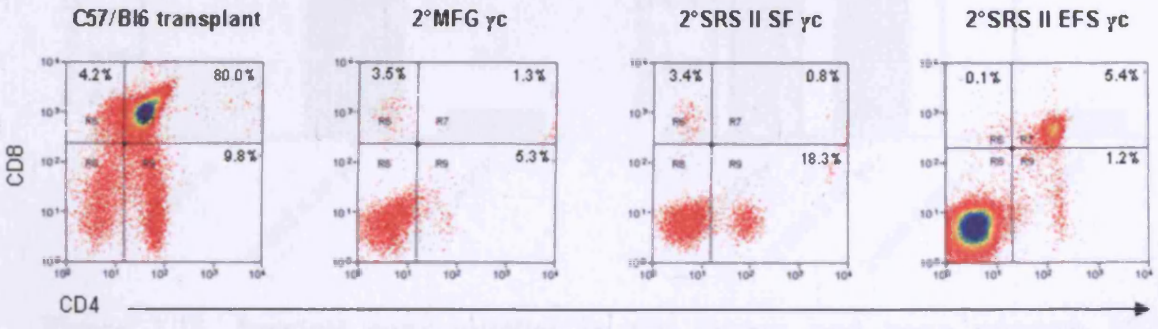

Figure 3.21. Flow cytometry of thymocytes from secondary transplant recipients. DP and SP thymocytes were detected only in the thymi of the SRS II EFS γc secondary transplant and C57/Bl6 transplanted mice.

3.3.10.3 Proviral Copy Number Analysis in the Spleens and Bone Marrow of Secondary Transplant Recipients

Proviral copy number was determined by quantitative real-time PCR for both the spleens and bone marrow of secondary transplant recipients using the same primers and standards as for the analysis of the initial transplanted mice. Integrated provirus was detected for all mice transplanted with bone marrow from γc-reconstituted primary animals, with proviral copy number detected at the same orders of magnitude in the bone marrow of both primary and secondary transplant recipients (proviral copy number in the bone marrow of the SRS II EFS γc secondary transplant recipient was not estimated due to poor genomic DNA yield) (figure3.22A). Proviral copy numbers in the spleens of the secondary transplant recipients were however found to be a log lower than estimated for the donor reconstituted mice (3.22B). These data likely reflect the decreased numbers of splenic lymphocytes in the secondary transplant recipients due to poor B cell reconstitution. These results also confirm the likelihood that the increased $CD11b^+$ myeloid populations observed in the spleens of the transplanted mice are not due to expansions of γc-transduced cells.

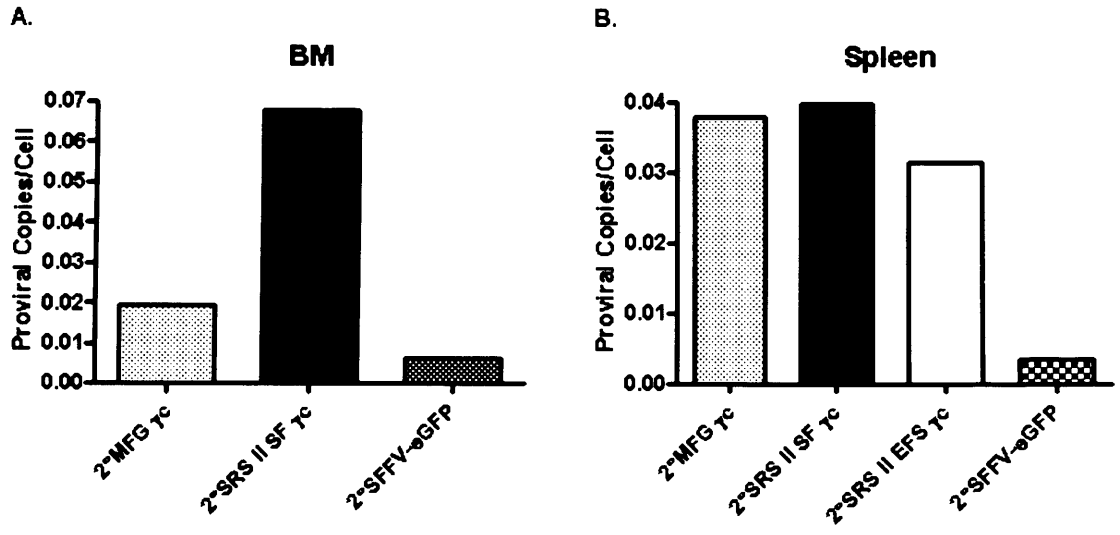

Figure 3.22. Proviral copy number in the spleens and bone marrow of secondary transplant recipients. Provirus was detected at low copy number in the (A) bone marrow and (B) spleens of secondary transplant recipients. Copy numbers in the spleens of the transplanted mice were found to be decreased as compared to the donor primary transplant recipients. Negligible γc-provirus was detected for SFFV-eGFP secondary transplant recipients.

3.3.11 Failure of T Cell Development in Older Engrafted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Transplantation of a cohort of older recipient *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice with SIN gammaretrovirally transduced cells failed to restore T lymphocyte populations in five out of six mice. This trend correlates well with results from the human SCID-X1 gene therapy trial, in which two older treated patients failed to undergo lymphoid reconstitution following transplantation of γc-retrovirally transduced HSCs (Thrasher et al., 2005).

3.3.11.1 Peripheral Blood Analysis of Transplanted Older *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Twelve week old sub-lethally irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice were transplanted with SIN gammaretrovirally transduced *Il2rg*$^{-/-}$ lin^{-} cells (recipient mice in all other cohorts were injected at six to ten weeks of age) the protocol and gammaretroviral preparations used to transduce the donor HSCs were identical to those used previously to successfully reconstitute the younger recipient mice. Samples of peripheral blood were taken for analysis three months post-transplant, stained for lymphocyte markers and analysed by flow cytometry for the reconstitution of circulating lymphoid cells.

In four of five SRS II EFS γc recipients and in one SRS II SF γc mouse, CD3^{+} T cells were absent from the periphery at the time of analysis. Circulating B220^{+} B lymphocytes were detected in all older recipient mice however, at levels comparable to wild-type mice indicating potential partial lymphoid reconstitution (figure 3.23). The remaining SRS II EFS γc transplanted mouse had both CD3^{+} and B220^{+} populations present in the peripheral blood (data not shown) and appeared to have undergone full lymphoid reconstitution as observed in younger recipient mice.

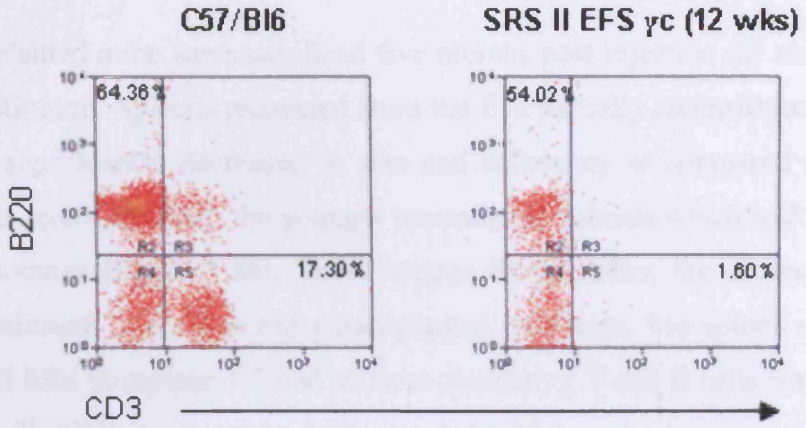

Figure 3.23. T lymphocytes are absent from the periphery following transplantation of older *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ recipients. Peripheral blood from transplanted mice was stained with antibodies to T (CD3) and B (B220) cell markers three months post-infusion of transduced cells. Only B cells were present in the periphery of five out of six older transplanted mice. An example flow cytometric plot of one of the SRS II EFS γc transplanted mice that failed to reconstitute circulating T lymphocytes is shown.

3.3.11.2 Decreased Splenic Weight and Cellularity in Older Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Transplanted mice were sacrificed five months post-injection for analysis of lymphoid reconstitution. Spleens recovered from the five partially reconstituted mice were found to be significantly decreased in size and cellularity as compared to the majority of spleens recovered from the younger transplanted animals which had recovered T and B lymphocytes (figure 3.24). These organs likely reflect the absence of T lymphoid reconstitution in the five older transplanted recipients. The spleen recovered from the SRS II EFS γc recipient found to have circulating T and B cells was also increased in size and cellularity as compared to remainder of the cohort.

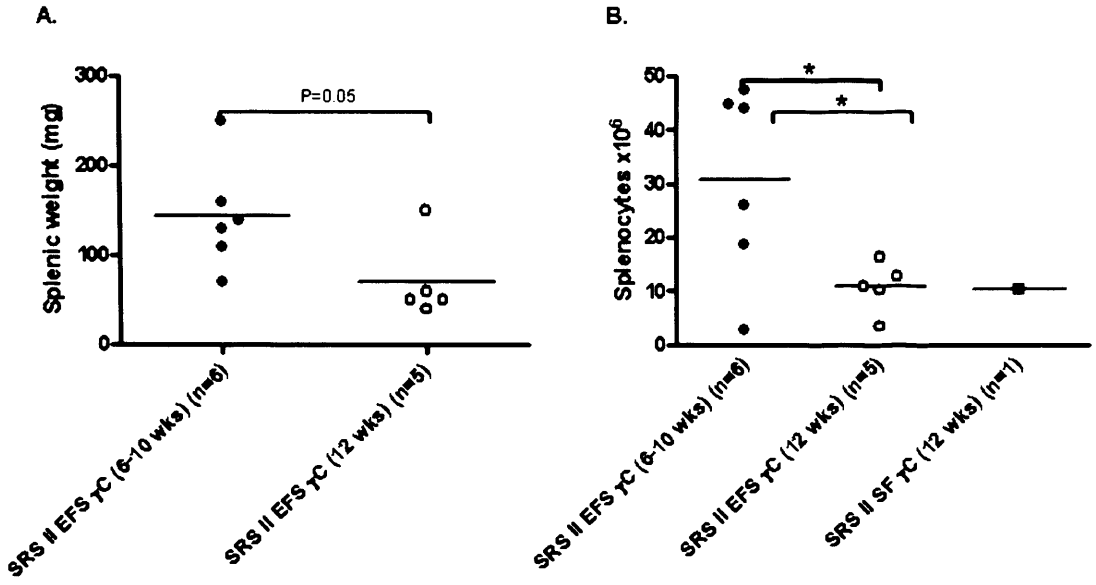

Figure 3.24. Splenic weight and cellularity is decreased in older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. (A) Splenic weights and (B) cellularity are on average decreased in recipient mice transplanted at twelve weeks of age due to the failure of T lymphoid reconstitution. Bars represent the means for each group. (*, P<0.05)

3.3.11.3 Flow Cytometric Analysis of Lymphocyte Reconstitution in the Spleens and Bone Marrow of Older Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Cells isolated from the spleens and bone marrow of the transplanted mice were stained with the same antibodies to lymphoid and myeloid cell surface markers as used previously and analysed by flow cytometry. In five of the six older transplanted mice, T cells were undetectable in both the spleens and bone marrow confirming the results from the peripheral blood analysis (figure 3.25). Concurrent with these observations, thymi were undetectable in all five of these mice. $B220^+$ B lymphocytes were detected in both organs for all six transplanted older mice however. In the five partially reconstituted animals mature $B220^+IgM^+$ B cells were present, but at significantly lower levels than in wild-type C57/Bl6 animals or the immunologically reconstituted mice. NK cells were also detectable in the bone marrow of the older recipient mice indicating that in this murine model, reconstitution of the T cell compartment appears to be directly related to age at transplantation. CD11b myeloid populations in all transplanted mice appeared normal (data not shown).

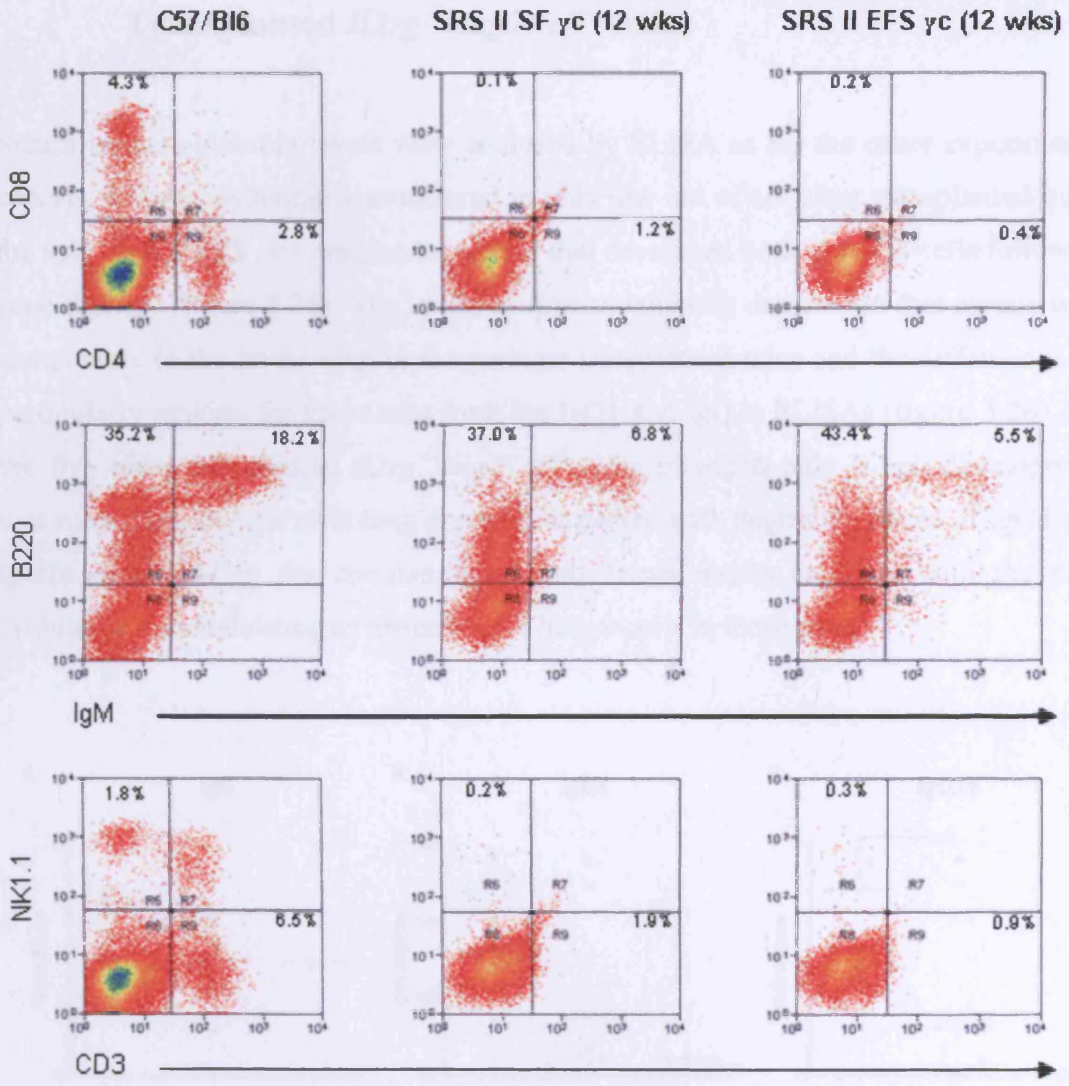

Figure 3.25. Flow cytometric analysis of bone marrow cells from older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. Bone marrow cells from transplanted mice were stained with antibodies to lymphocyte markers and analysed by flow cytometry. Five out of six older recipient mice failed to reconstitute the T cell compartment five months post-transplantation. Data from two representative mice

3.3.11.4 Failure of Immunoglobulin Isotype Switching in Older Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Serum immunoglobulin levels were analysed by ELISA as for the other experimental cohorts. Isotype switching was restored in only one out of six older transplanted mice, the sole SRS II EFS γc transplanted animal that developed both T and B cells following gene therapy (figure 3.26). The levels of immunoglobulin detected in this mouse were comparable to the levels seen in the younger transplanted mice and the differences are particularly striking for the results from the IgG1 and IgG2a ELISAs (figure 3.26). For the five older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice in which only B cell development was recovered, isotype switching appeared impaired with negligible levels of IgG1 and IgG2a detectable in the circulating plasma. These results correlate with the flow cytometric data indicating an absence of T helper cells in these mice.

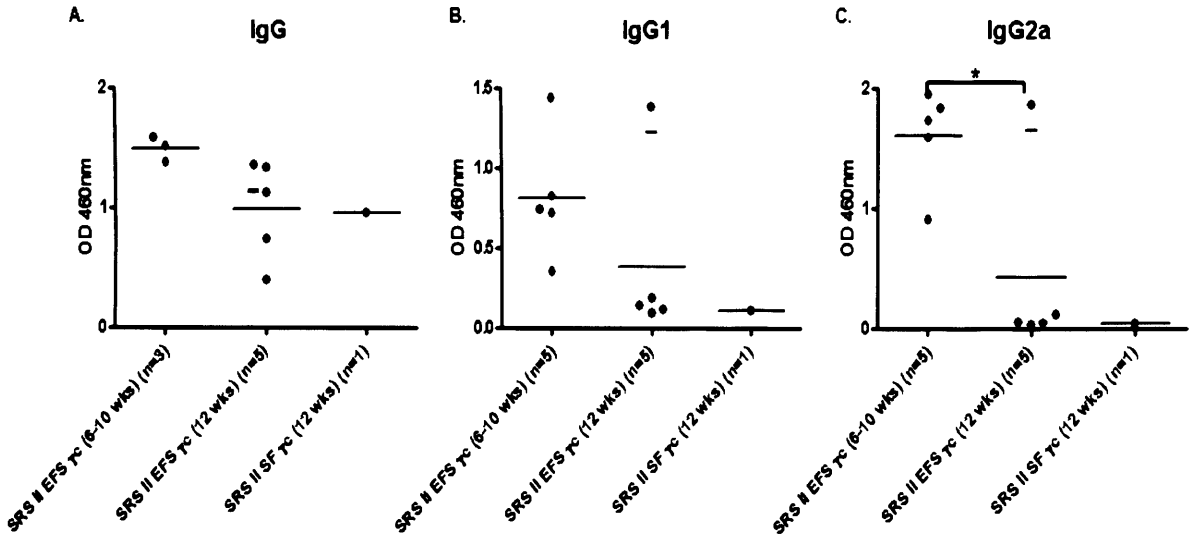

Figure 3.26. Immunoglobulin levels in the sera of older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. (A) Total IgG, (B) IgG1 or (C) IgG2a levels in the sera of the older transplant recipients was assayed by ELISA. Five out of six older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice were unable to undergo isotype switching. The results for the SRS II EFS γc older recipient that reconstituted both T and B lymphocytes are underlined in red. Data is compared to the serum levels from the SRS II EFS γc reconstituted younger mice that were analysed within the same assay. Bars represent the mean values for each group. (*, $P<0.05$)

3.3.11.5 Proviral Copy Number Analysis in the Spleens and Bone Marrow of Older Transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice

Quantitative real-time PCR was used to estimate proviral copy number in the spleens and bone marrow of transplanted older recipient mice using the same primer sets and standards as previously. The highest copy number observed for both tissues was from the sole SRS II EFS γc older transplant recipient that reconstituted both T and B lymphocytes (figure 3.27A&B). These results confirm that the development of both sets of lymphoid populations in this mouse is directly linked to increased proviral copy number and thus that the lymphocytes are likely to have originated from vector transduced precursors.

On the whole, proviral copy number correlates with efficacy of reconstitution, with the copy numbers detected in the spleens and bone marrow of the partially reconstituted five older transplanted mice on average lower than observed for the younger transplant recipients with restored T and B lymphocyte development (figure 3.27C).

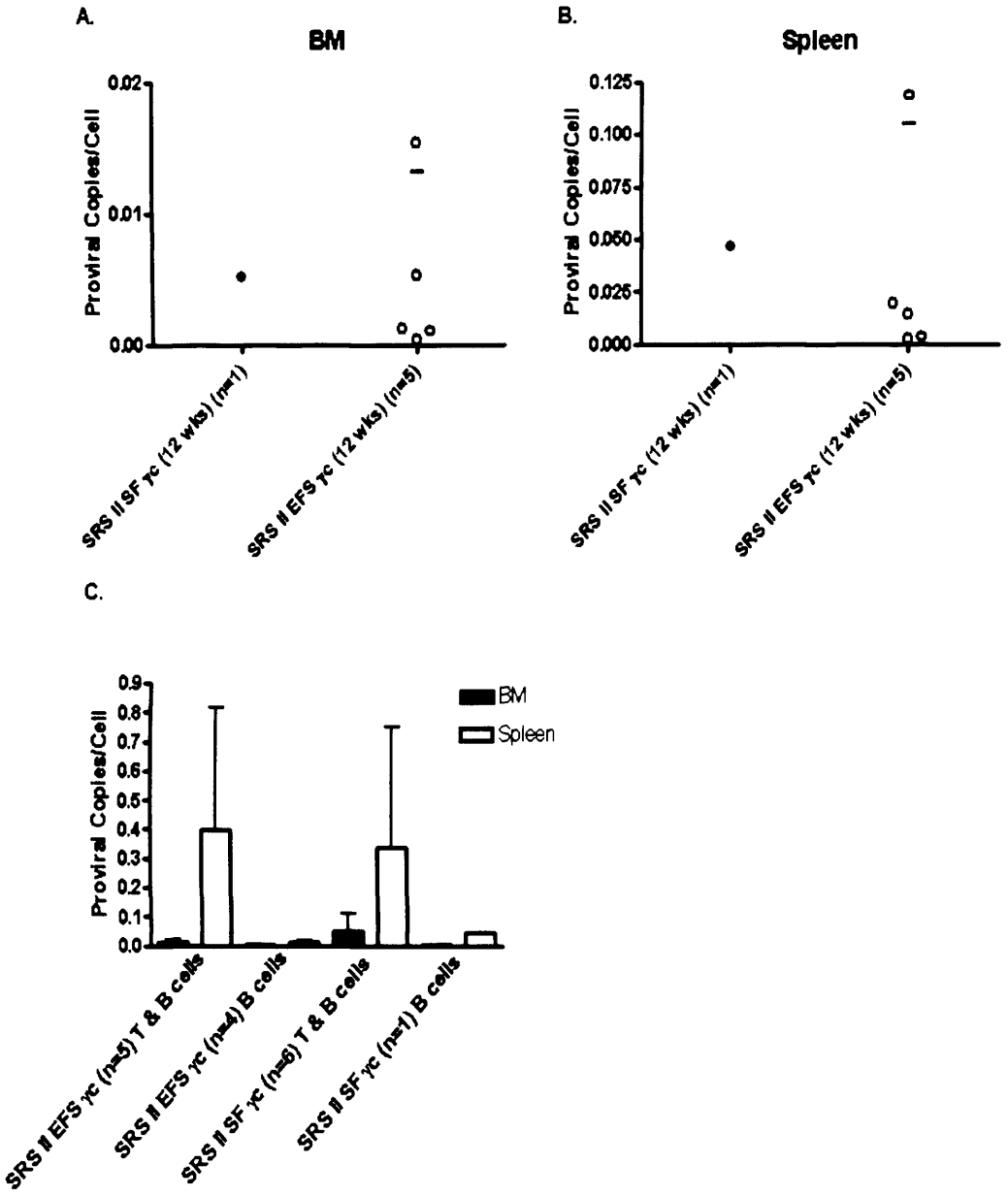

Figure 3.27. Proviral copy number in the spleens and bone marrow of older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-}$*/c5*$^{-/-}$ mice. Proviral copy number in the (A) bone marrow and (B) spleens of older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-}$*/c5*$^{-/-}$ mice. The results for the SRS II EFS γc older recipient that reconstituted both T and B lymphocytes are underlined in red. (C) Proviral copy number correlates with efficacy of reconstitution with the average vector copies being higher in both tissues in mice (younger transplant recipients and the one older transplant recipient) that developed both T and B lymphocytes post-transplantation as opposed to the five older transplant recipients which reconstituted B cells only. Columns represent the means (+/-SD) of each group. (SD, standard deviation; n, number of mice)

3.4 Conclusions

From the above data it is possible to conclude that the self-inactivating gammaretroviral SRS II vector series is able to efficiently transduce both immortalised and primary cells and express the human γc transgene on the cell surface. Both SRS II SF γc and SRS II EFS γc expressed the transgene to functional levels in murine γc-deficient haematopoietic precursors enabling *in vitro* lymphoid differentiation. Furthermore, the results demonstrate that transplantation of SIN vector transduced *Il2rg*$^{-/-}$ lin^{-} cells in alymphoid recipient mice enabled reconstitution of all lymphocyte lineages (for a summary of all *in vivo* experiments see Appendix 10). Immunity in these mice appeared restored; T cells were able to proliferate *ex vivo* in response to mitogens and at increased levels in the presence of a γc-dependent cytokine. SRS II EFS γc reconstituted mice had increased levels of serum immunoglobulin and the presence of IgG1 in the plasma demonstrated isotype switching in response to the γc-dependent cytokine IL-4.

The *ex vivo* lin^{-} transduction protocol resulted in high transduction efficiencies whilst maintaining stem cell pluripotency, as secondary transplant recipients exhibited complete or partial immunological reconstitution. Copy numbers in the spleens and bone marrow of both primary and secondary transplant recipients were comparable for all vectors and found to be low. Furthermore, proviral copies in homogeneous sorted cell populations from several experimental mice were less than one, indicating only a single vector integration per cell in the engrafted gene-corrected HSCs and their progeny. Low proviral copy number is an important factor in terms of safety of the gene therapy procedure, decreasing the likelihood of insertional mutagenesis by minimising the number of potentially hazardous integration events. The selective engraftment advantage of γc-corrected cells in the SCID-X1 model means that even in the setting of sub-lethal irradiation and low copy number phenotypic correction is acheived. This engraftment advantage was evident in reconstituted mice, since sorted myeloid CD11b^{+} populations were found to harbour negligible proviral DNA as compared to sorted lymphoid lineages.

Age at transplantation was found to be an important factor in terms of restoration of T cell immunity in the SCID-X1 murine model. Five out of six animals in a cohort of *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted at 12 weeks of age failed to reconstitute the T cell compartment, whilst B and NK cell populations were detected. These results correlate with the failure of gene therapy for SCID-X1 in two older patients enrolled in the clinical trial (Thrasher et al., 2005). It is postulated that in these patients the capacity to initiate thympoiesis is likely to be time-dependent since the absence of ongoing thymocyte and thymic epithelial cell interaction results in potentially irreversible disorganisation of thymic architecture and hypoplasia (Hale et al., 2004; Hollander et al., 1995; van et al., 2000). It is therefore likely that the T cell developmental failure in the older transplanted SCID mice is also attributable to irreversible disruption of the thymic architecture due to the prolonged absence of ongoing thymopoiesis in these animals; particularly given that other lymphoid lineages were restored.

In conclusion, the self-inactivating design does not appear to impact on efficacy of transgene expression or immunological reconstitution following engraftment of retrovially transduced *Il2rg*$^{-/-}$ lin^{-} cells in a SCID-X1 mouse model. Effective immunological reconstitution was observed for both viral (SFFV) and endogenous (EFS) promoter elements regulating human γc expression at comparable levels to those observed with the clinical LTR-regulated vector. Moreover, restoration of humoral immunity appeared most efficient in mice reconstituted with SRS II EFS γc transduced cells as compared to the MFG γc group. However future cohorts will need to be set up to confirm these findings and to analyse immunoglobulin levels in SRS II SF γc reconstituted mice. These SIN gammaretroviral vectors therefore present potentially safer alternative vectors for gene therapy of SCID-X1 having been demonstrated to be equally effective in this pre-clinical model of the disease. The less potent endogenous EFS promoter in particular provides a potentially less mutagenic alternative vector, being theoretically less likely to exert enhancer effects on nearby genes following proviral integration. Incorporation of this promoter, comprised of endogenous regulatory sequences, offers the further advantage that it is less likely cause an immune response, as opposed to viral promoters, that might result in transgene silencing (Qin et al., 1997).

3.5 Final Conclusions

- The SRS II series of gammaretroviral SIN vectors are able to efficiently transduce both haematopoietic and non-haematopoietic cell lines
- The SRS II vector series are able to restore the lymphoid differentiation potential of *Il2rg*$^{-/-}$ haematopoietic progenitor cells *in vitro* to similar levels as MFG γc
- The SRS II series of vectors were able to restore immunity in the SCID-X1 mouse model following *ex vivo* gene therapy
- Pluripotent HSCs were targeted during the *ex vivo* lin^{-} transduction protocol by the SIN gammaretroviral vectors
- Reconstitution of the T lymphoid compartment following gene therapy of the SCID-X1 murine model is time-dependent

Chapter Four

Cloning and Analysis of the Human *IL2RG* Promoter

4.0 Aims

- To identify the conserved regulatory sequences upstream of the human *IL2RG* gene and clone the promoter sequence into lentiviral vectors
- To analyse the tissue-specificity of the *IL2RG* promoter in the context of a lentiviral vector regulating eGFP expression in a variety of cell lines
- To analyse the tissue-specificity of the *IL2RG* promoter *in vivo* following injection of concentrated lentivirus into neonatal mice

4.1 Introduction

Whilst the SIN design confers a potential increase in vector safety by eliminating the potent dual viral promoter/enhancer LTR sequences, the choice of internal promoter remains an important factor when considering the mutagenic potential of retroviral vectors for gene therapy. Firstly, transgene expression must be regulated to a level that enables phenotypic correction of the disorder to be treated whilst avoiding aberrant transgene expression that may contribute towards cellular transformation. This was not considered to be a potential risk with regards the treatment of SCID-X1 however, since γc is unable to signal in the absence of other cytokine receptor components (Nakamura et al., 1994). A second consideration regarding the selection of an internal promoter is whether or not it is likely to exert enhancer effects on endogenous genes and thus trigger insertional mutagenesis. An ideal promoter for a gene therapy vector might therefore consist of endogenous regulatory elements, thus enabling physiological expression of the transgene following proviral integration. This might be particularly significant in HSC targeted gene therapy resulting in transgene expression being regulated to appropriate levels during the different stages of development and hence decreasing the likelihood that aberrant expression might lead to oncogenic events. In particular, this approach might be feasible in the SCID-X1 setting whereby gene-marked progenitors exhibit a selective growth advantage. It was therefore decided to develop and test a self-inactivating lentiviral vector for SCID-X1 incorporating the endogenous *IL2RG* regulatory elements as an additional safety feature.

4.2 Development of SIN Lentiviral Vectors Incorporating the *IL2RG* Promoter

4.2.1 Identification of *IL2RG* Regulatory Sequences Conserved Between Human and Mouse

Promoter activity within the sequences 5' to the *IL2RG* gene was first described by Noguchi *et al.* (Noguchi et al., 1993a); a 600bp fragment 5' to the transcription initiation site cloned upstream of a luciferase reporter gene exhibited increased levels of expression in Jurkat cells. Further characterisation of this region demonstrated minimal promoter activity within a fragment from 58 to 80 nucleotides upstream of the transcriptional start site incorporating a putative Ets binding site (figure 4.1) (Ohbo et al., 1995); the Ets family of transcription factors are particularly implicated in the control of expression of a large number of genes in haematopoietic cells, especially during lymphoid development (Anderson et al., 1999). A later paper confirmed this site as critical for promoter activity, and also present in the murine *Il2rg* promoter (Markiewicz et al., 1996). This work in addition characterised a -1053/+34 region and a series of 5' deletion constructs that exhibited promoter activity in cells of haematopoietic origin only. Three other potential regulatory motifs, a second putative Ets binding site located 55 bp upstream of the transcriptional start site, a GT-box and a TATA-like sequence at -88 were all characterised as non-essential for basal promoter activity (figure 4.1) (Markiewicz et al., 1996).

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1101 CTCTGTAAAG CCCTGGTTTA TAAGGTTCTT TCCACCGGAA GCTATGACAG
                          TATA like                Ets

1151 AGGAAACGTG TGGGTGGGGA GGGGTAGTGG GTGAGGGACC CAGGTTCCTG
      Ets          GT Box

          *  *    *
1201 ACACAGACAG ACTACACCCA GGGAATGAAG AGCAAGCGCC ATG
        +1
```

Figure 4.1. The minimal *IL2RG* promoter region. The -104/+39 region encompassing the *IL2RG* minimal promoter. The Ets binding site critical for promoter activity is shown in blue, all putative binding sites in black are non-essential for promoter activity. The three identified transcriptional start sites are marked with *, with the strong initiation start marked with +1. The ATG start codon is shown in red.

To ensure that all putative significant regulatory sequences were identified prior to incorporation of the promoter into lentiviral vectors, alignments between the region 5' of the human and murine γc genes were performed. The human and murine genes encoding γc exhibit 69% and 70% homology at the nucleotide and amino acid level respectively (Cao et al., 1993), it is therefore likely that important regulatory sequences upstream of these genes will be similarly conserved throughout evolution. Analyses were performed using a multiple sequence local alignment and visualisation tool (Mulan, http://mulan.dcode.org). The initial analysis aligned 20 kb of DNA directly upstream of the ATG start codons, identifying two areas of significant homology represented on the dot plot as diagonal lines (figure 4.2A). The first representing a stretch of DNA approximately 1.3 kb directly upstream of the γc genes and likely representing regulatory sequences comprising the *IL2RG* promoter, including those already identified (Noguchi et al., 1993a; Ohbo et al., 1995; Markiewicz et al., 1996). The second area of homology, located 7 kb downstream of the human and murine γc coding regions, represents an unrelated gene, *MED12*, encoding a transcriptional coactivator (Mediator of RNA polymerase II transcriptional subunit 12), and most likely its promoter elements, found in the reverse orientation within both genomes and also highly conserved. The area showing no significant homology between these two regions likely signifies that all non-redundant regulatory elements that comprise the endogenous γc promoters in both humans and mice are located proximal to the coding regions, within the first 1.3 kb directly upstream of the translational start sites.

To characterise the promoter region further, the 7 kb between the human *IL2RG* and *MED12* coding regions was analysed for both CpG content and the presence of Alu sequences (figure 4.2B). Approximately 1% of the human genome is comprised of methylation-free sequences rich in CpG dinucleotides referred to as CpG islands (Bird, 1986). CpG islands are associated with the promoter regions of most housekeeping genes, however these sequences have also been identified within the 5' flanking regions of tissue-specific genes such as human α-globin (Bird et al., 1987). Analysis of the 7 kb intergenic sequence using the online CpG plot tool (www.ebi.ac.uk/emboss/cpgplot) predicted a single 247 bp CpG island, approximately 3.2 kb upstream of *IL2RG*; consequently the sequences thus far characterised as the *IL2RG* promoter and those that retain cross-species homology are not CpG rich (figure 4.2B). Analysis of the

corresponding region of DNA from the murine genome failed to identify any putative CpG islands (data not shown). The equidistance of the putative CpG island between the *IL2RG* and *MED12* coding regions indicates potential functionality in the regulation of either or both genes, however, based on published evidence this sequence does not appear to be required for basal *IL2RG* promoter function (Ohbo et al., 1995; Markiewicz et al., 1996).

A significant fraction of DNA is comprised of short interspersed repeats (SINEs) such as those of the non-autonomous retroelement, Alu. The human genome contains over one million Alu sequences, the non-uniform distribution of which implies a potential role in gene regulation since regions upstream of transcriptional start sites are enriched with Alu elements and these sequences contain putative transcription factor binding sites (Polak and Domany, 2006). Using the online RepeatMasker tool (www.repeatmasker.org), the 7 kb stretch of DNA between the *IL2RG* and *MED12* genes was found to be enriched for these sequences, 41% of the DNA being comprised of 11 Alu elements (figure 4.2B). Of these sequences, 44% are located within the 1.5-3.5 kb of DNA closest to the *IL2RG* promoter, however, as for the putative CpG island, these elements do not appear crucial for basal promoter activity since they lie upstream of the sequences homologous between the human and murine genomes and of the regions previously characterised as containing promoter activity (Ohbo et al., 1995; Markiewicz et al., 1996).

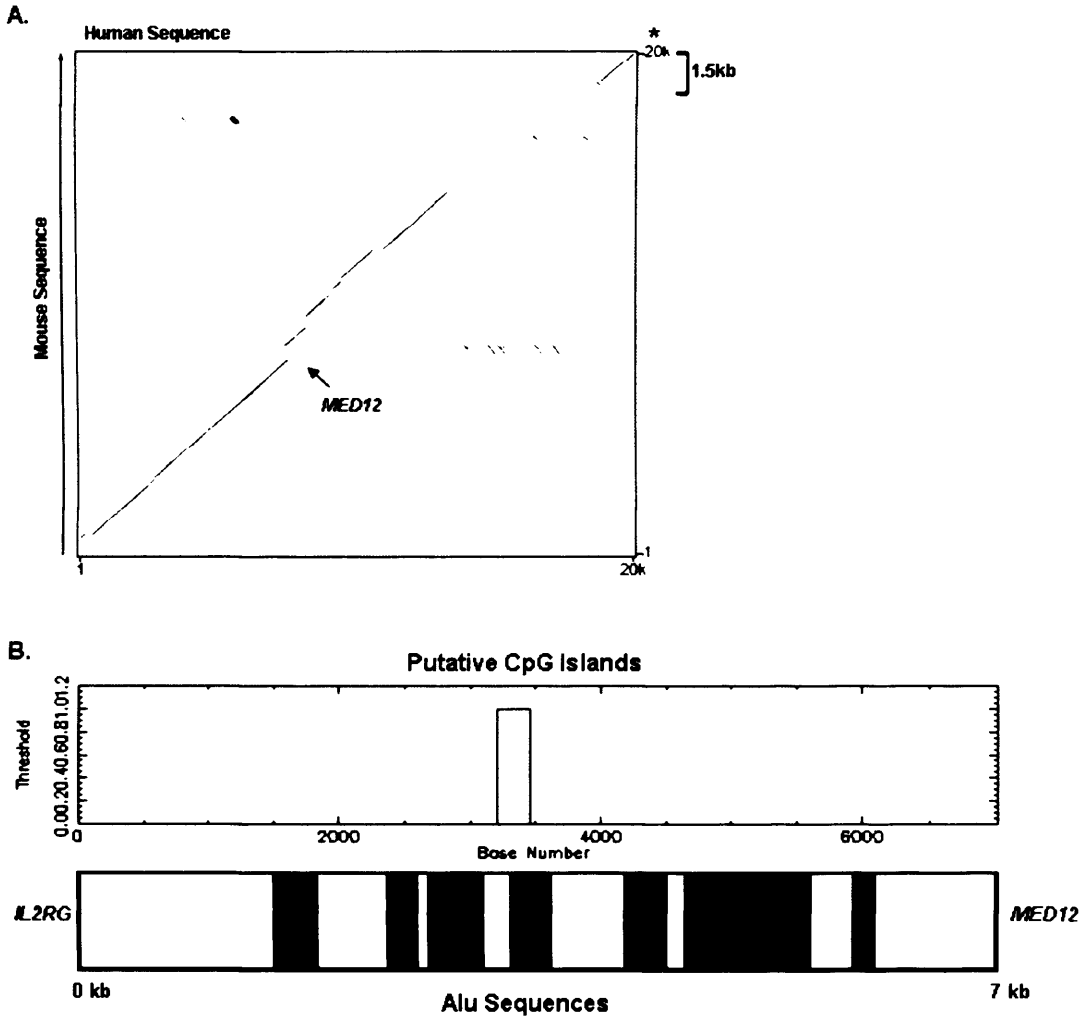

Figure 4.2. Homologous regions within 20 kb upstream of the human and murine γc genes. (A.) 20 kb of DNA upstream of the human and murine γc translational start sites was aligned using Mulan. The results are visualised as a dot plot, whereby homologous regions are represented by diagonal lines. Two areas of homology are observed; the first encompassing approximately 1.5kb upstream of the γc genes and likely representing the promoter regions and the second corresponding to an unrelated gene, *MED12*, in the reverse orientation to *IL2RG* (*, ATG start codons). (B.) A putative CpG island is located approximately 3.2 kb upstream of the *IL2RG* start codon in the human genome. The 7 kb of DNA between the human *IL2RG* and *MED12* coding regions is rich in Alu sequences.

In order to analyse the region upstream of the human and murine *IL2RG* genes in more detail, a second alignment was performed contrasting only 2 kb of sequence upstream of the translational start sites. This second analysis identified two regions of close homology 1.2 kb immediately upstream of the ATG start codons, and a third, smaller region found to be homologous to the reverse complement in the DNA possibly signifying a repeat sequence (figure 4.3A). In particular this alignment revealed two highly conserved regions of 94% and 92% homology within 91 bp upstream of the previously characterised transcriptional start site (Noguchi et al., 1993a). These almost identical sequences correlate with findings that this region incorporates the minimal *IL2RG* promoter in humans (Ohbo et al., 1995; Markiewicz et al., 1996), and most likely also in mice. The latter region also encodes the Ets binding site demonstrated to be crucial for promoter activity (figure 4.3A) (Markiewicz et al., 1996). To date, experiments to characterise the *IL2RG* promoter have used constructs encompassing between 669 and 1089 bp of sequence upstream of translational start site (Ohbo et al., 1995; Markiewicz et al., 1996), however this alignment reveals a further 98 bp of sequence 5' to the γc gene that shows significant homology to the corresponding region in the murine genome and hence implies a potential regulatory role. Analysis of conserved transcription factor binding sites between the two species was performed using the MultiTF tool (http://multitf.dcode.org) (figure 4.3B). Several putative binding sites were identified within the regions of homology along the 2kb upstream of the γc genes. Within the 98 bp of sequence thus far omitted in *IL2RG* promoter studies, five conserved potential transcription factor binding sites were identified, of which one, the putative MZF1 site, is implicated in early haematopoiesis (Gaboli et al., 2001). Furthermore, in the 40 bp directly upstream of this stretch of DNA within the area homologous to the reverse complement in the murine sequence, a further five potential transcription factor binding sites were identified. These binding sites also imply a regulatory role for this third region of homology; in particular an Ets2 binding site is conserved, which, together with Ets1 is thought to be expressed during T cell development (Anderson et al., 1999). It was therefore decided to clone the entire 1.24 kb region upstream of the *IL2RG* gene translational start site (-1164/+36 relative to the transcriptional start site) into lentiviral vectors representing potentially all of the endogenous sequence regulating γc expression in humans.

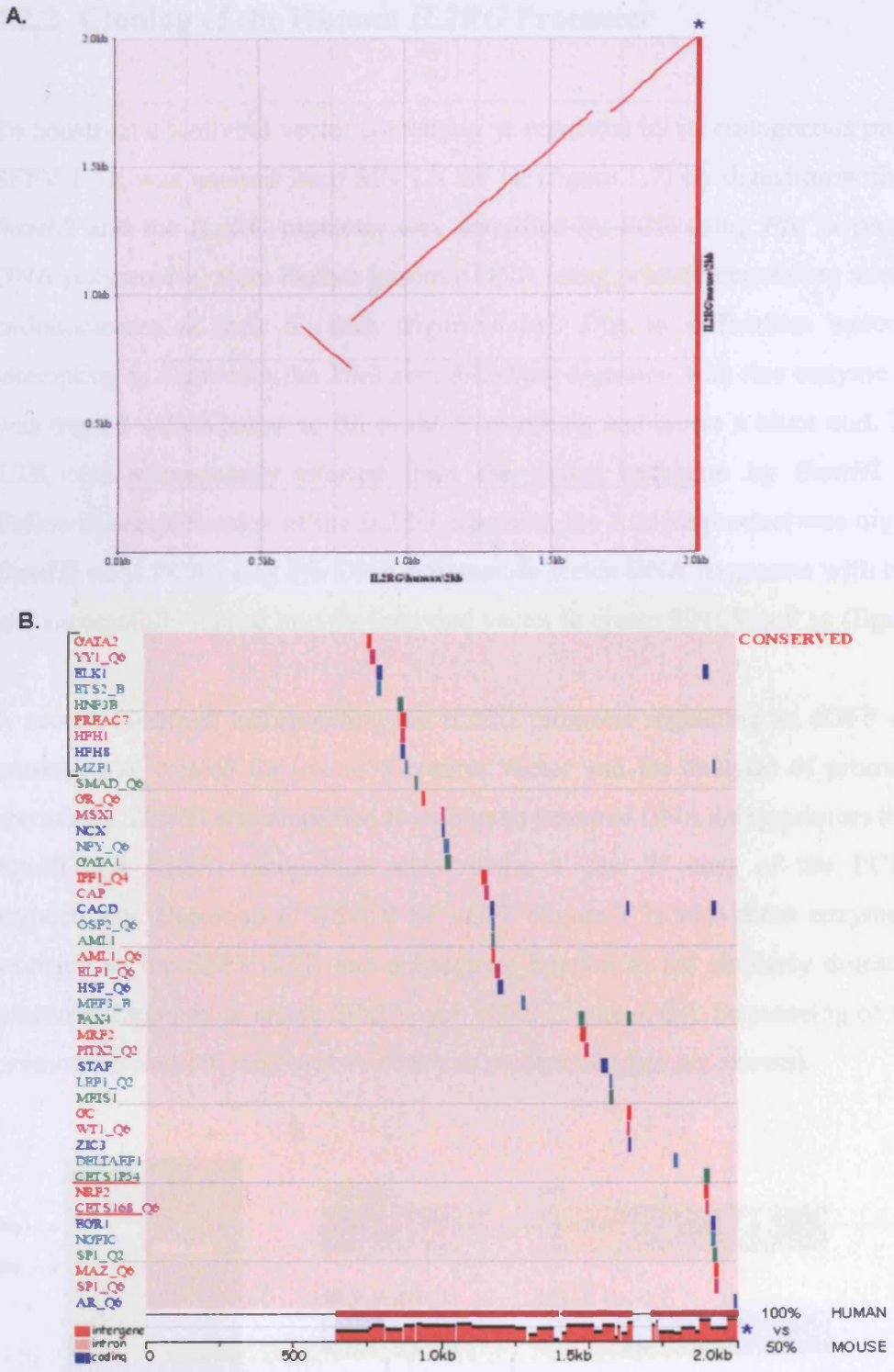

Figure 4.3. The homology between the 2 kb upstream of the human and murine γc genes. (A) Alignment of the 2 kb of DNA immediately upstream of the human and murine γc coding regions reveals two regions of close homology directly upstream of the ATG start codons, and a third region homologous to the murine reverse complement. (B) Putative transcription factor binding sites conserved within the homologous regions were identified using MultiTF. The critical Ets binding site in the basal promoter (recognised here as two potential overlapping sites) is underlined in red. The additional binding sites incorporated into the 1.24 kb *IL2RG* promoter for use in lentiviral vectors are bracketed. (*, ATG start codons).

4.2.2 Cloning of the Human *IL2RG* Promoter

To construct a lentiviral vector containing γc regulated by its endogenous promoter the SFFV LTR was excised from SIN LV SF γc (figure 1.7) by digestion with *Xho*I and *BamH*I and the *IL2RG* promoter was amplified by PCR using *Pfu* (a proof-reading DNA polymerase) from human genomic DNA using primers containing sites for these endonucleases at their 5' ends (figure 4.4A). Due to difficulties encountered in attempting to clone into the *Xho*I site, following digestion with this enzyme the vector was treated with Klenow to fill in the 5' overhang and create a blunt end. The SFFV LTR was subsequently excised from the vector backbone by *BamH*I digestion. Following amplification of the *IL2RG* promoter, the 1.24 kb product was digested with *BamH*I only, PCR using *Pfu* DNA polymerase yields DNA fragments with blunt ends, and successfully ligated into the lentiviral vector to create SINLV γcP γc (figure 4.4B).

A second construct incorporating the *IL2RG* promoter regulating an eGFP expression cassette was created for use as a control vector and for analysis of promoter tissue specificity. *IL2RG* was amplified from human genomic DNA using primers that created *EcoR*I and *BamH*I recognition sites at the 5' and 3' ends of the PCR product respectively. Digestion of SINLV SF eGFP (figure 1.7) with these enzymes enabled excision of the SFFV LTR and subsequent ligation to the similarly digested *IL2RG* promoter fragment to create SINLV γcP eGFP (figure 4.4B). Sequencing of the *IL2RG* promoter from both lentiviral vectors was performed (data not shown).

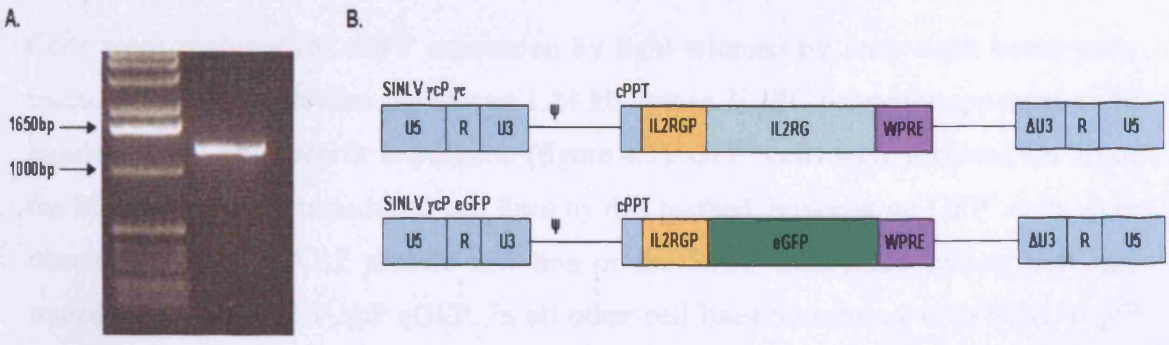

Figure 4.4. Lentiviral constructs incorporating the *IL2RG* promoter. (A) PCR of the 1.24 kb *IL2RG* promoter region from human genomic DNA. (B) Lentiviral constructs containing the cloned *IL2RG* promoter.

4.3 Analysis of the Tissue-Specificity of the *IL2RG* Promoter *In Vitro*

To assess the tissue-specificity of the *IL2RG* promoter *in vitro* a variety of haematopoietic and non-haematopoietic cell lines were transduced with VSV-G pseudotyped SINLV γcP eGFP or SINLV SF eGFP and analysed for transgene expression. The eGFP transgene enables convenient direct identification of transduced cells by both fluorescence microscopy and flow cytometry negating the requirement of antibody staining.

Each cell line was transduced using three different MOIs (0.1, 1 and 10) in triplicate. The haematopoietic cell lines used included the γc-deficient T lymphoid cell line ED-7R, SCID-X1 LCL (immortalised B cells) and the myeloid monocytic cell line U937. The non-haematopoietic cell lines consisted of the fibroblastoid cell line HT1080, an embryonic kidney cell line (293T), the C2C12 muscle cell line and the HepG2 liver carcinoma cell line. All cell lines used, except for C2C12, are originally derived from human cells.

4.3.1 Analysis of Transduced Cell lines by Fluorescence Microscopy

Cells were analysed for eGFP expression by light microscopy forty-eight hours post-transduction, at which time the cloned 1.24 kb human *IL2RG* promoter appeared to be regulating cell line-specific expression (figure 4.5). GFP^+ cells were detected for all of the SINLV SF eGFP transduced cell lines by this method, however no GFP^+ cells were observed for the C2C12 muscle cell line or the 293T embryonic kidney cell line transduced with SINLV γcP eGFP. In all other cell lines transduced with SINLV γcP eGFP, faint GFP^+ cells were visible by microscopy, however the low levels of GFP observed mean that these cells are difficult to distinguish following photographic capture (figure 4.5). The SINLV SF eGFP transduced cells appeared brighter under the fluorescence microscope than those transduced with the *IL2RG* promoter vector

demonstrating the potency of the viral promoter. For both viruses the highest levels of eGFP expression were observed in transduced ED-7R and U937 cells, both of haematopoietic origin.

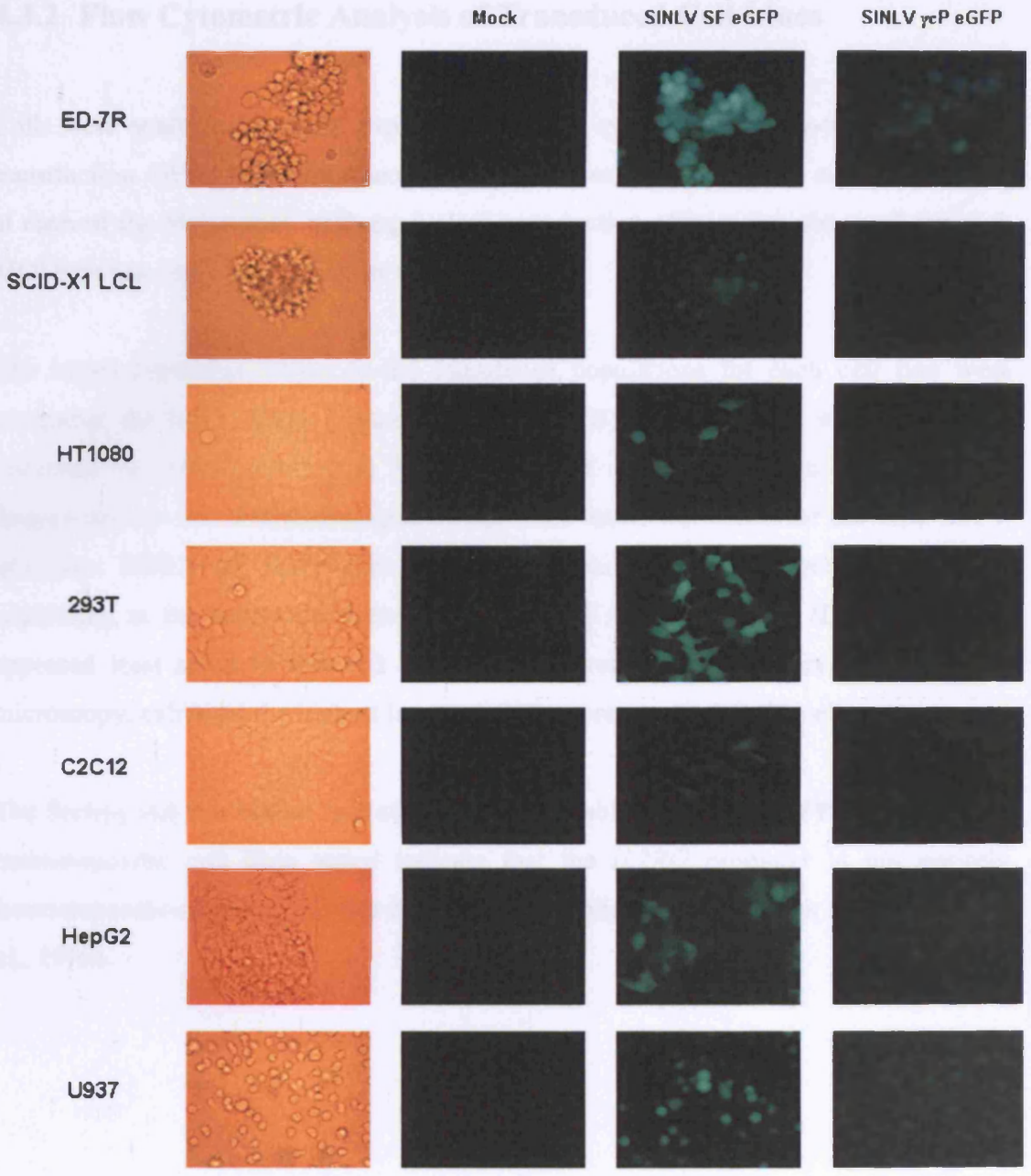

Figure 4.5. Analysis of GFP expression in transduced cell lines by light microscopy. A variety of haematopoietic and non-haematopoietic cell lines were transduced with SINLV SF eGFP or SINLV γcP eGFP and analysed by light microscopy for eGFP expression after forty-eight hours. No eGFP expression was observed for 293T or C2C12 cells transduced with SINLV γcP eGFP, whilst GFP^+ cells were detected for all cell lines transduced with SINLV SF eGFP. Photos are representative of three transductions of each cell line at an MOI of 10.

4.3.2 Flow Cytometric Analysis of Transduced Cell Lines

Cells were analysed for eGFP expression by flow cytometry seventy-two hours post-transduction. GFP^+ cells were detected in all cell lines transduced with either vector and at each of the MOIs used, with equivalent transduction efficiencies observed for each MOI between lentiviruses (figures 4.6A and 4.7A).

The mean expression levels in the transduced populations for each cell line were compared for both vectors (figures 4.6B and 4.7B). In correlation with the results observed by light microscopy, higher levels of eGFP expression in all of the haematopoietic and non-haematopoietic cell lines tested were seen for the viral SFFV promoter. SINLV SF eGFP was found to regulate the highest levels of transgene expression in the embryonic kidney cell line 293T; conversely the *IL2RG* promoter appeared least active in this cell line and, in correlation with observations by light microscopy, exhibited the highest levels of GFP expression in ED-7R cells.

The finding that the SINLV γcP eGFP vector was able to express eGFP in all the non-haematopoietic cell lines tested indicate that the *IL2RG* promoter is not entirely haematopoietic-restricted *in vitro* in contrast to published observations (Markiewicz et al., 1996).

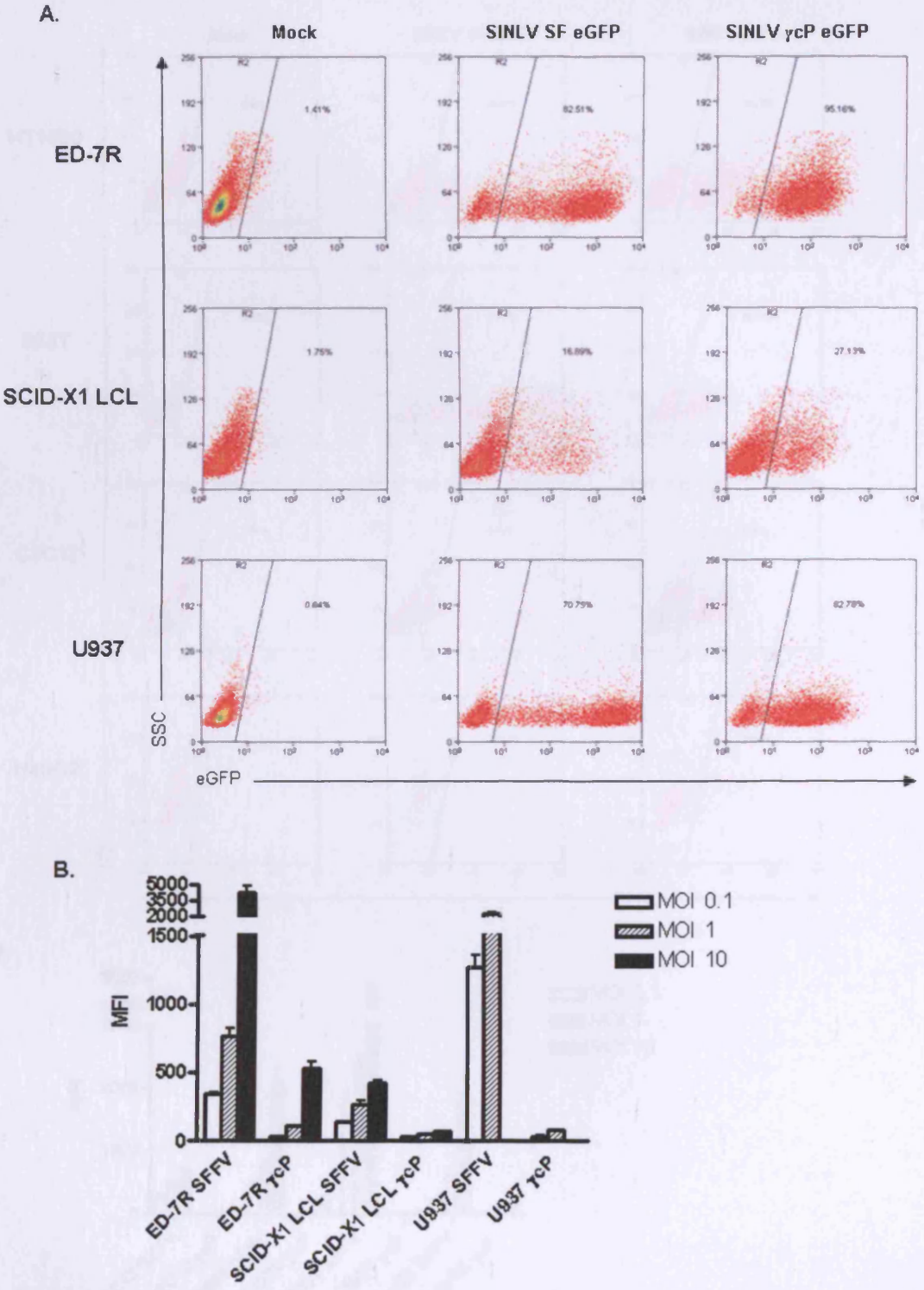

Figure 4.6. Analysis of GFP expression in transduced haematopoietic cell lines by flow cytometry. Cells were analysed for eGFP expression seventy-two hours post-transduction by flow cytometry. (A) Representative plots of eGFP expression in cell lines transduced at an MOI of 1. (B) Mean (+/-SD) eGFP expression levels in each cell line at each MOI for both vectors. The *IL2RG* promoter is less potent than the viral SFFV promoter, both promoters are most active in ED-7R cells. (SD, standard deviation)

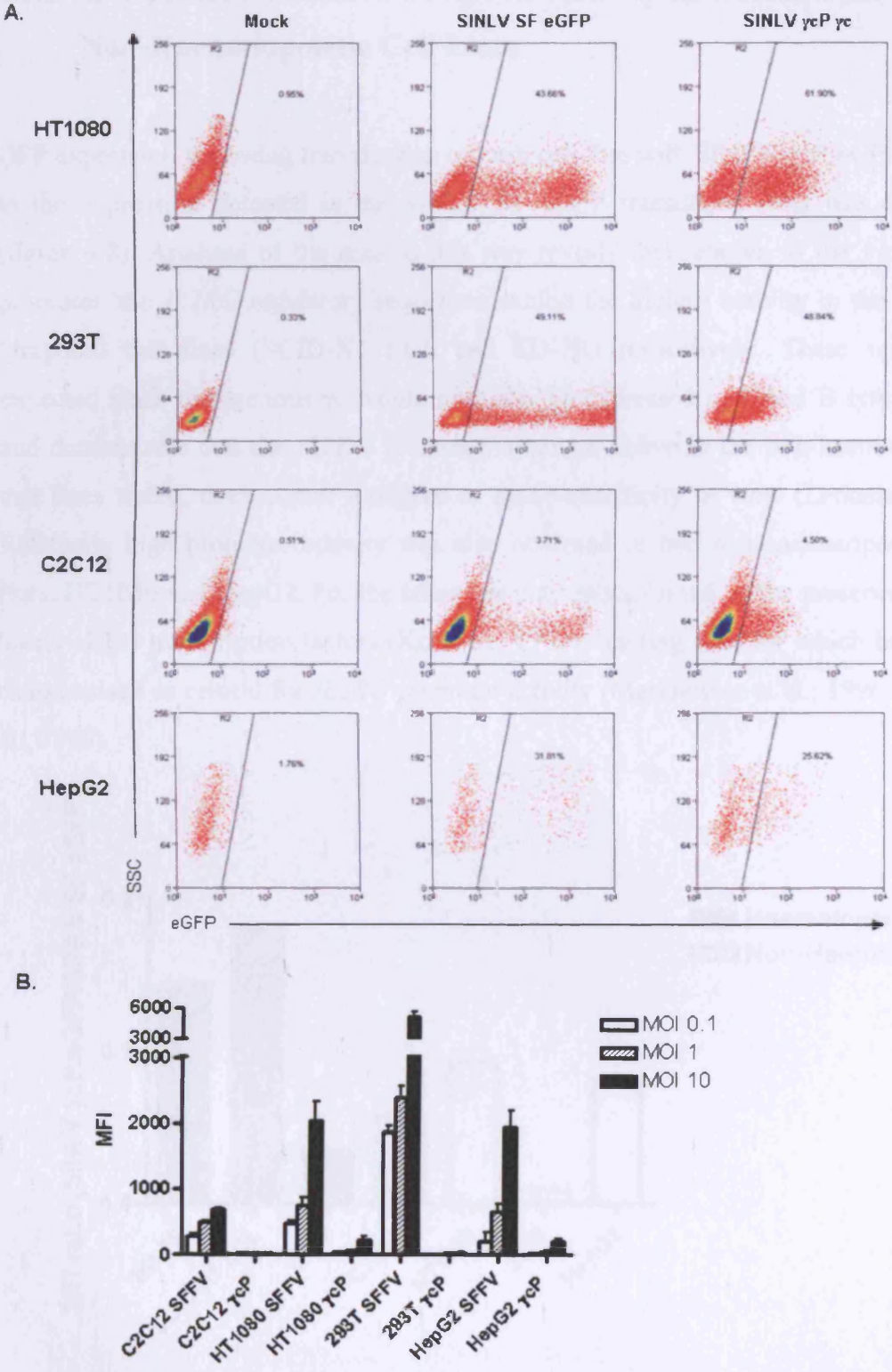

Figure 4.7. Analysis of GFP expression in transduced non-haematopoietic cell lines by flow cytometry. Cells were analysed for eGFP expression seventy-two hours post-transduction by flow cytometry. (A) Representative plots of eGFP expression in cell lines transduced at an MOI of 1. (B) Mean (+/-SD) eGFP expression levels in each cell line at each MOI for both vectors. The *IL2RG* promoter is able to regulate low levels of expression in non-haematopoietic cell lines. (SD, standard deviation)

4.3.3 Comparison of *IL2RG* Promoter Activity in Haematopoietic and Non-Haematopoietic Cell Lines

GFP expression following transduction of each cell line with SINLV γcP eGFP relative to the expression detected in the SINLV SF eGFP transduced cells was compared (figure 4.8). Analysis of the data in this way reveals that relative to the viral SFFV promoter, the *IL2RG* regulatory sequences exhibit the highest activity in the B and T lymphoid cell lines (SCID-X1 LCL and ED-7R) respectively. These results are expected since endogenous γc would normally be expressed in T and B lymphocytes and demonstrate that the *IL2RG* promoter, although active in the non-haematopoietic cell lines tested, does confer a degree of tissue-specificity *in vitro* (Leonard, 1996). Relatively high promoter activity was also observed in two non-haematopoietic cell lines, HT1080 and HepG2. For the latter this may be explained by the presence of high levels of Ets transcription factors (Ko et al., 1999), binding sites for which have been characterised as critical for *IL2RG* promoter activity (Markiewicz et al., 1996; Ohbo et al., 1995).

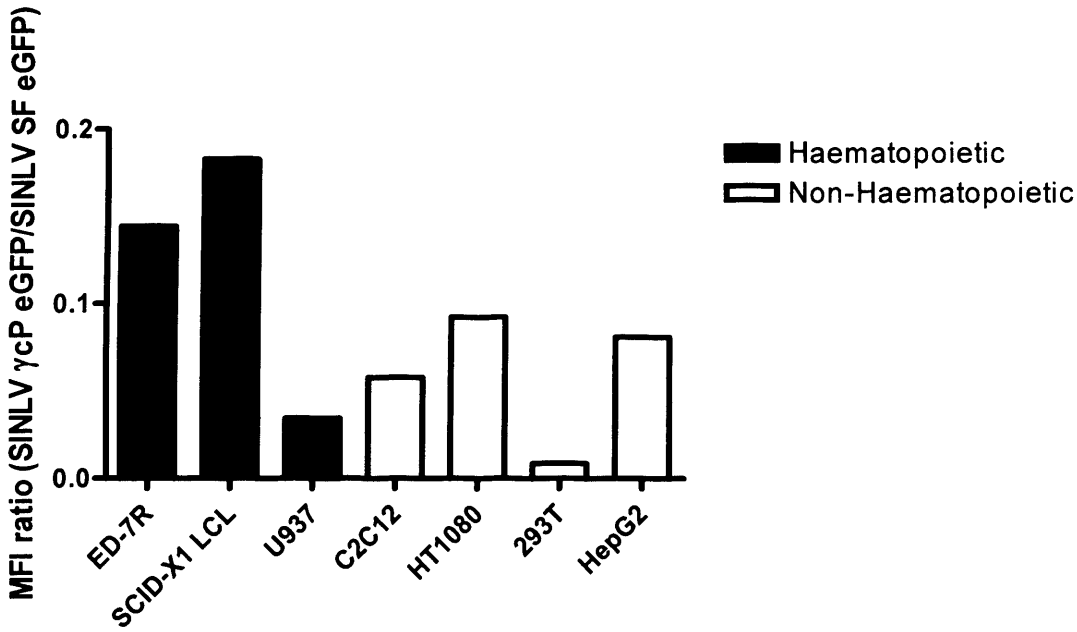

Figure 4.8. GFP expression in SINLV γcP eGFP transduced cell lines relative to expression from the SFFV promoter. The relative GFP expression in all cell lines transduced with SINLV γcP eGFP relative to expression in SINLV SF eGFP transduced cells is compared. The *IL2RG* promoter exhibits the highest relative activity in the two lymphoid cell lines, SCID-X1 LCL and ED-7R. Results shown represent the means of three transductions with each virus at an MOI of 1.

4.4 Analysis of Tissue Specificity of the *IL2RG* Promoter *In Vivo* – Intraveneous Injection of Lentiviral vectors into Neonatal Mice

Due to the aberrant phenotype of some immortalised cell lines, the SINLV γcP eGFP vector was also tested in an *in vivo* setting. Direct injection of concentrated lentiviral supernatant into the superficial temporal vein of one-day old neonatal outbred mice has been shown to permit transduction of a variety of different organs and cell types *in vivo* (VandenDriessche et al., 2002; Kobayashi et al., 2005; Carbonaro et al., 2006) (Dr S Waddington personal communication). Analysis of GFP localisation one month post-injection therefore enables characterisation of tissue-specificity conferred by a promoter in an *in vivo* setting.

Equal volumes of VSV-G pseudotyped SINLV γcP eGFP or SINLV SF eGFP matched-titre supernatant was intravenously injected into the superficial temporal vein of one-day old neonatal mice (injections performed by Simon Waddington). A total of three neonates were injected per vector. One month post-injection, mice were sacrificed and analysed for GFP expression by fluorescence microscopy and flow cytometry.

4.4.1 Fluorescence Microscopic Analysis of Lentivirally Injected Mice

Whole organs from the injected mice were analysed for GFP^+ cells by fluorescence microscopy. Mice injected with SINLV SF eGFP had detectable GFP expression in both lymphoid and non-lymphoid tissues, with particularly strong expression detected in the livers from all three mice and widespread expression observed in the spleens (figure 4.9A). GFP expression in all three mice injected with SINLV γcP eGFP however appeared restricted to the spleens only, with no detectable GFP^+ cells in the liver. Furthermore, GFP expression in the spleens of these mice appeared localised to the periphery of the white pulp in each animal, potentially within the marginal zone known to contain mainly B cells and macrophages (figure 4.9B) (Nolte et al., 2004).

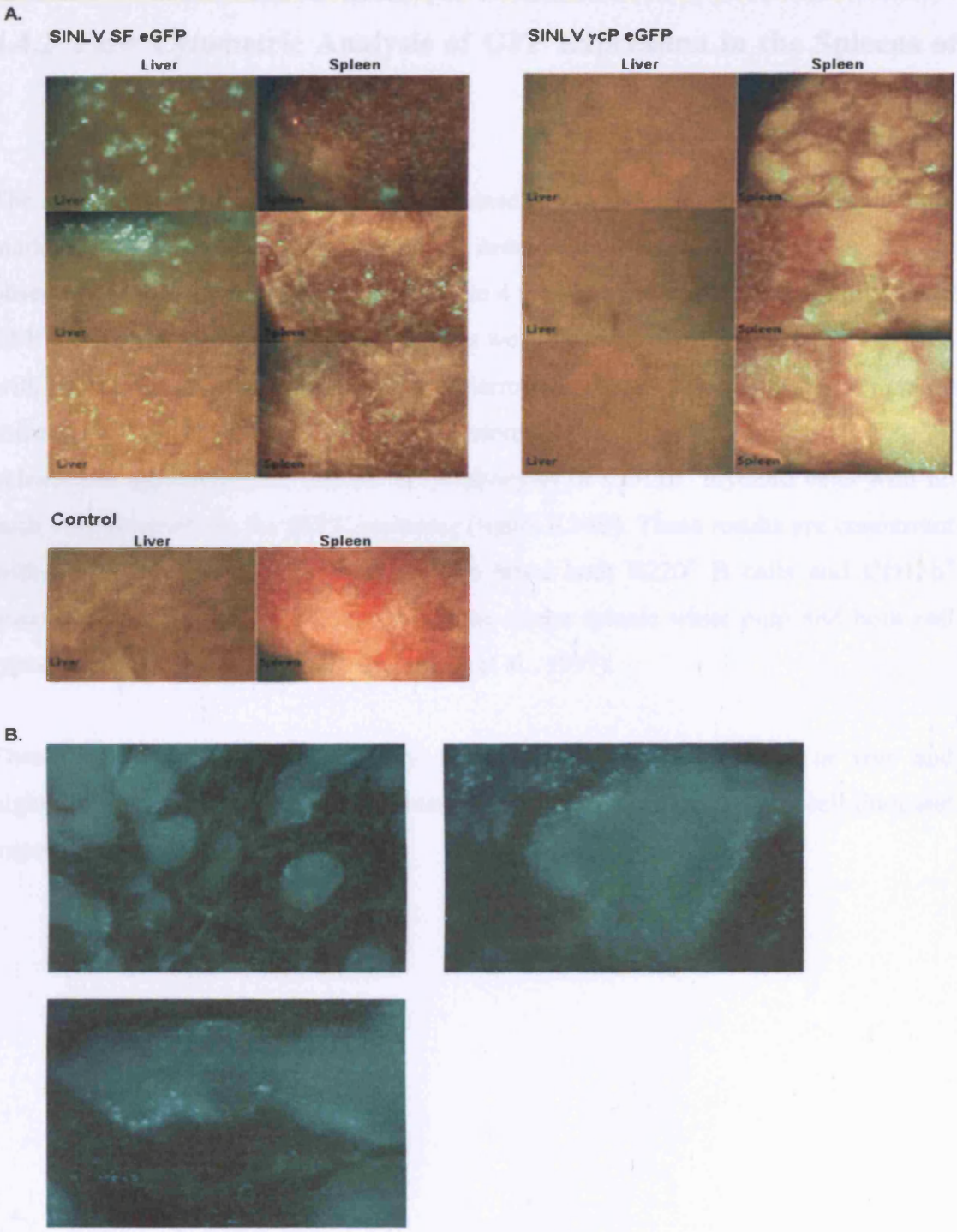

Figure 4.9. GFP expression in the livers and spleens from mice neonatally-injected with SINLV SF eGFP or SINLV γcP eGFP. (A) Fluorescence microscopic analysis of whole livers and spleens from mice one-month post-neonatal injection of lentiviral supernatant (30x magnification). Mice injected with SINLV SF eGFP had detectable GFP expression in the livers and spleens, whilst mice injected with SINLV γcP eGFP had GFP^+ cells detectable in the splenic white pulp only. (B) Higher magnification of GFP expression in the spleens of mice injected with SINLV γcP eGFP (90x magnification). The GFP^+ cells are mainly located in the periphery of the white pulp. Photographs are representative of three mice for each vector. The control represents an uninjected mouse. (Photographs taken by Dr S Waddington)

4.4.2 Flow Cytometric Analysis of GFP Expression in the Spleens of Lentivirally Injected Mice

The spleens from the injected mice were stained for a variety of lymphoid and myeloid markers and analysed by flow cytometry in order to characterise the GFP^+ populations observed by fluorescence microscopy (figure 4.9). For mice injected with either virus, GFP^+ T, B and $CD11b^+$ myeloid populations were detected in the spleen (figure 4.10A) with no GFP^+ cells detected in the bone marrow or thymi (data not shown). For the mice injected with SINLV γcP eGFP lentivirus the highest proportions of GFP^+ splenocytes appeared to be B220lo B lymphocytes or $CD11b^+$ myeloid cells with no such bias observed for the SFFV promoter (figure 4.10B). These results are concurrent with the fluorescence microscopic analysis since both $B220^+$ B cells and $CD11b^+$ macrophages are found in the marginal zone of the splenic white pulp and both cell types express γc (Leonard, 1996; Andersson et al., 1997).

These results imply tissue-specificity of the cloned *IL2RG* promoter *in vivo* and highlight the differences that can be obtained between *in vitro* analyses in cell lines and experiments utilising *in vivo* models.

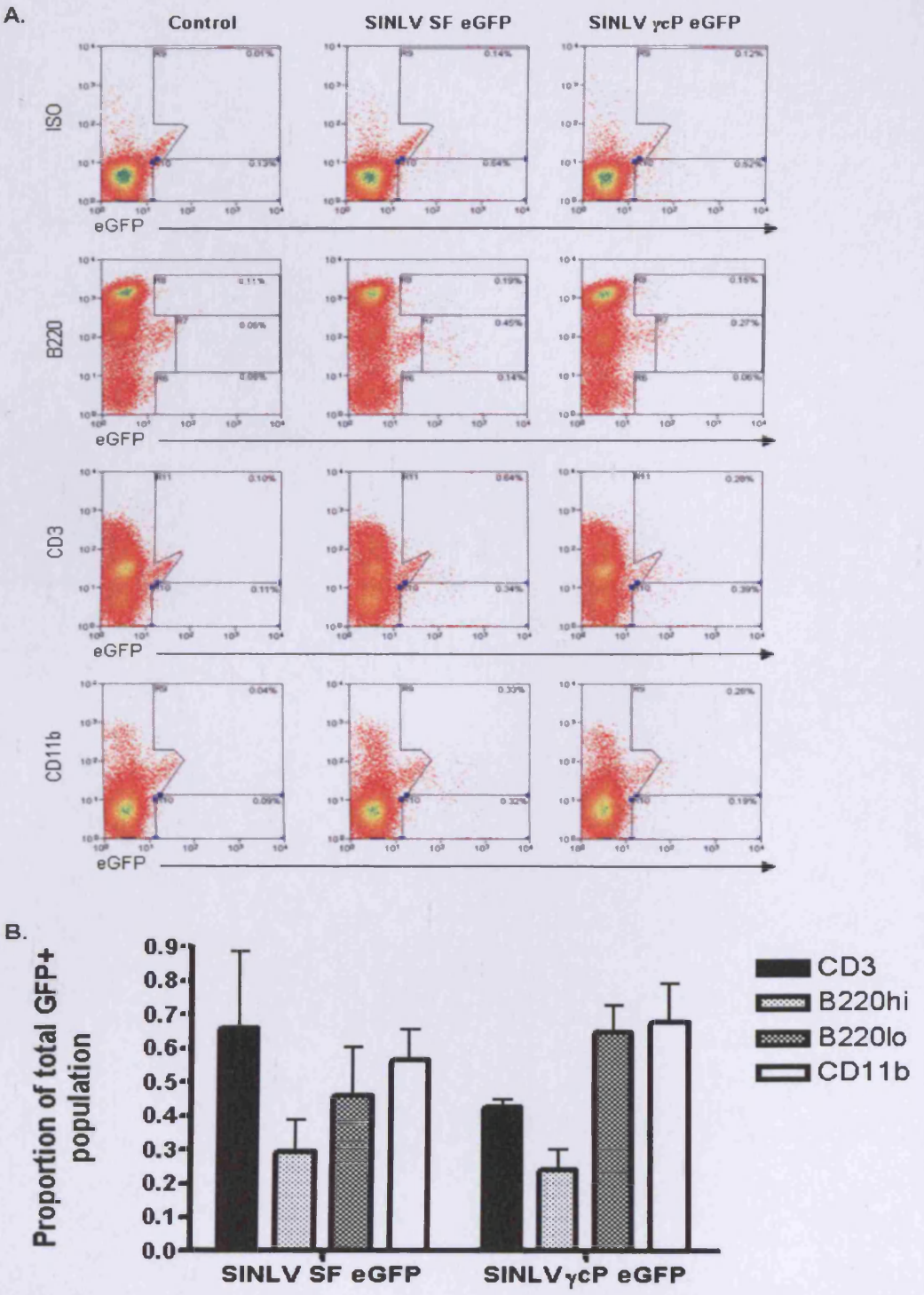

Figure 4.10. Flow cytometric analysis of splenocytes from mice neonatally-injected with SINLV SF eGFP or SINLV γcP eGFP. (A) Splenocytes from the injected mice were stained with lymphoid and myeloid cell surface markers and analysed by flow cytometry. GFP^+ B and T lymphocytes and $CD11b^+$ myeloid cells were observed in all mice. Representative plots from one mouse in each group are shown. (B) The mean (+/-SD) proportions of GFP^+ cells that co-stained with the cell surface markers. Mice injected with SINLV γcP eGFP had high proportions of B220lo and $CD11b^+$ GFP^+ cells in the spleen. (SD, standard deviation)

4.5 Conclusions

Analysis of regulatory sequences upstream of the human and murine genes for γc identified a previously uncharacterised region of DNA that showed significant evolutionary conservation and led to the construction of lentiviral vectors incorporating a 1.24 kb segment 5' of the human *IL2RG* gene that might potentially enable physiological transgene expression.

To analyse tissue-specificity of the cloned *IL2RG* promoter region *in vitro*, a variety of haematopoietic and non-haematopoietic cell lines were transduced with SINLV γcP eGFP or SINLV SF eGFP for comparison. GFP^+ cells were detectable by light microscopy forty-eight hours post-transduction in all haematopoietic cell lines and in all but two of the non-haematopoietic cell lines tested for SINLV γcP eGFP. All cell lines transduced with SINLV SF eGFP had detectable GFP^+ cells at this stage however, with noticeably brighter expression regulated by the SFFV promoter. Seventy-two hours post-transduction transgene expression was detected by flow cytometry in all cell lines transduced with either SINLV γcP eGFP or SINLV SF eGFP, with much higher levels of GFP observed in all cell lines tested for the latter vector. Analysis of *IL2RG* promoter activity relative to that from the SFFV promoter revealed the highest levels of transgene expression in the T and B lymphoid cell lines, derived from cells in which γc is normally expressed. Although monocytes have been documented to express γc and respond to γc-dependent cytokines (Alderson et al., 1991; Bonder et al., 1999), much lower relative expression was observed in the U937 monocytic cell line. This result may reflect either that γc is not ubiquitously expressed in this cell type, a study investigating γc expression in monocytes from SCID-X1 patients suggests that γc is not essential for monocyte development or function (Ariga et al., 2002), or differences between the phenotype of wild-type and immortalised cells. Despite published results that the *IL2RG* promoter is haematopoietic-specific *in vitro* (Markiewicz et al., 1996), transgene expression was detected in all non-haematopoietic cell lines transduced with SINLV γcP eGFP lentivirus, with the highest expression seen in the fibroblastoid cell line HT1080 and the liver carcinoma cell line HepG2. The promoter activity detected in HepG2 cells might be explained by the high levels of Ets-1 and Ets-2 transcription factors expressed in this cell line (Ko et al., 1999), since a crucial Ets binding site has

been identified within the basal *IL2RG* promoter (Markiewicz et al., 1996; Ohbo et al., 1995). The differences observed between this *in vitro* assay and published work using a plasmid transfection system may be attributed to the use of a lentiviral vector to characterise promoter activity in this instance. Lentiviral vectors integrate into the host genome and therefore may be affected by neighbouring regulatory elements resulting in potentially enhanced or decreased expression levels, effects that episomally-maintained plasmid DNA would not be subjected to. Whilst the vectors incorporate the self-inactivating design, it has been reported that the inactivated LTR retains a degree of promoter activity, thus it is possible that transgene expression regulated by the viral enhancer elements has been detected in cell lines in which the *IL2RG* promoter is inactive (Logan et al., 2004; Hanawa et al., 2005). It must also be noted that all of the non-haematopoietic cell lines tested differ from those used in a previous study to characterise this promoter (Markiewicz et al., 1996).

To analyse tissue-specificity of the *IL2RG* promoter in an *in vivo* setting one-day old neonatal mice were injected with concentrated lentiviral supernatant in a method demonstrated to target a wide variety of cell types (VandenDriessche et al., 2002; Kobayashi et al., 2005; Carbonaro et al., 2006). Analysis of the injected animals one month post-injection revealed *IL2RG* promoter activity to be restricted to the spleen and localised to the periphery of the white pulp, whilst transgene expression was widespread in the spleens and livers of animals injected with SINLV SF eGFP lentivirus. These data highlight the tissue-specificity of the *IL2RG* promoter *in vivo* since integrated SINLV γcP eGFP provirus was detectable in both organs by PCR (data not shown) and injection of lentiviral supernatant via this method into neonatal mice has been demonstrated to primarily target the liver (VandenDriessche et al., 2002; Kobayashi et al., 2005; Carbonaro et al., 2006). Flow cytometric analysis of splenocytes from the injected mice characterised the highest proportions of GFP^+ cells in the SINLV γcP eGFP mice to stain positive for B220 B lymphocyte or CD11b myeloid cell surface markers. These populations are found in high number in the splenic marginal zone located in the periphery of the white pulp and would explain the localisation of transgene positive cells observed by fluorescence microscopy (Nolte et al., 2004). The high proportion of GFP^+ B cells also correlates with the results observed from the *in vitro* analyses in which the highest relative activity from the *IL2RG*

promoter was observed in SCID-X1 LCLs. It is therefore possible to conclude that the *IL2RG* promoter exhibits tissue-specificity *in vivo*, with promoter activity restricted to cells of haematopoietic origin only in the model used. This promoter might therefore be suitable for use in a gene therapy vector for SCID-X1, increasing vector safety by restricting transgene expression to the required cell types only. These results also highlight the importance of *in vivo* experiments in the assessment of regulatory sequences, in particular due the aberrant phenotypes of immortalised and cancer cell lines, since promoter activity was detected in a liver carcinoma cell line following *in vitro* transduction, whilst no transgene expression whatsoever was observed in the livers of three injected mice.

4.6 Final Conclusions

- High homology is observed between 1.24 kb of sequence upstream of the human and murine γc genes
- In the context of a lentiviral vector, the human 1.24 kb *IL2RG* promoter region exhibits activity in both haematopoietic and non-haematopoietic cell lines
- *In vivo*, expression from the *IL2RG* promoter is restricted to cells of haematopoietic origin in the periphery of the splenic white pulp

Chapter Five

Self-Inactivating Lentiviral Vectors for SCID-X1

5.0 Aims

- To assess the performance of the SIN lentiviral vectors incorporating the SFFV, EFS and *IL2RG* promoters *in vitro*
- To assess the ability of the SIN lentiviral vector series to restore immunity in the SCID-X1 murine model following *ex vivo* gene therapy

5.1 Introduction

The integration phase of the lentivirus life cycle differs from that of the gammaretroviruses in that the pre-integration complex (PIC) may be transported into the nucleus by the nuclear import machinery of the target cell (Bukrinsky et al., 1992). Following infection, reverse transcription of the lentiviral RNA genome occurs in the cytoplasm of the host cell and the resultant double-stranded DNA subsequently assembles with proteins to form the PIC. In wild-type lentiviruses these include matrix, integrase and vpr proteins, all of which have been implicated in nuclear import (Sherman and Greene, 2002). In addition, a triple-stranded DNA 'flap' structure created by the initiation of reverse transcription at the cPPT acts as a nuclear import signal for the PIC (Zennou et al., 2000). In the context of lentiviral vectors for gene therapy, the central polypurine tract (cPPT) sequence has been shown to be required for improved transduction of both human primary cells (including HSCs) and rat neurons, whilst the role of vpr in nuclear translocation is thought to be redundant since the viral accessory proteins were omitted during the production of these vectors (Follenzi et al., 2000; Zennou et al., 2001).

Vectors derived from lentiviruses such as HIV-1 therefore offer the advantage that they are able to transduce non-dividing cells. This ability may be of significant benefit in HSC gene therapy given that the cycling of progenitor cells required for *ex vivo* transduction with gammaretroviral vectors may result in loss of self-renewal and engraftment capabilities. A series of second generation self-inactivating lentiviral

vectors derived from HIV-1 (Zufferey et al., 1998) have therefore been developed for the treatment of SCID-X1 incorporating identical SFFV and EFS promoter elements to those present in the SRS II series of gammaretroviral vectors (figure 1.7). These vectors, together with the SINLV γcP γc vector incorporating the *IL2RG* promoter, were therefore assessed both *in vitro* and *in vivo* for their potential as gene therapy vectors for the treatment of SCID-X1.

5.2 *In Vitro* Analysis of SIN Lentiviral Vectors

5.2.1 Transduction of Fibroblast and SCID-X1 Lymphoid Cell lines

In order to test the SINLV γcP γc vector and the lentiviral vectors incorporating the SFFV and EFS promoters, VSV-G pseudotyped lentiviral supernatant was produced by transient transfection of 293T cells and concentrated by ultracentrifugation. The murine fibroblast cell line SC-1 and the two aforementioned human SCID-X1 cell lines, ED-7R and SCID-X1 LCL, were consequently transduced with equivalent vector doses to analyse performance of the SIN lentiviral vector series *in vitro*. Transgene cell surface expression following lentiviral transduction was assessed by flow cytometry subsequent to staining with an antibody to human γc (anti-CD132-PE).

The SIN lentiviral vector series was found to efficiently transduce the murine fibroblast cell line and express γc transgene to detectable levels on the cell surface (figure 5.1). Markiewicz *et al.* reported tissue specificity of the human *IL2RG* promoter using a luciferase reporter system with little or no luciferase activity detected in non-haematopoietic cell lines including fibroblasts (Markiewicz et al., 1996). In the context of a lentiviral vector however, the *IL2RG* promoter appears active in all three of the cell lines tested, with γc expression detected in fibroblasts and lymphoid cell lines transduced with SINLV γcP γc but not in mock-transduced cells (figure 5.1). SINLV SF γc and SINLV EFS γc were also demonstrated capable of restoring γc expression in lymphoid SCID-X1 cell lines.

In all three of the cell lines tested, the highest expression levels were observed for the viral SFFV promoter (figure 5.1B) correlating with *in vitro* data gained from SIN gammaretroviral vector analysis (see figures 3.1 and 3.2). Whilst the levels of γc expression were consistently lower for SINLV EFS γc, the difference in expression levels as compared to SINLV SF γc appeared much smaller in the lymphoid SCID-X1 cell lines, correlating with published data that the elongation factor 1α promoter is suitable for use in haematopoietic cells (Salmon et al., 2000). The lowest expression levels in the SC-1 and ED-7R cell lines were observed for the *IL2RG* promoter vector, however expression of γc in the SCID-X1 LCLs following transduction with this vector was comparable to the levels detected for the other lentiviral vectors. These data confirm that the 1.24 kb sequence upstream of the endogenous *IL2RG* gene exhibits promoter activity in the lymphoid and fibroblastoid cells tested and that the SINLV γcP γc lentiviral vector is capable of restoration of γc on the cell surface of SCID-X1 B and T cell lines. The levels of γc expression observed following transduction of fibroblast and T lymphoid cell lines with this vector were lower than seen for SINLV SF γc and SINLV EFS γc at an equivalent MOI; however a degree of tissue specificity was conferred by SINLV γcP γc.

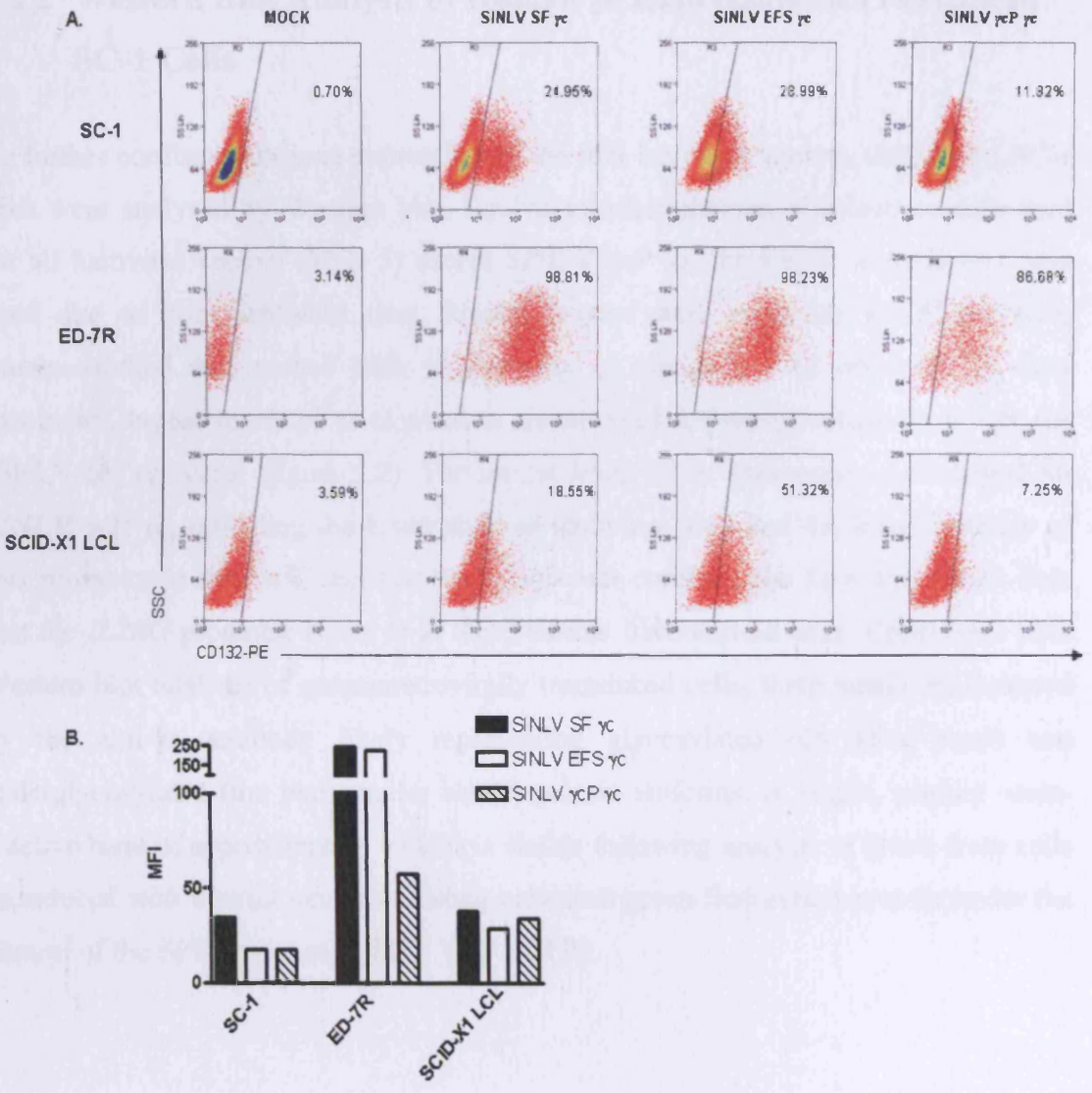

Figure 5.1. Lentiviral transduction of fibroblast and lymphoid SCID-X1 cell lines. (A) Flow cytometric analysis of SC-1 (fibroblast), ED-7R and SCID-X1 LCL (lymphoid) cells transduced with the SIN lentiviral vector series at an MOI of 1. (B) The mean fluorescent intensity (MFI) of each transduced cell line was compared for both vectors, the highest expression levels being observed for SINLV SF γc.

5.2.2 Western Blot Analysis of Human γc Expression in Transduced SC-1 Cells

To further confirm transgene expression by the SIN lentiviral vectors, transduced SC-1 cells were analysed by Western blot. Equivalent multiplicities of infection were used for all lentiviral vectors (MOI 5) except SINLV γcP γc, for which an MOI of 1 was used due to poor lentiviral titre. Protein lysates were separated by SDS-PAGE, immunoblotted and probed with an antibody to human γc. As observed by flow cytometry, higher levels of γc expression are detected following transduction with the SINLV SF γc vector (figure 5.2). The lowest levels of γc expression are detected for SINLV γcP γc, reflecting the lower dose of lentivirus used and the lower potency of this promoter in this cell line; this result however confirms the flow cytometric data that the *IL2RG* promoter is active in these murine fibroblastoid cells. Concurrent with Western blot analysis of gammaretrovirally transduced cells, three bands are detected by the anti-γc antibody likely representing glycosylated (64 kDa band) and underglycosylated (the two smaller bands) protein isoforms. A single, smaller cross-reactive band of approximately 49 kDa is visible following analysis of lysate from cells transduced with control vector encoding enhanced green fluorescent protein under the control of the SFFV promoter (SINLV SF eGFP).

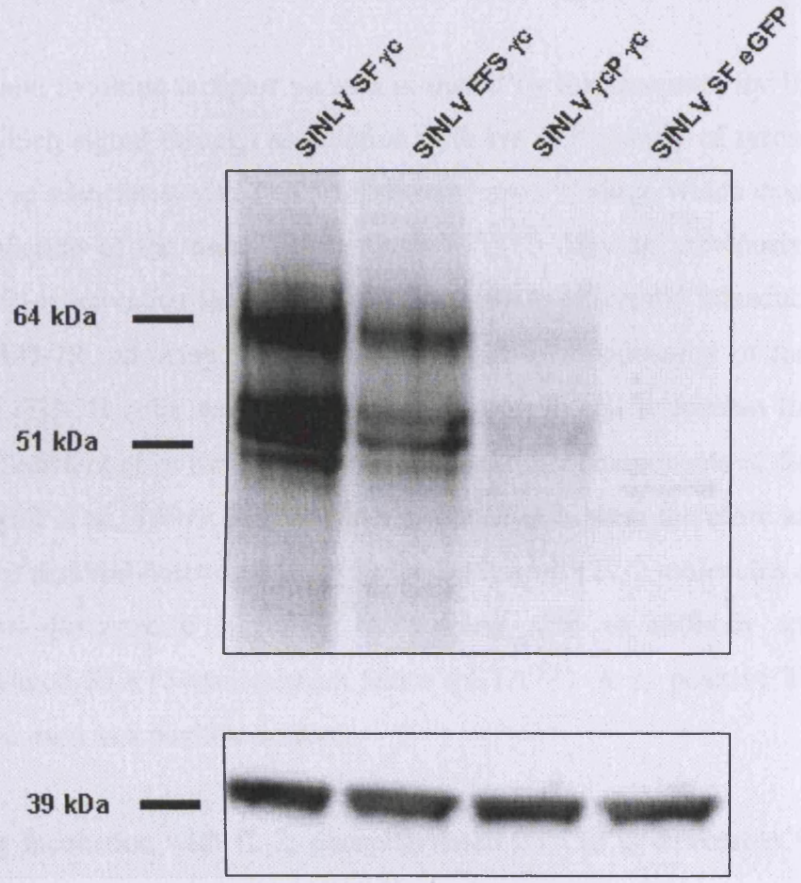

Figure 5.2. Western blot analysis of SC-1 cells transduced with the SIN lentiviral vectors. SC-1 fibroblast cells were transduced with the SIN lentiviral vector series at an MOI of 5 (an MOI of 1 was used for SINLV γcP γc); Western blot analysis of cell lysates confirmed that the *IL2RG* promoter is active in SC-1 cells and that the highest levels of transgene expression are detected in cells transduced with SINLV SF γc. To confirm antibody specificity and as a vector control, SC-1 cells were also transduced with SINLV SF eGFP. The blot was also probed with an antibody to the 41 kDa β-actin protein (lower lane) to demonstrate equivalent loading in each lane.

5.2.3 Reconstitution of Functional IL-2 Receptor Complexes Following Lentiviral Transduction of ED-7R Cells

The common cytokine receptor γ-chain is shared by the receptors for IL-2, 4, 7, 9, 15 and 21, which signal through association with the JAK family of tyrosine kinases. In particular γc associates with JAK3 following ligand binding, which causes subsequent phosphorylation of the transcription factor STAT5. Having previously demonstrated that the self-inactivating lentiviral vectors are able to efficiently transduce the human T cell line ED-7R, an assay was performed to assess functionality of the expressed γc transgene. ED-7R cells are derived from a human T cell leukaemia line (ED40515$^-$) naturally deficient in γc however they express all other components of the IL-2 receptor (IL2R) (Ishii et al., 1994); lentivirally-transduced cells were therefore analysed for the presence of restored functional IL2R by incubation with IL-2 molecules and subsequent analysis of downstream signalling by staining with an antibody specific for the phosphorylated STAT5 transcription factor (pSTAT5). A γc positive T-cell line, ED-7R-γc, was used as a positive control.

Following incubation with IL-2, phosphorylated STAT5 is detectable for each of the SIN lentivirally-transduced ED-7R cells at levels comparable to the γc-expressing control ED-7R-γc cells (figure 5.3) indicating that the γc transgene expressed by the lentiviral vectors is able to restore functional IL-2 receptor complexes in this cell line. No aberrant downstream JAK-STAT signalling was observed in the transduced cells in the absence of IL-2. Mock transduced ED-7R cells incubated with the cytokine had no detectable pSTAT5.

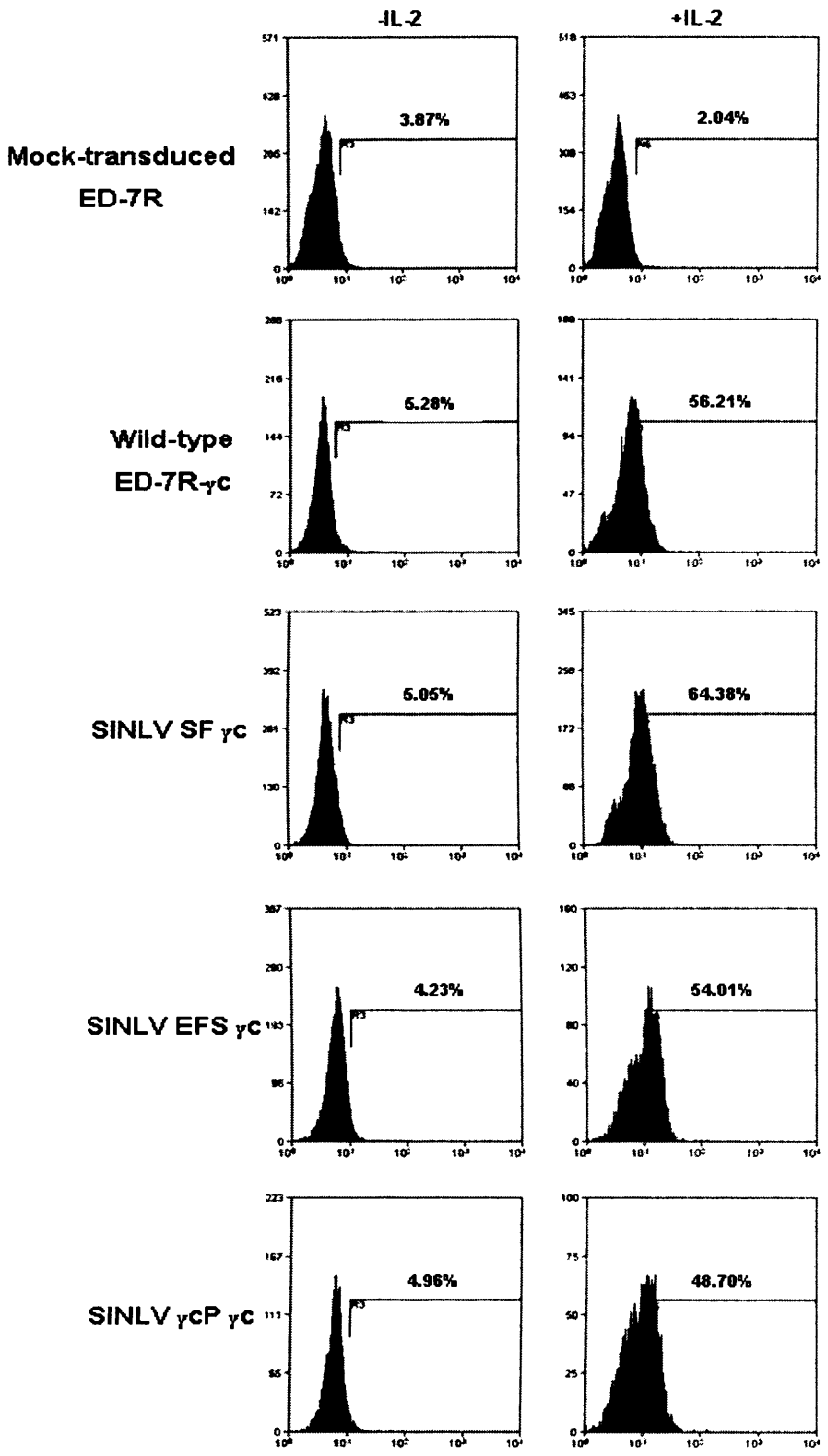

Figure 5.3. The restoration of functional IL-2 receptors on lentivirally transduced ED-7R cells. Lentivirally transduced γc-deficient ED-7R cells (MOI 5) were incubated with IL-2 and assessed for downstream signalling by intracellular staining with an anti-pSTAT5 antibody. pSTAT5 was detected by flow cytometry in cells transduced with either SINLV SF γc, SINLV EFS γc or SINLV γcP γc but not in mock transduced ED-7R cells, indicating that the γc transgene expressed by each of the promoters within the SIN lentiviral vectors is functional.

5.2.4 *In Vitro* B cell differentiation of *Il2rg*$^{-/-}$ HSCs Transduced with the SIN Lentiviral Vectors

To assess the ability of the SIN lentiviral vectors to transduce and form functional γc-containing receptor complexes on primary murine progenitor cells the OP9 system was used. As for the analysis of the self-inactivating gammaretroviral vectors, lin$^-$ haematopoietic progenitors isolated from γc-deficient mice were transduced *ex vivo*. The protocol used for the VSV-G pseudotyped lentiviral vectors however involved only a single round of transduction, in which the lin$^-$ cells were incubated overnight with concentrated lentiviral supernatant at an MOI of 25 in serum-free media in the presence of cytokines. Following transduction the lin$^-$ cells were seeded onto OP9 stromal layers and co-cultured for a total of thirteen days in the presence of IL-7 and Flt-3. The co-cultures were analysed by light microscopy and flow cytometry subsequent to staining with antibodies to differentiated lymphocyte cell surface markers.

Following six days of co-culture on OP9-eGFP stromal layers, cobblestone colonies formed from primitive progenitor populations were visible for both the lentivirally-transduced and mock transduced *Il2rg*$^{-/-}$ lin$^-$ cells (figure 5.4A). A large amount of cell death was apparent for the SINLV SF γc transduced cells however, potentially indicating toxicity of the lentiviral preparation used to transduce the cells. On day six, the haematopoietic cells were removed from the stromal layers, stained with antibodies to B (CD19) and NK (NK1.1) cell markers and analysed by flow cytometry (figure 5.4B). Wild-type C57/Bl6 cells were found to have undergone B cell differentiation with the majority of cells being CD19$^+$. The lentivirally and mock transduced cells however appeared to be differentiating at a slower rate, with between 15 and 31% of cells staining positive for the NK cell marker NK1.1 and only a smaller percentage of cells found to be CD19$^+$. As observed by light microscopy, only a small proportion of the cells transduced with SINLV SF γc remained viable and could thus be analysed by flow cytometry.

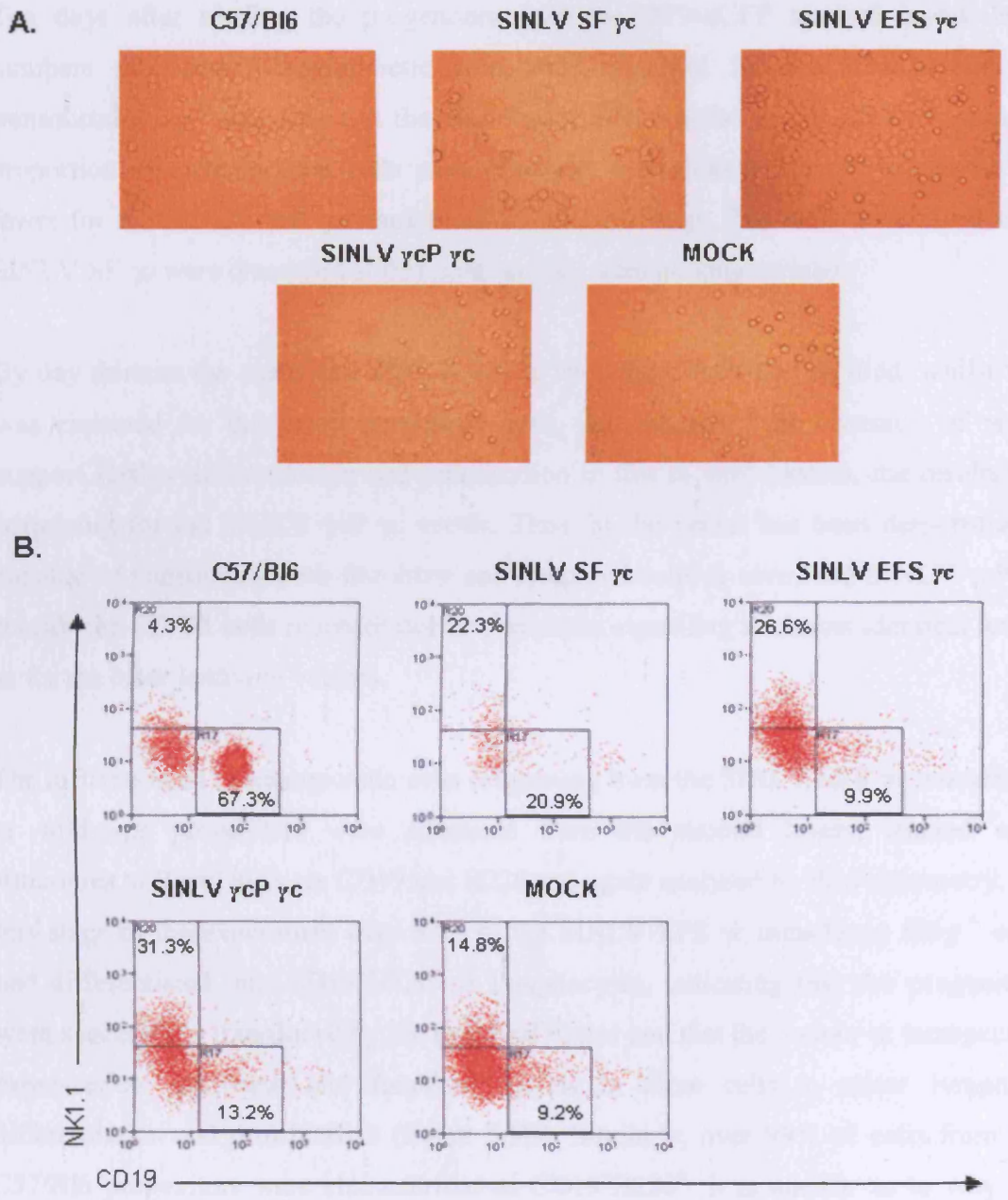

Figure 5.4. Analysis of OP9-eGFP co-cultures on days five and six.
(A) Cobblestone colonies representing early progenitor cell populations are visible after five days co-culture of transduced lin$^-$ cells on the OP9-GFP stromal cell line. (B) Cells were stained with antibodies to NK cell marker NK1.1 and B cell marker CD19 and analysed by flow cytometry on day six. A large population of CD19$^+$ early B lymphocytes are observed for C57/Bl6 wild-type cells, with smaller CD19$^+$ populations present for the *Il2rg*$^{-/-}$ cells transduced with the SIN lentiviral vectors.

Ten days after seeding the progenitors onto the OP9-eGFP stromal layers large numbers of round haematopoietic cells were observed for the SINLV EFS γc transduced *Il2rg*$^{-/-}$ lin^{-} cells and the wild-type C57/Bl6 cells (figure 5.5A). A smaller proportion of differentiated cells were observed in the mock treated well and even fewer for the SINLV γcP γc transduced cells at this stage. The cells transduced with SINLV SF γc were discarded at this point as they were no longer viable.

By day thirteen the mock and SINLV γcP γc transduced cells had all died; whilst this was expected for the mock transduced cells, demonstrating the necessity of γc to support further differentiation and proliferation in this *in vitro* system, the results are surprising for the SINLV γcP γc vector. Thus far the vector has been demonstrated capable of transducing both fibroblast and lymphoid cells *in vitro*, and SINLV γcP γc transduced ED-7R cells reconstituted γc-dependent signalling to almost identical levels as for the other lentiviral vectors.

The differentiated haematopoietic cells originating from the SINLV EFS γc transduced or wild-type progenitors were separated from the stromal layers, stained with antibodies to B cell markers CD19 and B220 and again analysed by flow cytometry. By this stage of the experiment over 97% of the SINLV EFS γc transduced *Il2rg*$^{-/-}$ cells had differentiated into CD19^{+}B220^{+} B lymphocytes, indicating that the progenitors were successfully transduced by the lentiviral vector and that the human γc transgene is expressed to sufficient and functional levels in these cells to allow lymphoid differentiation and proliferation (figure 5.5B). Similarly, over 99% of cells from the C57/Bl6 progenitors were characterised as CD19^{+}B220^{+}. It is unclear as to why the SINLV SF γc transduced progenitors died at such an early stage of the experiment, in particular since the mock transduced cells remained healthy and viable for a longer period of time and that thus far this vector has performed in a similar fashion to SINLV EFS γc. These results represent a single experiment using the OP9 system due to time constraints within the project; hence repetition of this work using a different lentiviral preparation would confirm whether the results observed are due to toxicity of the preparation or due to an inherent property of the vector itself.

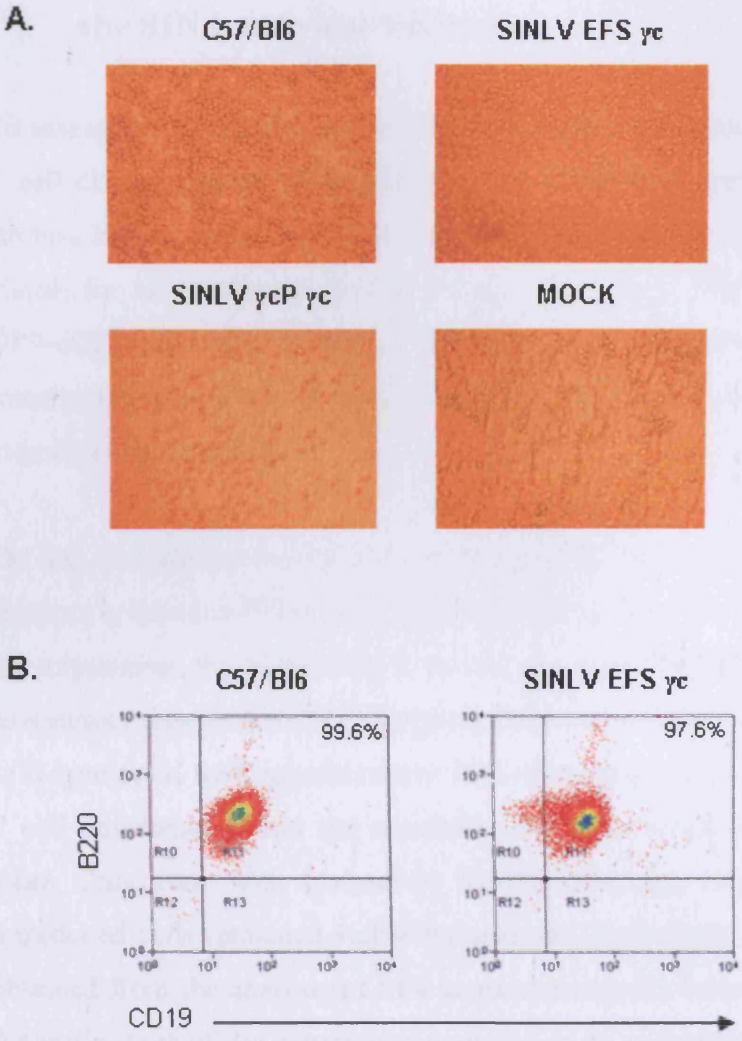

Figure 5.5. Analysis of OP9-eGFP co-cultures on days ten and thirteen.
(A) After ten days co-culture large numbers of round haematopoietic cells are observed resting on top of the stromal OP9 layers for the wild-type and SINLV EFS γc transduced cells. The SINLV γcP γc transduced cells however appeared to be arrested from undergoing further differentiation or proliferation. (B.) Cells were stained for B cell surface markers CD19 and B220 on day thirteen and analysed by flow cytometry. Over 97% of wild-type and SINLV EFS γc transduced *Il2rg*$^{-/-}$ HSCs cells had differentiated into CD19^{+}B220^{+} B lymphocytes by this stage.

5.2.5 *In Vitro* T cell differentiation of *Il2rg*$^{-/-}$ HSCs Transduced with the SIN Lentiviral Vectors

To assess the propensity for the SIN lentivirally transduced *Il2rg*$^{-/-}$ lin$^-$ cells to undergo T cell differentiation, a proportion of the progenitors were also seeded on OP9-DL1 stromal layers. As observed for the OP9-eGFP co-cultures, cobblestone colonies were visible for all conditions on day 5 (data not shown). The cells used to seed both the OP9-eGFP and the OP9-DL1 stromal layers originated from the same initial transduction and hence, as expected, poor viability of the SINLV SF γc transduced cells was also observed at this stage.

On day six, haematopoietic cells from each co-culture were stained with antibodies to thymocyte markers CD44 and CD25 and analysed by flow cytometry. By this stage of the experiment the SINLV EFS γc and the surviving SINLV SF γc transduced lin$^-$ progenitors appeared to be undergoing differentiation at the same rate as the C57/Bl6 wild-type cells, with approximately 90% of the population at the CD44$^+$CD25$^-$ stage of T cell development and the remainder having proceeded to the CD44$^+$CD25$^+$ DN2 stage. Concurrent with analysis by light microscopy, very few of the SINLV SF γc transduced cells remained viable for analysis (figure 5.6). These results contrast those obtained from the analysis of SIN gammaretrovirally transduced progenitors, which at the same stage of the experiment appeared to be undergoing T cell differentiation at a faster rate than the C57/Bl6 wild-type cells (see figure 3.6B). These data may be indicative of the shorter transduction protocol required for lentiviral vectors resulting in decreased differentiation of the lin$^-$ progenitors. The majority of the mock transduced cells appeared arrested at the DN1 stage indicating a requirement for γc-mediated signalling for further T cell development. The SINLV γcP γc transduced cells also appeared arrested at the DN1 stage correlating with the results observed from the OP9-eGFP co-cultures.

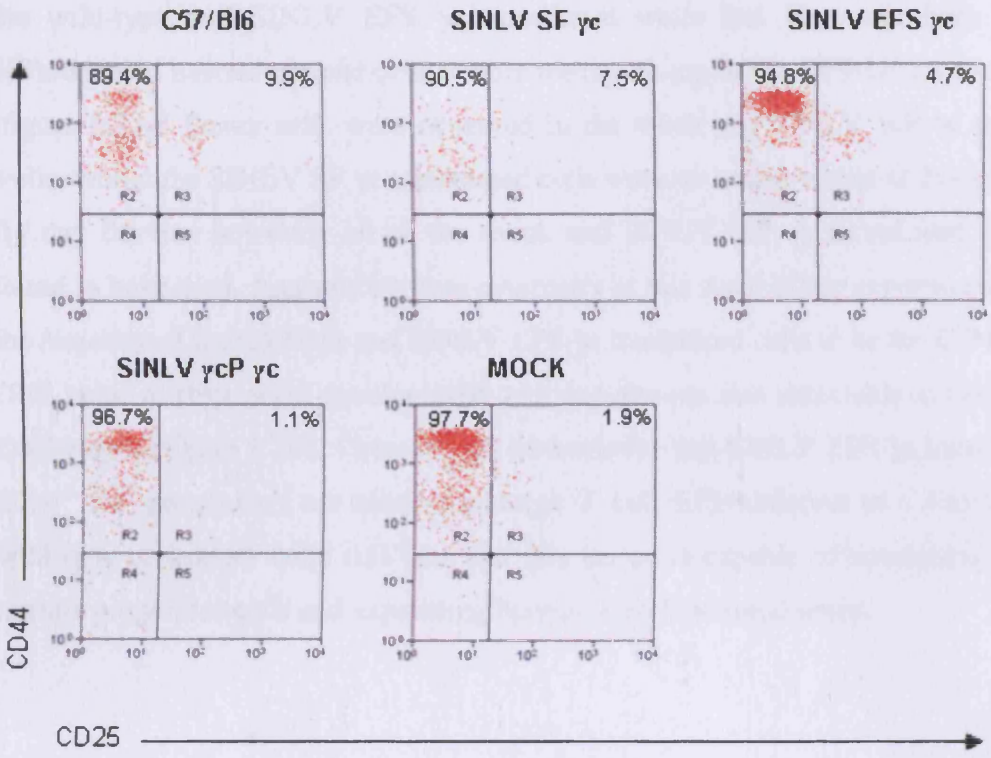

Figure 5.6. Analysis of OP9-DL1 co-cultures on day six. After six days co-culture on OP9-DL1 stromal layers, haematopoietic cells were stained with antibodies to early T cell markers (CD44 and CD25) and analysed by flow cytometry. Both the C57/Bl6 and SINLV EFS γc transduced cells were found to be differentiating from the DN1 to the DN2 stage of thymocyte development, whilst the mock and SINLV γcP γc transduced cells appeared arrested at DN1.

Similarly to the results observed for the OP9-eGFP co-culture, after ten days incubation the wild-type and SINLV EFS γc transduced wells had large numbers of round differentiated haematopoietic cells visible resting on top of the OP9-DL1 stromal layers (figure 5.7A). Fewer cells were observed in the mock and SINLV γcP γc transduced wells, whilst the SINLV SF γc transduced cells were no longer viable at this time-point. By day thirteen however, all of the mock and SINLV γcP γc transduced cells were found to have died. Analysis by flow cytometry at this stage of the experiment revealed the majority of the C57/Bl6 and SINLV EFS γc transduced cells to be the $CD44^{+}CD25^{+}$ DN2 stage of thymocyte development with populations also detectable at the DN1 and DN3 stages (figure 5.7B). These results demonstrate that SINLV EFS γc transduced *Il2rg*$^{-/-}$ lin^{-} progenitors are able to undergo T cell differentiation at a similar rate to wild-type progenitor cells and thus that this vector is capable of tranducing primitive murine progenitor cells and expressing human γc to functional levels.

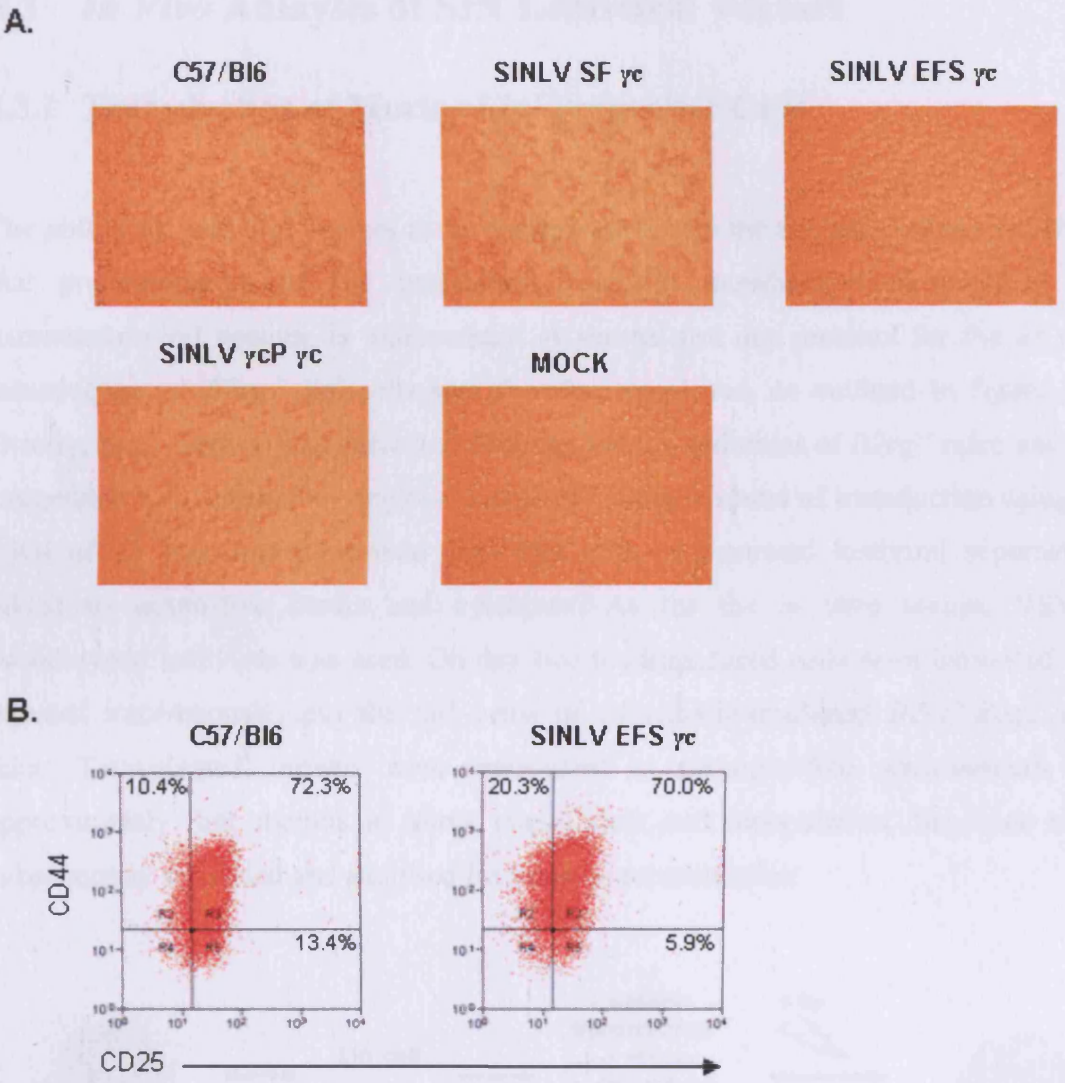

Figure 5.7. Analysis of OP9-DL1 co-cultures on days ten and thirteen. (A) After ten days co-culture, large numbers of round haematopoietic cells are visible resting on top of the stromal layers in the C57/Bl6 and SINLV EFS γc transduced wells. (B) Following thirteen days of co-culture the wild-type C57/Bl6 and SINLV EFS γc transduced cells appeared to be differentiating further towards DN4. No viable cells remained for all other conditions.

5.3 *In Vivo* Anlaysis of SIN Lentiviral Vectors

5.3.1 Transduction of Murine Lin$^-$ Progenitor Cells

The ability of lentiviral vectors to be transported across the nuclear membrane means that pre-stimulation of lin$^-$ progenitors prior to transduction, as required for gammaretroviral vectors, is unnecessary. A shorter two-day protocol for the *ex vivo* transduction of *Il2rg*$^{-/-}$ lin$^-$ cells was therefore employed, as outlined in figure 5.8. Briefly, bone marrow was harvested from the femurs and tibias of *Il2rg*$^{-/-}$ mice and lin$^-$ progenitor cells isolated by negative selection. A single round of transduction using an MOI of 25 was then performed overnight with concentrated lentiviral supernatant added to serum-free media and cytokines. As for the *in vitro* assays, VSV-G pseudotyped lentivirus was used. On day two the transduced cells were harvested and injected intravenously into the tail veins of sublethally-irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice. Transplanted animals were maintained in pathogen-free environments for approximately four months to allow engraftment and repopulation, the mice were subsequently sacrificed and analysed for immune reconstitution.

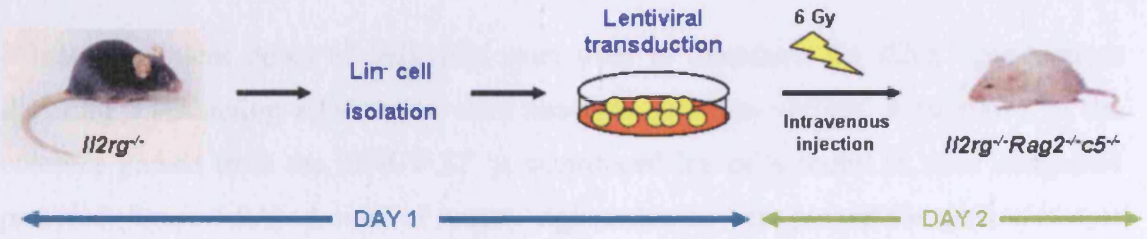

Figure 5.8. *Ex vivo* lin$^-$ transduction protocol. A schematic of the SIN lentiviral *ex vivo* transduction protocol; lin$^-$ cells isolated from *Il2rg*$^{-/-}$ mice were transduced overnight with VSV-G pseudotyped lentivirus and subsequently injected into sub-lethally irradiated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice.

5.3.2 Lentiviral Lin$^-$ Transduction Levels

A cohort of mice injected with *Il2rg*$^{-/-}$ lin$^-$ cells transduced with SINLV SF γc, SINLV γcP γc, SINLV γcP eGFP or with mock-transduced *Il2rg*$^{-/-}$ progenitors was set up. A different preparation of SINLV SF γc than was used for the *in vitro* OP9 co-culture assay was used for this study. To assess pre-transplant lentiviral transduction levels, a proportion of transduced lin$^-$ cells were also seeded in semi-solid medium supplemented with murine cytokines. Following two weeks incubation at 37°C, colonies formed from the transduced progenitor cells were isolated and genomic DNA harvested for analysis. Colonies were assessed for the presence of integrated vector by polymerase chain reaction (PCR) using primers directed to the γc transgene for SINLV SF γc transduced lin$^-$ cells, whilst the cells transduced with the *IL2RG* promoter vectors were analysed using primers to the *IL2RG* regulatory sequences. Analysis of the PCR products by agarose gel electrophoresis enabled calculation of the proportion of transduced progenitor cells that were injected into the recipient mice. Lin$^-$ cells transduced with the SINLV γcP eGFP vector were also analysed by flow cytometry to estimate transduction levels by assessing the proportion of eGFP-expressing cells present within the population.

Whilst equivalent doses of lentivirus were used to transduce the *Il2rg*$^{-/-}$ progenitors differing transduction efficiencies were observed between vectors, with 100% of the colonies picked from the SINLV SF γc transduced lin$^-$ cells found to have integrated provirus (figure 5.9A). A total of twenty-eight colonies were picked for each vector, it is therefore possible that had a larger number been analysed, transgene negative colonies may have been detected and thus the true transduction efficiency might be slightly lower than calculated. The lowest transduction efficiency was observed for SINLV γcP γc, with only 41% of the colonies picked found to contain integrated provirus; the same titration method was used for both lentiviral preparations, hence it was surprising that such low transduction efficiency was achieved for this vector. Repetition of the PCR using primers to the γc transgene yielded a similar result (data not shown) hence the data does not reflect a discrepancy between the efficiencies of the two primer sets used. Flow cytometric analysis of the SINLV γcP eGFP transduced progenitors revealed approximately 60% of cells to be eGFP$^+$, indicating that this

promoter is active in early murine progenitor cells (figure 5.9B); analysis of the colonies derived from these cells by PCR gave a transduction of 70%, potentially reflecting a degree of transgene silencing in 10% of the population or a discrepancy between the two assays (figure 5.9A).

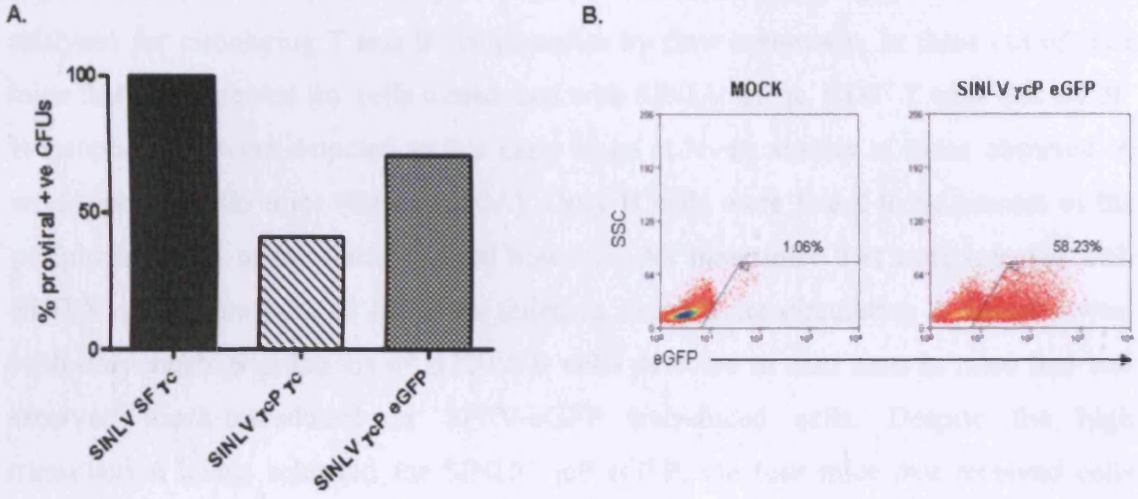

Figure 5.9. Lin⁻ transduction efficiency. (A) Pre-transplant CFU transduction efficiencies of the lentiviral cohort of transplanted animals. (B) The transduction efficiency of SINLV γcP eGFP transduced lin⁻ cells was also assayed by flow cytometric analysis.

5.3.3 Detection of Circulating Lymphoid Cells in the Peripheral Blood of *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with Lentivirally Transduced Cells

Eight weeks post-transplant samples of peripheral blood from repopulated mice were analysed for circulating T and B lymphocytes by flow cytometry. In three out of four mice that had received lin$^-$ cells transduced with SINLV SF γc, CD3$^+$ T cells and B220$^+$ B lymphocytes were detected at this early stage at levels similar to those observed in wild-type C57/Bl6 mice (figure 5.10A). Only B cells were found to be present in the peripheral blood of the fourth animal however. All three mice that were injected with SINLV γcP γc transduced lin$^-$ cells failed to reconstitute circulating T lymphocytes, with only small populations of B220lo B cells detected as also seen in mice that had received mock-transduced or SFFV-eGFP transduced cells. Despite the high transduction levels achieved for SINLV γcP eGFP, the four mice that received cells transduced with this vector had no detectable eGFP$^+$ cell populations in the peripheral blood (figure 5.10B). These data indicate either that the transduced progenitors failed to engraft in the transplanted mice, that the promoter was silenced *in vivo* or that it is inactive in the differentiated myeloid and B220lo B cell lineages in these animals.

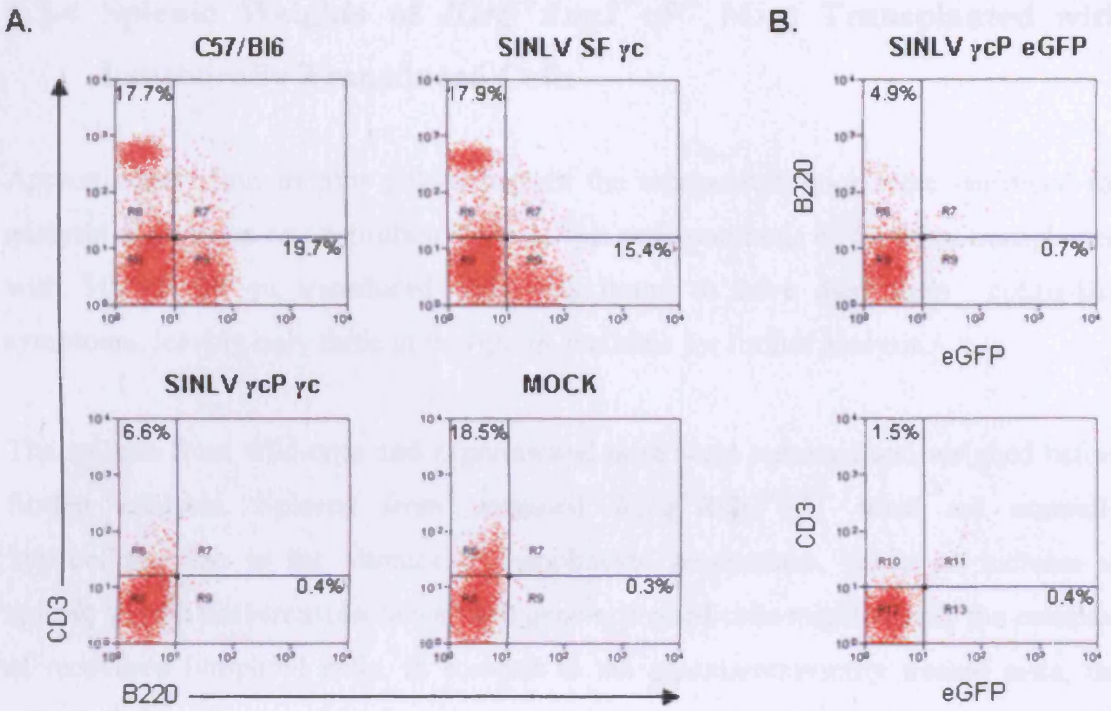

Figure 5.10. Peripheral blood analysis of transplanted *Il2rg*$^{-/-}$*Rag2*$^{-}$/*c5*$^{-/-}$ mice. (A) Peripheral blood mononuclear cells from repopulated mice were stained with antibodies to CD3 (T cell marker) and B220 (B cell marker) and analysed by flow cytometry. Circulating T and B lymphocytes were detected in *Il2rg*$^{-/-}$*Rag2*$^{-}$/*c5*$^{-/-}$ mice eight weeks post-transplantation of SINLV SF γc transduced *Il2rg*$^{-/-}$ lin^{-} cells. Mice that had received cells transduced with SINLV γcP γc or mock-transduced cells failed to reconstitute circulating lymphocytes. (B) No detectable eGFP^{+} or lymphocyte cell populations were present in the SINLV γcP eGFP mice.

5.3.4 Splenic Weights of *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with Lentivirally Transduced Cells

Approximately four months post-transplant the repopulated mice were sacrificed for analysis of immune reconstitution. Prior to this time-point one of the mice transplanted with SINLV SF γc transduced cells was found to have died from colitis-like symptoms, leaving only three in this group available for further analysis.

The spleens from wild-type and experimental mice were removed and weighed before further analysis. Spleens from untreated *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice are normally hypocellular due to the absence of lymphocyte populations, hence an increase in splenic weight post-transplantation with gene-corrected cells might signify the presence of recovered lymphoid cells. In contrast to the gammaretrovirally treated mice, the spleens from animals that had received lentivirally transduced lin$^-$ cells were uniformly smaller and lower in weight than observed for wild-type animals (figure 5.11). Surprisingly, the spleens removed from the SINLV SF γc mice, that had detectable circulating lymphocytes eight weeks post-injection, were similar in weight to the spleens from the SINLV γcP eGFP or mock-treated animals. The mice that had received cells transduced with SINLV γcP γc were found to have the largest spleens, which were noted to be significantly heavier than those from the SINLV γcP eGFP mice, even though no detectable lymphocyte populations were present in the peripheral blood of these animals. No cases of splenomegally were observed in any of the experimental animals.

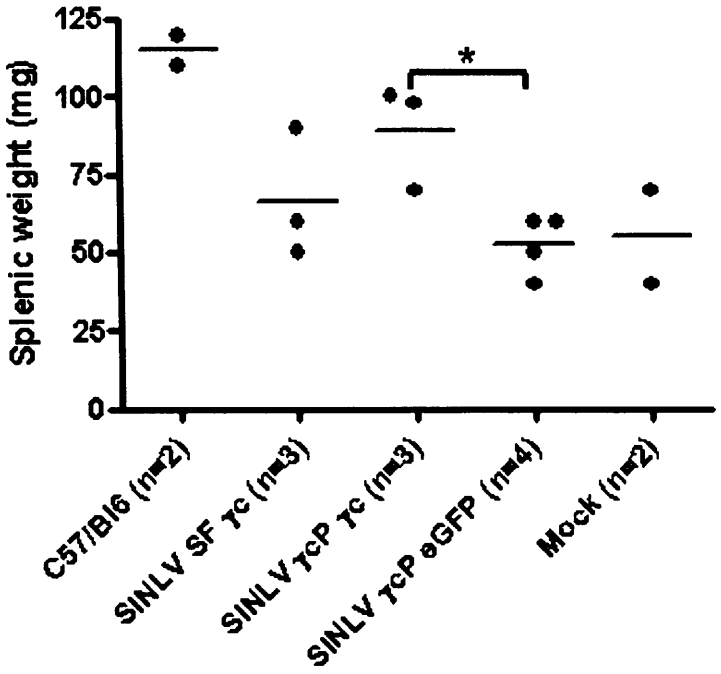

Figure 5.11. The splenic weights of *Il2rg*$^{-/-}$*Rag2*$^{-}$*/c5*$^{-/-}$ mice transplanted with lentivirally transduced lin^{-} cells. The splenic weights from all transplanted mice were observed to be lower than for wild-type C57/Bl6 animals. The heaviest spleens were those removed from SINLV γcP γc mice, found to be significantly increased in weight as compared to SINLV γcP eGFP animals despite lymphocytes being absent from the periphery. (*, $P<0.05$)

5.3.5 Flow Cytometric Analysis of Lymphoid Populations in the Spleen and Bone Marrow of *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with Lentivirally Transduced Cells

Cells isolated from the spleens and bone marrow of transplanted mice were stained with antibodies to lymphoid and myeloid populations and analysed by flow cytometry. In correlation with the results from the peripheral blood analysis, two out of three SINLV SF γc mice had detectable T, B and NK cell populations in the spleens and bone marrow (figure 5.12A). The third mouse failed to reconstitute the T cell compartment; however mature B220^{+}IgM^{+} B cells and NK cell populations were detected in both tissues (data not shown). The partial immunological reconstitution observed in this animal might be due to less successful intravenous injection resulting in a lower dose of lentivirally-transduced cells as compared to the remainder of the cohort. All three mice transplanted with SINLV γcP γc transduced lin^{-} cells failed to undergo immune reconstitution, with similar staining observed in the two *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with mock-transduced cells (figure 5.12A). T and NK cells remained absent in these animals, however approximately 30% of cells in the spleen were found to have an immature B220lo phenotype. These results likely reflect the ability of *Il2rg*$^{-/-}$ lin^{-} cells to undergo partial B cell development in the absence of γc signalling (DiSanto et al., 1995; Cao et al., 1995).

Concurrent with the peripheral blood analysis, all four mice transplanted with SINLV γcP eGFP transduced cells had no detectable eGFP^{+} populations in either tissue (figure 5.12B). As observed for the mock-transduced and SINLV γcP γc mice, T and NK cells failed to develop in these animals due to the absence of γc-mediated signalling, however a proportion of B220^{+} cells were detected.

An increase in the proportion of myeloid CD11b^{+} cells in both the spleen and bone marrow was observed for all transplanted mice as compared to wild-type animals. It is therefore likely that this increase is due to the *ex vivo* manipulation of the murine HSCs and/or the repopulation setting in this experiment as opposed to relating to the γc transgene itself.

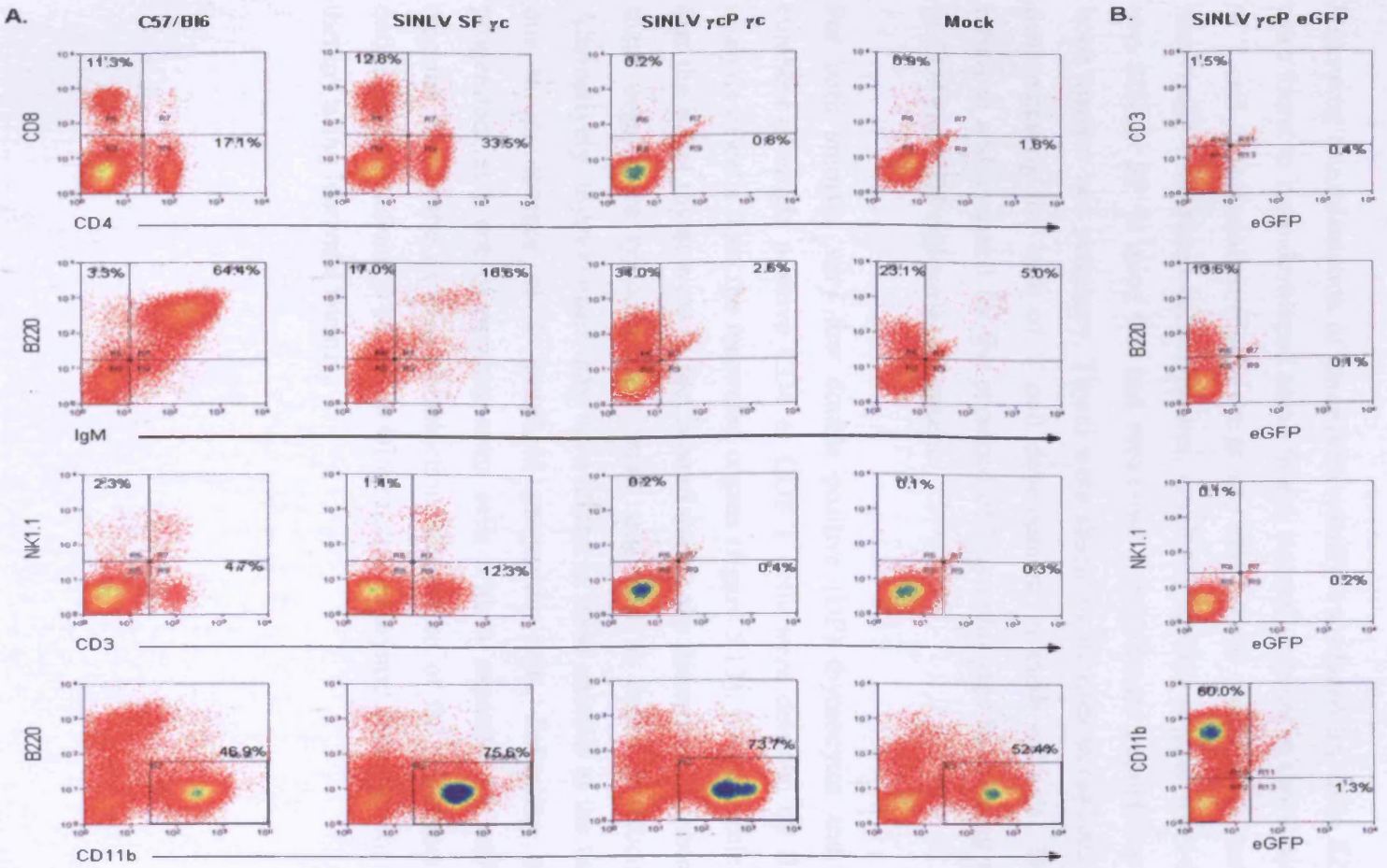

Figure 5.12. Flow cytometric analysis of lymphoid reconstitution in the spleens and bone marrow of engrafted animals. (A) Representative flow cytometry plots from the analysis of lymphocyte restoration in the spleen and bone marrow of *Il2rg*$^{-/-}$*Rag2*$^{-}$/*c5*$^{-/-}$ mice transplanted with γc-lentivirally or mock transduced *Il2rg*$^{-/-}$ lin^{-} cells. CD4^{+} and CD8^{+} T cells and mature B220^{+}IgM^{+} B cells are present only in the spleens of SINLV SF γc mice (first two rows). Similarly, CD3^{+} T lymphocytes and NK1.1^{+} NK cells are also only detected in the bone marrow of these animals (third row). CD11b^{+} myeloid populations in the bone marrow are increased following repopulation (fourth row). (B) Mice transplanted with SINLV γcP eGFP transduced cells had no detectable eGFP^{+} populations and failed to develop any T, NK or mature B cells.

5.3.6 Thymic Development in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with Lentivirally Transduced Cells

Following transplantation of gammaretrovirally-transduced lin$^-$ cells *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ were found to have developed small thymi, normally absent in this strain due to a lack of T cell differentiation (Goldman et al., 1998). Four months post-transplantation of lentivirally-transduced cells however, small thymic-like organs were detected in the two SINLV SF γc mice that had recovered T lymphocyte populations in the spleen, bone marrow and periphery. Thymi were absent in all other transplanted mice, further demonstrating the lack of T cell development in these animals. The thymi were removed and analysed for the presence of T lymphocytes by staining with antibodies to CD4 and CD8 cell surface markers.

For both animals, very few double positive (DP) thymocytes and relatively low numbers of single positive CD4 or CD8 T cells were detected by flow cytometric analysis of cells from the recovered organs (figure 5.13). One possible explanation is that the intact thymi were in fact missed during the dissection of these animals since these organs are typically very small and hard to detect in reconstituted mice. Alternatively, thymopoiesis may have halted in these animals at the time of sacrifice due to an absence of T lymphoid progenitor cells, indicating that committed progenitors and not pluripotent stem cells with a capacity for self-renewal were targeted during the *ex vivo* transduction. Repetition of the experiment might allow definitive conclusions to be drawn with regards to thymic development following gene therapy using lentiviral vectors.

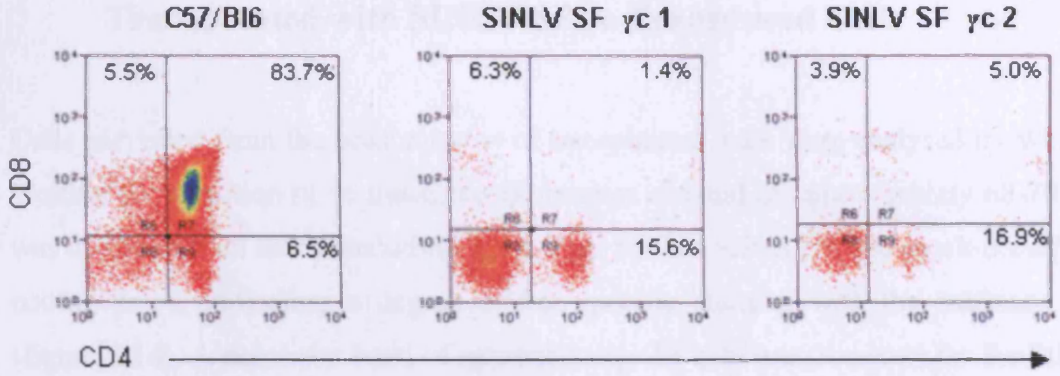

Figure 5.13. Flow cytometric analysis of thymocytes from *Il2rg*$^{-/-}$*Rag2*$^{-}$/*c5*$^{-/-}$ mice transplanted with lentivirally transduced lin^{-} cells. Thymi removed from the two SINLV SF γc mice that had undergone T cell development and from a wild-type C57/Bl6 mouse were stained with antibodies to CD4 and CD8 and analysed by flow cytometry. Very few DP cells were detected for the transplanted mice.

5.3.7 Detection of Human γc Expression in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with SINLV SF γc Transduced Cells

Cells harvested from the bone marrow of transplanted mice were analysed by Western blotting for detection of γc transgene expression. A band of approximately 68-70 kDa was detected in all lanes, including those from SINLV γcP eGFP and mock-transduced control mice, indicating a degree of non-specific staining with the antibody used (figure 5.14). A dominant band of approximately 58 kDa was observed for the SINLV SF γc mice only however, confirming expression of the γc transgene in these animals. No such bands were observed in the lanes representing two mice transplanted with SINLV γcP γc transduced cells, indicating a lack of transgene expression in the bone marrow of these animals and thus explaining the failure of T and B cell differentiation.

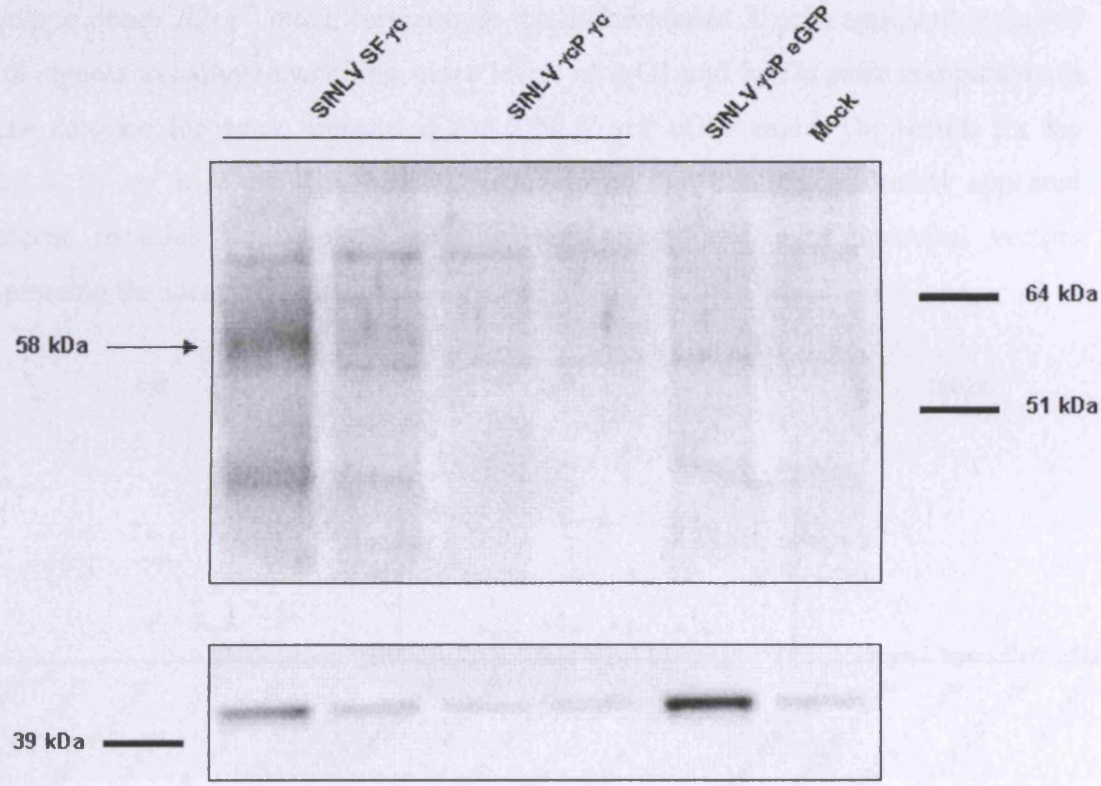

Figure 5.14. γc expression in the bone marrow of *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with lentivirally transduced lin$^-$ cells. Protein isolated from the bone marrow of transplanted mice was analysed by Western blotting using an antibody to human γc. A band of approximately 58 kDa, likely representing human γc, was detected for two SINLV SF γc mice only. The blot was also probed with an antibody to β-actin (41 kDa) to confirm protein loading (bottom lane).

5.3.8 Immunoglobulin Levels in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with Lentivirally Transduced Cells

Samples of sera taken from the transplanted mice at the time of sacrifice were analysed by ELISA to assess immunoglobulin levels and thus reconstitution of humoral immunity (figure 5.15). Levels of IgG in the sera of mice transplanted with SINLV γcP γc, SINLV γcP eGFP or mock transduced cells were found to be uniformly low, reflecting the absence of mature B cells in these animals. Levels of isotype subclasses IgG1 and IgG2a also remained diminished in these mice post-transplantation, reflecting the failure of immunological reconstitution in these animals. Although two of the three SINLV SF γc mice analysed had recovered T and B cell populations in the bone marrow and spleen, humoral immunity remained impaired in these animals at the time of sacrifice. Levels of IgG in the sera of these animals were detected at similar levels to donor *Il2rg*$^{-/-}$ mice; furthermore the differentiated T cells appeared impaired with regards to isotype-switching, since levels of IgG1 and IgG2a were comparable to those detected for mock transduced and SINLV γcP eGFP mice. The results for the SINLV SF γc mice are therefore surprising given that humoral immunity appeared restored in mice transplanted with lin$^-$ cells transduced with retroviral vectors expressing the identical transgene.

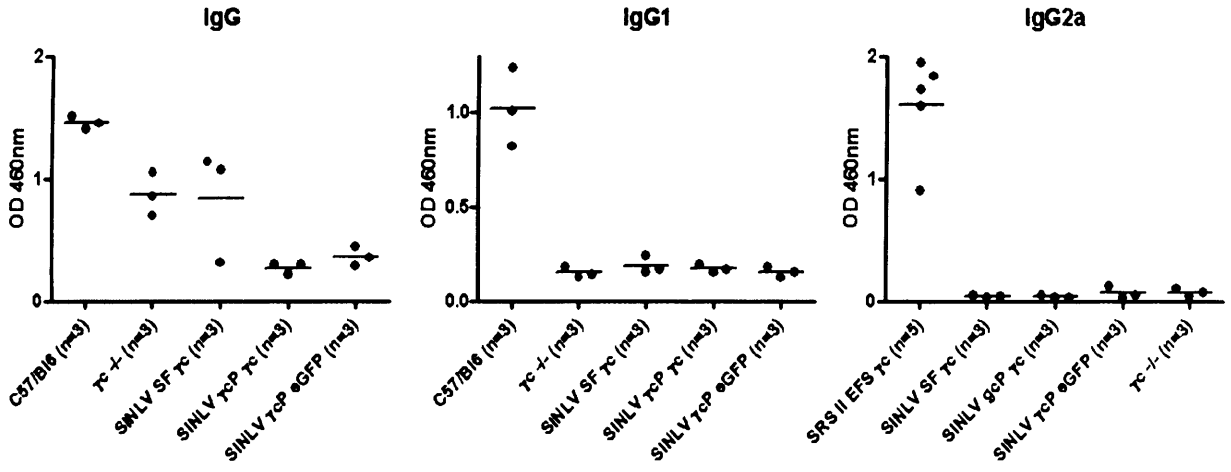

Figure 5.15. Serum immunoglobulin levels in *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted with lentivirally transduced lin$^-$ cells. Serum immunoglobulin levels in transplanted and control mice were analysed by ELISA. Humoral immunity remained impaired in all transplanted animals.

5.3.9 Proviral Copy Number in the Spleens and Bone marrow of *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ Mice Transplanted with Lentivirally Transduced Cells

Genomic DNA extracted from splenocytes and bone marrow cells from transplanted mice was analysed for integrated provirus by real-time quantitative PCR. Primers and probes to the γc cDNA and mouse *titin* housekeeping gene were used as previously. Provirus was detected in the spleens of all mice that had received SINLV SF γc transduced *Il2rg*$^{-/-}$ lin^{-} cells, with copy number reflecting efficacy of reconstitution (figure 5.16). The lowest proviral copy number was observed for SINLV SF γc 3, the sole animal in the group that failed to develop T lymphocytes. This mouse also had negligible provirus detected in the bone marrow, reflecting the low proportion of mature B cells detected by flow cytometric analysis of this tissue (data not shown) and the likelihood that few transduced pluripotent progenitor cells with self-renewal capacity engrafted post-transplantation. The highest proviral copy number was for SINLV SF γc 1, correlating with the highest proportion of both T and mature B cells detected by flow cytometric analysis of the spleens and bone marrow and indicating that these cell populations developed from lentivirally-transduced, engrafted progenitor cells. Low levels of integrated provirus were only detected for one of the mice transplanted with SINLV γcP γc in both tissues. Since this animal failed to reconstitute T cells or mature B lymphocytes, these data likely signify that although transduced cells engrafted post-transplant, the *IL2RG* promoter might have been silenced. The SINLV γcP eGFP mice and those that had received mock-transduced cells were found to contain negligible proviral copies, demonstrating the specificity of the primer sets used.

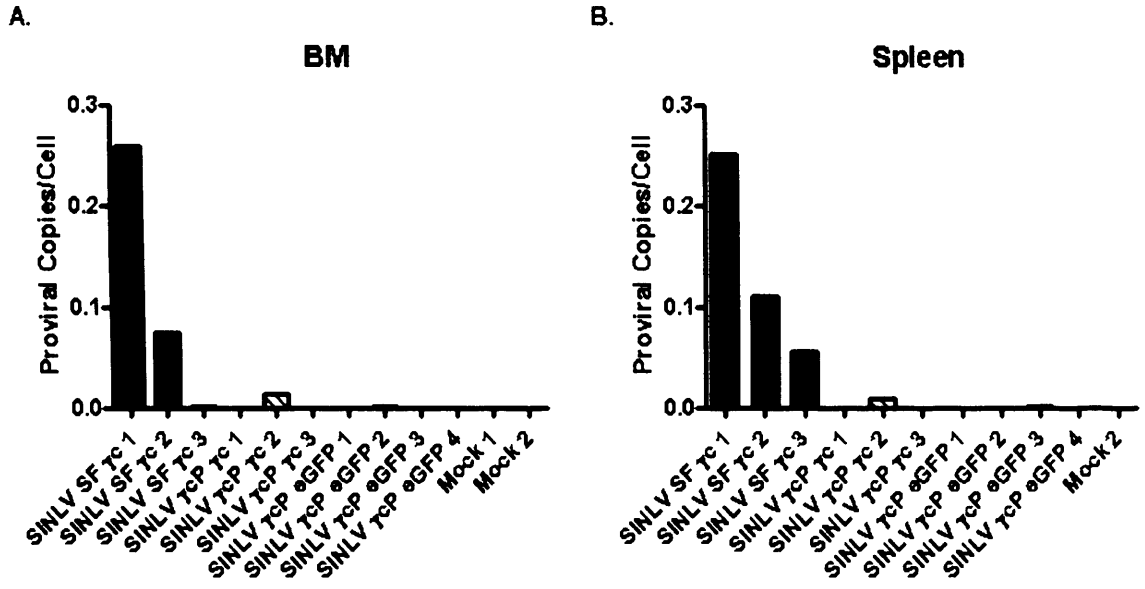

Figure 5.16. Proviral copy number in the spleens and bone marrow of *Il2rg*$^{-/-}$ *Rag2*$^{-}$*/c5*$^{-/-}$ mice transplanted with lentivirally transduced lin^{-} cells. Proviral copy number detected in the (A) bone marrow and (B) spleens of transplanted mice. Integrated provirus was detected for all mice transplanted with SINLV SF γc transduced cells and SINLV γcP γc 2.

5.4 Conclusions

In vitro analyses of SINLV γcP γc together with SIN lentiviral vectors incorporating SFFV and EFS promoters demonstrated these that vectors were capable of transducing both lymphoid and fibroblast cell lines and expressing human γc on the cell surface to detectable levels. Expression of the γc transgene in the murine fibroblastoid cells was further confirmed by Western blot analysis, verifying that the *IL2RG* promoter is active in this cell line. This data conflicts with a previous report that the *IL2RG* regulatory sequences exhibit little or no promoter activity in non-haematopoietic cell lines (Markiewicz et al., 1996). Functionality of γc expressed from each of the lentiviral vectors was confirmed following transduction of a γc-deficient human T cell line, enabling reconstitution of functional γc-containing IL-2 receptor complexes and subsequent downstream signalling.

The OP9 system was used to assess the potential of the SIN lentiviral vectors to transduce primary murine progenitor cells and express γc to functional levels that enable lymphoid differentiation *in vitro*. The lentiviral vector SINLV EFS γc performed in a similar manner to the SRS II series of gammaretroviral vectors in this system (see chapter 3.2.3) with transduced cells undergoing T and B cell development at similar rates as the wild-type C57/Bl6 progenitors. Lin^- cells transduced with SINLV SF γc however exhibited poor viability, potentially attributed to toxicity of the lentiviral preparation used and hence few results were gained from this assay. Following transduction with SINLV γcP γc, *Il2rg*$^{-/-}$ progenitors were found to behave in much the same way as mock transduced cells, with little or no lymphoid differentiation seen *in vitro* leading to loss of viability by day thirteen. Several reasons might explain why the SINLV γcP γc vector does not function in this system – firstly, the cloned *IL2RG* promoter region might not function in primary murine haematopoietic progenitors; although all sequences upstream of the human *IL2RG* gene that displayed significant homology to the corresponding region in the murine genome were incoporated into the vector, it is possible that a regulatory motif critical for expression in primitive cell populations was omitted. Secondly the initial transduction of the *Il2rg*$^{-/-}$ progenitors may have been unsuccessful, although the same preparation has been shown to successfully transduce cell lines *in vitro*. Finally, as the

IL2RG promoter is neither a strong viral promoter nor that of a housekeeping gene, it might be more prone to silencing following integration either due to positional effects or downregulation by cellular factors. Further work to characterise the *IL2RG* promoter using a vector encoding eGFP may lead to more definite conclusions being drawn.

Analysis of the SIN lentiviral vectors *in vivo* confirms the hypothesis that the failure of the OP9 experiment using SINLV SF γc transduced *Il2rg*$^{-/-}$ progenitors was probably due to toxicity associated with the particular lentiviral preparation used. A different preparation of this lentiviral vector was demonstrated capable of efficiently transducing murine lin$^-$ cells using an overnight protocol (without prior stimulation of the progenitors) and expressing human γc to levels that enabled detection of circulating T and B lymphocyte populations in the peripheral blood of three out of four animals just eight weeks post-transplantation (for a summary of the *in vivo* experiments see Appendix 11). Furthermore, five months post-gene therapy T, B and NK cell populations were restored in the spleens and bone marrow of these mice. Despite the high transduction levels and presence of recovered lymphocyte populations, spleens from the transplanted mice remained hypocellular and thus T cell proliferation could not be assessed. Furthermore, it is debatable whether or not thymi were present in these animals since normal thymocyte staining was not observed. Failure of transduced HSCs with self-renewal capacity to engraft post-injection may have resulted in degeneration of the thymi due to an absence of seeding by T cell progenitors. Humoral immunity, as assessed by serum immunoglobulin levels, also remained impaired in these mice with levels of IgG in the plasma comparable to levels detected in *Il2rg*$^{-/-}$ donor mice and no evidence of isotype switching observed. Integrated provirus was detected in splenocytes and bone marrow cells from the reconstituted mice and γc expression also detected in the latter by Western blot analysis; hence it is unclear why the spleens in these mice remained hypocellular and immunoglobulin isotype switching remained defective.

One of the three SINLV SF γc mice was found to have recovered only the B cell compartment post-transplantation. It is likely that this animal received a lower dose of transduced progenitors than the other transplanted mice due to unsuccessful injection and thus received fewer γc-corrected, pluripotent lin$^-$ cells.

Analysis of the self-inactivating lentiviral vectors incorporating the cloned *IL2RG* promoter region correlates with the results observed following co-culture of transduced *Il2rg*$^{-/-}$ lin$^-$ cells on the OP9 stromal layers. Despite lin$^-$ transduction levels of approximately 40%, recipient mice failed to develop T or mature B lymphocytes post-transplantation of SINLV γcP γc transduced cells. These data indicate that either the human *IL2RG* promoter is inactive in murine haematopoietic progenitors, that the transduced progenitors failed to engraft post-injection or that the promoter was silenced *in vivo*. The first hypothesis was disproved by the presence of eGFP$^+$ cells detected by flow cytometric analysis of the SINLV γcP eGFP transduced lin$^-$ cells; however no eGFP$^+$ cells were detected eight weeks post-transplantation of these cells. Although eGFP$^+$ cells would have no proliferative advantage in this setting, two out of four mice that had received cells transduced with a gammaretroviral vector encoding eGFP had detectable transgene positive cells in the peripheral blood post-transplant. It therefore seems likely that whilst the *IL2RG* promoter appears active in murine lin$^-$ progenitors, the promoter is somehow silenced, potentially during differentiation, resulting in a failure of progenitor cells to respond to cytokine signals either *in vitro* or *in vivo*. In the case of the SINLV γcP γc transduced progenitors, promoter silencing at an early stage might also lead to loss of selective proliferative advantage which might explain why negligible proviral copies were detected in the spleens and bone marrow from two of the transplanted mice. The detection of low levels of integrated provirus in the spleen and bone marrow of the third SINLV γcP γc mouse also supports the theory that the human *IL2RG* promoter is inactive since this mouse failed to develop T cells. γc-deficient human cells transduced with SINLV γcP γc were able to signal in response to IL-2 *in vitro*, thus differences between the human and murine regulatory sequences may result in the promoter being inactivated. This hypothesis could be tested by co-culture of human *Il2rg*$^{-/-}$ CD34$^+$ cells transduced with SINLV γcP γc on the OP9 stromal layers. It is also possible however that the lin$^-$ transduction levels for the SINLV γcP γc *in vivo* experiments were below the threshold required for lymphoid reconstitution, since repopulation experiments with all other vectors (both lentiviral and gammaretroviral) were performed with transduction levels of over 70%.

5.5 Final Conclusions

- The SIN lentiviral vector series incorporating the SFFV, EFS and *IL2RG* promoters are able to transduce both lymphoid and fibroblastoid cells *in vitro* and restore functional IL-2 receptor complexes on a SCID-X1 T cell line
- The SINLV EFS γc vector restored the lymphoid differentiation potential of *Il2rg*$^{-/-}$ haematopoietic progenitor cells *in vitro*
- *Il2rg*$^{-/-}$ lin^{-} cells transduced with SINLV γcP γc failed to undergo lymphoid development *in vitro* or reconstitute the murine SCID-X1 defect *in vivo*
- The SINLV SF γc vector was able to restore T, B and NK cell populations in the SCID-X1 murine model following *ex vivo* gene therapy

Chapter Six

Discussion

Gene therapy for SCID-X1 has proven an effective treatment in two separate clinical trials (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2002; Gaspar et al., 2004). Out of a total of twenty-one treated infants, nineteen have successfully restored cellular and humoral immunity, enabling them to return to normal social environments and cope with environmental pathogens (Gaspar et al., 2004; Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2002; Ginn et al., 2005). Given the morbidity and mortality associated with haploidentical stem cell transplantation and the recurrent failure to restore humoral immunity following these transplants, gene therapy for this condition offers an effective alternative treatment with superior immunological reconstitution. The recent development of lymphoproliferative diseases in three patients enrolled in the French SCID-X1 gene therapy trial however, has underlined the potential risks of such a treatment and reinforced the necessity of continued research into vector design and safety (Hacein-Bey-Abina et al., 2003b; Hacein-Bey-Abina et al., 2003c; AFSSAPS, 2005).

Insertional mutagenesis is an inherent risk associated with integrating retroviral vectors used in gene therapy, highlighted by the severe adverse events observed in the French SCID-X1 trial (Hacein-Bey-Abina et al., 2003b; Hacein-Bey-Abina et al., 2003c). Insertional activation of growth-promoting genes has since also been reported in a gene therapy trial for X-linked chronic granulomatous disease (X-CGD), however in this instance, the *in vivo* expansion of the insertionally activated cell clones is thought to have attributed to therapeutic benefit of the two affected patients (Ott et al., 2006). Whilst retroviral integration was initially considered an essentially random process, the completion of the human genome sequence has led to the identification of integration site preferences of lentiviral and gammaretroviral vectors. Studies in immortalised cell lines and in cells derived from transduced simian HSCs *in vivo*, determined the preferential integration of MLV-based gammaretroviral vectors near transcriptional start sites, whilst lentiviral vector integrants (derived from either HIV-1 or simian immunodeficiency virus) were located predominantly within transcriptional units (Schroder et al., 2002; Wu et al., 2003; Mitchell et al., 2004; Hematti et al., 2004). These studies indicate that the integration preferences of retroviral vectors may be independent of cell-type; however the targeted coding regions may be dependent on the set of genes expressed in the particular transduced cell type.

One advance in vector technology is the self-inactivating (SIN) design (Yu et al., 1986); the duplication of the LTR promoter-enhancer elements in retroviral vectors following reverse transcription may increase the risk of insertional mutagenesis, therefore enhanced safety may be achieved by a SIN deletion within the U3 region of the 3' LTR, leading to inactivation of both LTR sequences following entry into the host cell. Evaluation of these vectors in an *in vitro* toxicity assay demonstrated their capacity to immortalise murine haematopoietic cells, by insertional activation of growth-promoting genes, to be significantly reduced as compared to their LTR-regulated counterparts (Modlich et al., 2006). Expression of the transgene in SIN vectors is therefore regulated by an internal promoter, the selection of which confers a further degree of safety to this vector design, since a less-potent or even tissue-specific promoter may be incorporated into the vector backbone, thus further decreasing the likelihood of insertional mutagenesis and potentially restricting transgene expression to relevant tissues only.

The SRS II series of self-inactivating gammaretroviral vectors for SCID-X1 were developed to incorporate advances in vector technology and include the deletion of all known promoter-enhancer sequences within the U3 region of the 3' LTR (Kraunus et al., 2004). Whilst the LTR-regulated vector used in SCID-X1 clinical trials retained portions of MLV *gag-pol* coding sequence to enable splicing and thus enhanced transgene expression, the SIN vectors are devoid of any gammaretroviral coding regions so that the probability of recombination events leading to replication competent retrovirus (RCR) production is decreased. Additional features of these vectors include a Rous sarcoma virus (RSV) promoter in the 5' LTR and the incorporation of a Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to increase vector titre (Schambach et al., 2006d; Schambach et al., 2006a). The internal promoters, consisting of either the U3 region of the murine Spleen focus forming virus (SFFV) or the short form of the human elongation factor 1α promoter (EFS), are located immediately upstream of the γc transgene and were selected due to their high activity levels in haematopoietic cells, including human $CD34^+$ progenitors (Demaison et al., 2002; Baum et al., 1995; Tumas et al., 1996; Salmon et al., 2000; Dardalhon et al., 2001).

In this study the SRS II series of gammaretroviral vectors encoding the human *IL2RG* cDNA, were assessed both *in vitro* and *in vivo* and compared to the LTR-regulated vector, MFG γc, used in both the French and UK clinical gene therapy trials for SCID-X1 (chapter three). Both SRS II SF γc and SRS II EFS γc, incorporating the SFFV and EFS internal promoters respectively, were demonstrated capable of transducing both fibroblastoid and SCID-X1 lymphoid cell lines and efficiently expressing the human γc transgene to detectable levels on the cell surface. Furthermore, the SIN gammaretroviral vectors were able to restore the T and B lymphoid differentiation potential of *Il2rg*$^{-/-}$ murine lin^{-} progenitors *in vitro* post-transduction, and to equivalent levels to those achieved with the clinical vector, MFG γc. Immunological reconstitution of the SCID-X1 murine model following engraftment of γc-deficient HSCs transduced with LTR-regulated gammaretroviral vectors encoding the human *IL2RG* cDNA has been previously described (Lo et al., 1999; Aviles Mendoza et al., 2001). Therefore, to assess the feasibility of gene therapy of SCID-X1 using the SRS II series of self-inactivating gammaretroviral vectors, *in vivo* reconstitution experiments using the SCID-X1 murine model were performed. T and NK cell reconstitution post-gene therapy with either of the SIN gammaretroviral vectors encoding γc was achieved to the same levels as for MFG γc. Furthermore, restoration of humoral immunity in transplanted animals appeared more efficient for SRS II EFS γc than for the LTR-regulated vector. The selective proliferative advantage of transduced, γc-corrected HSCs in the SCID-X1 setting was evident by the negligible proviral copies detected in sorted myeloid populations from reconstituted mice, as compared to the low proviral copy numbers detected in sorted lymphoid populations. Maintaining low proviral copy number is desirable with regards to safety of the gene therapy procedure by minimising the number of potentially hazardous integration events. That cellular and humoral reconstitution was observed in these animals following gene therapy with low proviral copy number (less than one in sorted lymphocytes) and in the setting of only sub-lethal irradiation, indicates that phenotypic correction of the disease is achievable by gene therapy with a low vector dose. Immunological reconstitution observed in secondary transplant recipients demonstrated that pluripotent haematopoietic stem cells were successfully targeted by the SIN vectors during the initial transduction protocol and that both the SFFV and EFS promoters remained active over an extended period of time *in vivo* (approximately seven months for the EFS promoter and approximately

twelve months for the SFFV promoter). A requirement for SCID-X1 gene therapy is that the promoter remains active over the duration of the patient's lifespan and not provoke an immunological response *in vivo* that may result in transgene silencing, such has been observed for vectors incorporating the Cytomegalovirus (CMV) promoter (Qin et al., 1997). These results demonstrate that the SIN vector design in the context of a potent viral or endogenous promoter, whilst potentially increasing vector safety, does not impact on efficacy of immune reconstitution following gene therapy of the SCID-X1 murine model.

Failure of gene therapy in two older treated SCID-X1 patients has been reported (Thrasher et al., 2005), indicating a potential time-dependence on the capacity to re-initiate thympoiesis. Results gained from reconstitution experiments in older recipient mice support this theory (chapter three). Five out of six recipient *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice transplanted at twelve weeks of age failed to reconstitute the T cell compartment, whilst B and NK cell populations were detected in both the spleens and bone marrow. In contrast, only one out of twenty-one recipient mice transplanted at between six to ten weeks of age failed to develop T cells post-gene therapy. Analysis of the older transplanted mice in which T lymphopoiesis failed to develop revealed an absence of detectable thymic organs. The integrity of the thymic microenvironment is dependent upon the presence of developing thymocytes such that the absence of ongoing thymopoiesis results in potentially irreversible disorganisation of the thymic architecture (van et al., 2000; Hollander et al., 1995). It is therefore likely that in the older treated SCID-X1 patients and the older transplanted *Il2rg*$^{-/-}$*Rag2*$^{-/-}$*c5*$^{-/-}$ mice, T cell development failed post-gene therapy due to the prior breakdown of the thymic microenvironment.

Lentiviruses rely on active transport of the pre-integration complex though the host cell nuclear membrane and thus vectors derived from these viruses offer the advantage of being able to transduce non-dividing cells (Sherman and Greene, 2002). This property is particularly desirable for the transduction of quiescent haematopoietic stem cells, enabling shorter *ex vivo* transduction protocols without the requirement for pre-stimulation and thus potentially avoiding loss of progenitor pluripotency and/or engraftment capabilities. These vectors have consequently been widely used in gene

transfer studies (Vigna and Naldini, 2000) and the development of second and third generation vectors, in which the accessory genes are abolished and the required coding genes are provided *in trans* during vector production, have increased the safety of such vectors (Dull et al., 1998; Zufferey et al., 1998).

A series of lentiviral vectors derived from HIV-1 were developed for the treatment of SCID-X1. As for the gammaretroviral series of vectors, the lentiviruses incorporate the self-inactivating design and either the SFFV or EFS internal promoters for regulation of γc transgene expression. Transduction of a SCID-X1 T lymphoid cell line with these vectors enabled restoration of functional γc-containing IL-2 receptor complexes and the SINLV EFS γc vector, incorporating the EFS promoter, restored the lymphoid differentiation potential of murine γc-deficient lin^- progenitor cells *in vitro* post-transduction (chapter five). Results for the SINLV SF γc vector, incorporating the SFFV promoter, were not obtained for the latter assay since the vector preparation used appeared toxic to the primary murine cells, a problem associated with VSV-G pseudotyped vector supernatants. High transduction levels of murine *Il2rg*$^{-/-}$ lin^- cells were observed following an overnight transduction protocol with the SINLV SF γc vector and the transduced progenitors successfully reconstituted T and B lymphoid lineages in three out of four transplanted mice (chapter five). Gene therapy using the SINLV SF γc vector appeared less efficient than for the gammaretroviral vectors; the spleens from the SINLV SF γc reconstituted mice remained hypocellular five months post-transplantation and immunoglobulin isotype switching remained impaired. Furthermore, both single and double-positive T cells were undetectable in the recovered thymi from these animals, potentially due to degeneration of these organs as a result of an absence of seeding by T cell progenitors. This might have occurred had transduced pluripotent haematopoietic stem cells failed to engraft post-transplantation. It was postulated that the overnight transduction protocol used in this experiment, as compared to the five-day protocol required for gammaretroviral transduction, might result in an increased pool of transduced, pluripotent progenitors prior to injection into the recipient mice; hence these results are unexpected. There are several possible explanations for these data however; firstly, it is possible that the gammaretrovirally-treated mice received an increased number of transduced, pluripotent progenitors due to the prolonged transduction protocol, involving forty-eight hours pre-stimulation,

having expanded this primitive cell population. Consequently, the proportion of lentivirally transduced progenitors in the setting of only sub-lethal irradiation may have been insufficient for the equivalent, prolonged phenotypic correction seen with SRS II SF γc and SRS II EFS γc. It is also possible that the lentiviral supernatant used in this experiment triggered the differentiation of the murine lin$^-$ cells during the *ex vivo* transduction protocol, so that an increased proportion of committed lymphoid precursors engrafted in the recipient mice. The lentiviral vector used in this experiment was pseudotyped with the VSV-G envelope and concentrated by ultracentrifugation, hence it is possible that cellular debris and components of the media used to culture the transfected 293T cells for viral production were also present in the lentiviral preparation. It must be noted however that prior to ultracentrifugation, lentiviral supernatants were filtered through 0.22μm sterile filters, hence any contaminating particles would be extremely small in diameter.

The use of tissue-specific promoters in the context of SIN retroviral vectors may act as an additional safety feature by restricting transgene expression *in vivo* to the required tissues for therapeutic benefit only and hence avoid aberrant expression that may potentially result in cellular transformation. This is particularly desirable with regards to cancer gene therapy whereby the expression of toxic gene products in healthy tissues must be prevented. Much research has therefore focussed on the development of viral vectors incorporating tissue-specific regulatory elements and progress has included the use of the α1-antitrypsin promoter and the human Wiskott Aldrich Syndrome protein (WASP) promoter for liver and haematopoietic-specific expression respectively (Kramer et al., 2003; Dupre et al., 2006). For gene therapy of SCID-X1, restriction of human γc expression to cells of haematopoietic origin only might confer a further degree of safety to a SIN retroviral vector. The human *IL2RG* evolutionarily-conserved regulatory sequences were consequently identified and incorporated into SIN lentiviral vectors upstream of γc or eGFP transgenes (chapter four). A further desirable property of utilising such a promoter is physiological transgene expression *in vivo*, particularly in the context of HSC gene therapy, ensuring regulation of γc expression to the appropriate levels during differentiation.

Previous studies have characterised the region immediately upstream of the human and murine *IL2RG* genes to contain promoter activity and demonstrated haematopoietic-specificity of these sequences when regulating luciferase expression in transfected cell lines (Noguchi et al., 1993a; Ohbo et al., 1995; Markiewicz et al., 1996; Cao et al., 1993). Analysis of the 1.24 kb of sequence immediately upstream of the human *IL2RG* translational start site in the context of a lentiviral vector regulating eGFP transgene expression (SINLV γcP eGFP) yielded contradictory results *in vitro* (chapter four). Transgene expression was detected by flow cytometry in all haematopoietic and non-haematopoietic cell lines transduced with SINLV γcP eGFP, however analysis of transgene expression relative to that from the SFFV promoter in each of the cell lines tested revealed the highest relative *IL2RG* promoter activity in the T and B lymphoid cell lines. The discrepancy between these results and those from published studies may reflect the differences between plasmid and lentivirus-based assays; whilst plasmids are maintained episomally, integrated lentiviral vectors may be subjected to enhanced or decreased expression levels due to neighbouring endogenous regulatory sequences. Furthermore, despite the vectors used in this study being of the self-inactivating design, it has been reported that SIN LTR sequences retain a degree of promoter activity and thus the transgene expression observed in the non-haematopoietic cell lines transduced with SINLV γcP eGFP may have been regulated by non-specific viral promoter-enhancer elements within the LTR (Logan et al., 2004; Hanawa et al., 2005). Injection of SINLV γcP eGFP into neonatal mice however demonstrated tissue-specificity of the *IL2RG* promoter *in vivo*; transgene expression in three SINLV γcP eGFP injected mice was restricted to the spleens in all animals, despite detection of proviral DNA in both the livers and spleens by PCR (data not shown) and published observations that the liver is primarily targeted by lentivirus injected using this technique (VandenDriessche et al., 2002; Kobayashi et al., 2005). These results underline the importance of *in vivo* models for comprehensive assessment of regulatory sequences (chapter four).

The lentiviral vector incorporating the *IL2RG* promoter upstream of the γc transgene (SINLV γcP γc) efficiently transduced a SCID-X1 T lymphoid cell line and reconstituted functional IL-2 receptor complexes on the cell surface (chapter five). In contrast to SINLV EFS γc however, SINLV γcP γc transduced murine *Il2rg*$^{-/-}$ lin^{-} cells

retained their inability to undergo T or B lymphoid differentiation *in vitro* and behaved in the much the same way as the mock-transduced *Il2rg*$^{-/-}$ progenitors. Analysis of this vector *in vivo* revealed a similar result; despite lin$^-$ transduction levels of approximately 40%, the SINLV γcP γc transduced progenitors failed to restore lymphopoiesis in the murine model of SCID-X1 and proviral DNA was undetectable in the bone marrow and spleens of two out of three transplanted animals. The absence/low levels of integrated provirus in the SINLV γcP γc mice implies the lack or low levels of engraftment and proliferation of transduced lin$^-$ cells post-injection, thus explaining the failure of immune reconstitution post-gene therapy. These results are difficult to interpret since data from SINLV γcP eGFP transduced *Il2rg*$^{-/-}$ lin$^-$ cells confirms that the promoter is active in these murine progenitors. Furthermore, analysis of promoter specificity following injection of SINLV γcP eGFP into neonatal mice demonstrated promoter activity in splenocytes *in vivo* (figure 4.9). It is therefore likely that the *IL2RG* promoter incorporated into the lentiviral vector is silenced *in vivo*, potentially at an early stage of differentiation, leading to the loss of proliferative advantage conferred upon γc-expressing cells in this model. Furthermore, had only low numbers of pluripotent HSCs with self-renewal capacity been initially transduced using the overnight transduction protocol (as hypothesised for SINLV SF γc reconstitution experiments) any engrafted committed lymphoid progenitors would have been lost over time, thus explaining the lack of detectable proviral sequence in two out three mice. It is possible that silencing may have occurred due to differences in the human and murine *IL2RG* regulatory sequences, a hypothesis that would be confirmed by transduction and *in vitro* differentiation of human SCID-X1 CD34$^+$ cells. It must also be noted however that failure of immune reconstitution in these mice may have been simply due to the SINLV γcP γc transduction levels being lower than required for phenotypic correction in this model, particularly in the setting of sub-lethal irradiation.

One abstacle to successful long-term correction of inherited disorders by gene therapy is transcriptional silencing of retroviral vectors, in particular following the transduction of primitive cell types such as HSC (Challita and Kohn, 1994). Retrovirus vector transgene silencing has been associated with cytosine methylation of CpG dinucleotides and consequent histone deacetylation leading to chromatin condensation

(Pannell and Ellis, 2001) as observed in a clinical gene therapy trial for CGD in which silencing of the therapeutic transgene is coupled with methylation of the SFFV LTR promoter sequences (M.Grez personal communication). To counteract silencing, modified retroviral vectors, such as the SRS II series of SIN gammaretroviral vectors used in this study, containing deletions or mutations of viral silencing elements have been developed; in addition progress has been made with the inclusion of insulator elements, such as the hyper-sensitive site 4 (HS4) of the chicken β-globin locus, into gene therapy vectors (Recillas-Targa et al., 2004). These elements are being exploited to reduce gene expression variability seen with integrated vectors due to chromosomal position effects (Puthenveetil et al., 2004); moreover, the enhancer blocking activity of insulator elements may protect endogenous genes from insertional activation (Burgess-Beusse et al., 2002). More recently, we have shown that the "ubiquitously-acting chromatin opening element" (UCOE) from the human *HNRPA2B1-CBX3* genes (Antoniou *et al.*, 2003; Williams *et al.*, 2005) is able to provide reproducible, therapeutically relevant and stable transgene expression from within a lentiviral vector context and therefore shows great promise as part of future developments to provide efficacious and safe gene therapy (Zhang et al., 2007).

Another strategy to improve upon vector safety is the incorporation of suicide genes such as thymidine kinase that permits the elimination of transduced cells in the event of neoplastic transformation. In a murine model of insertional mutagenesis, this approach was recently demonstrated effective in eliminating leukaemia (Blumenthal et al., 2007). Progress in the mapping of integration site preferences allows identification of viral vectors that might potentially be less likely to cause insertional mutagenesis, such as those derived from avian sarcoma leukosis virus (ASLV) and human foamy virus, which demonstrate little bias for integration within transcriptionally active regions (Narezkina et al., 2004; Mitchell et al., 2004; Trobridge et al., 2006; Nowrouzi et al., 2006). A desirable property of a gene therapy vector would be the ability to integrate into a precise location within the human genome, thus mediating efficient transgene expression without perturbing neighbouring genes. The use of bacteriophage integrases such as phi C31, chimeric transposases and the AAV Rep protein are addressing this issue (Ishikawa et al., 2006; Liu et al., 2006; Maragathavally et al.,

2006), however more research is needed before these systems might be suitable for clinical application.

The results gained from this study indicate that self-inactivating gammaretroviral vectors for the treatment of SCID-X1 are able to reconstitute the immune defect in the SCID-X1 murine model to equivalent levels as the LTR-regulated vector used in clinical trials for gene therapy of this disease. Furthermore, improved humoral immunity in the murine model was observed following gene therapy with the SRS II EFS γc vector incorporating a human house-keeping gene promoter. SIN lentiviral vectors encoding the human γc transgene were found to efficiently correct defective γc-dependent signalling *in vitro* following transduction of a human SCID-X1 T cell line, however *in vivo* immune reconstitution of the SCID-X1 murine model with SINLV SF γc appeared less effective than for the SIN gammaretroviral vectors. On the basis of these experiments, and data obtained in Professor Christopher Baum's laboratory in which a SIN gammaretroviral vector incorporating the EFS promoter was significantly less mutagenic than an equivalent vector containing an internal SFFV promoter (unpublished observations), it has been proposed that the SRS II EFS γc vector replace MFG γc in the clinical trial of gene therapy for SCID-X1 at Great Ormond Street Hospital. Long-term *in vivo* safety studies have therefore been initiated, as well as pilot scale production of good manufacturing practice (GMP) GALV pseudotyped vector. The self-inactivating design of this vector, together with the less potent, endogenous EFS promoter, will potentially decrease the likelihood of insertional mutagenesis following transplantation of gene-corrected cells *in vivo* whilst maintaining the therapeutic efficacy thus far seen in this clinical trial.

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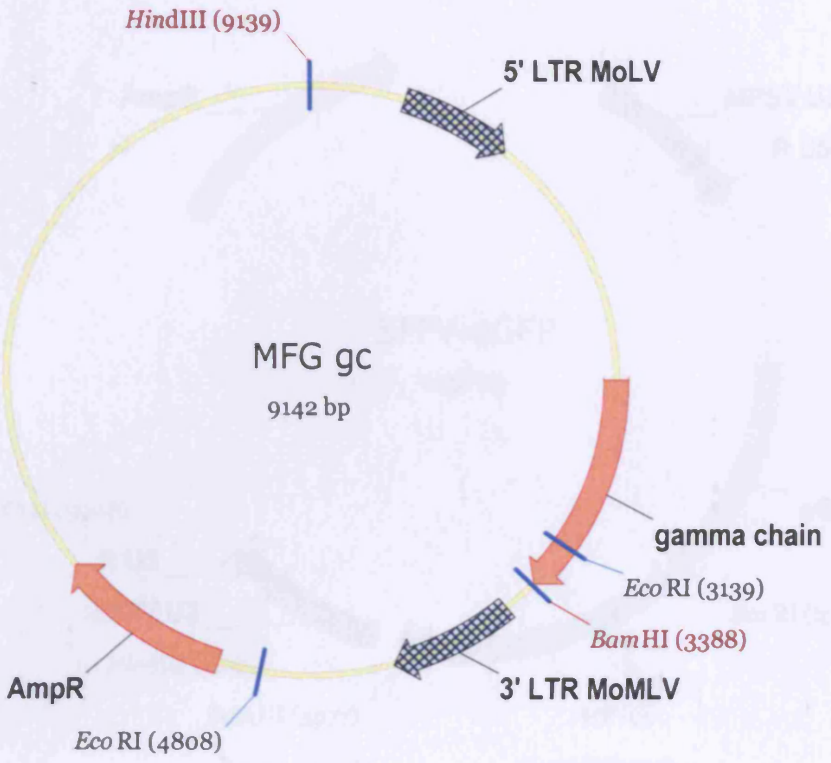
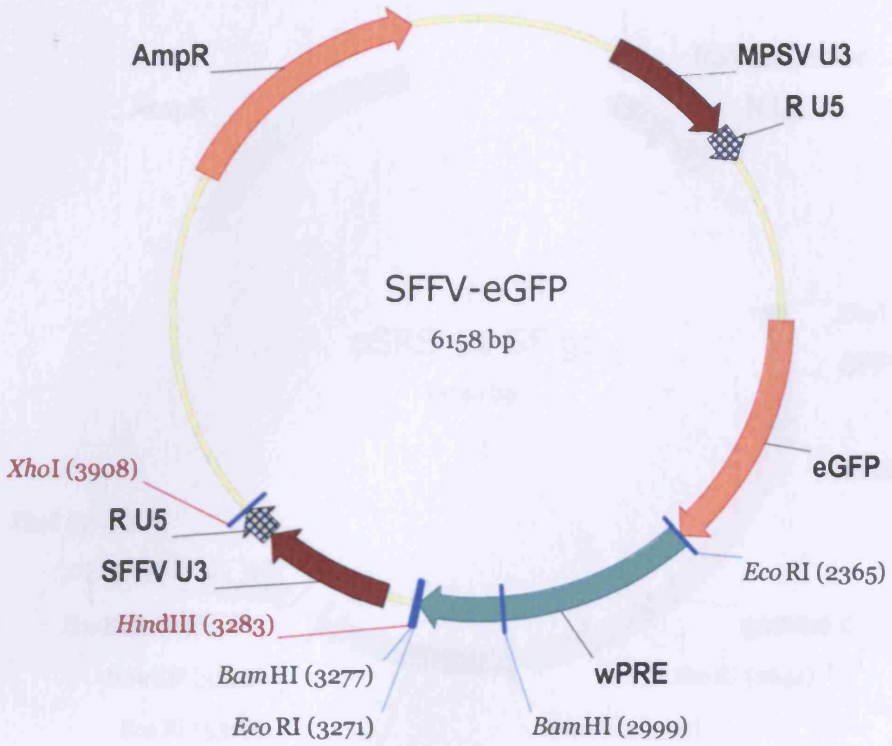
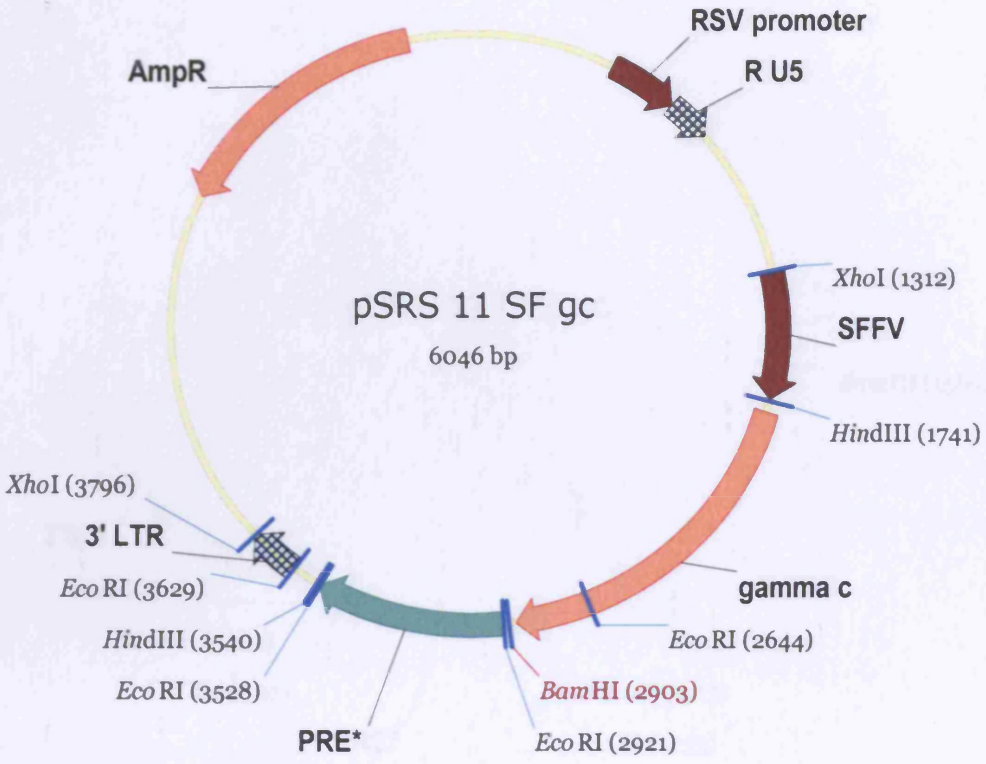
Appendices



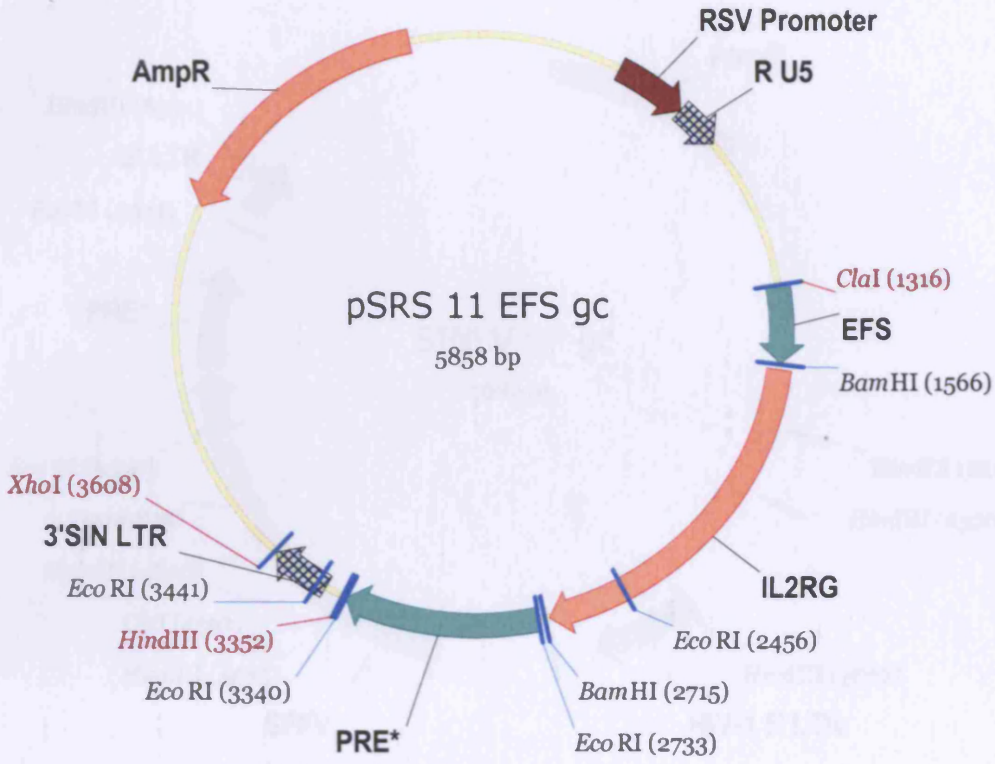
Appendix 1. MFG γc gammaretroviral vector map.



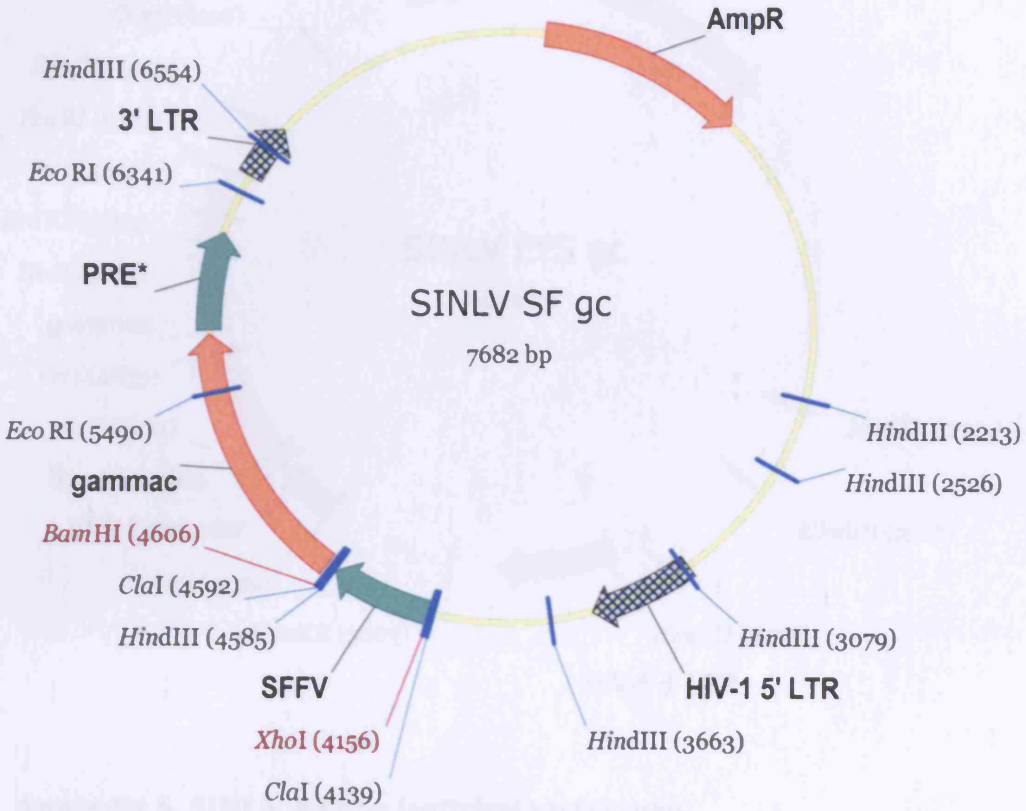
Appendix 2. SFFV-eGFP gammaretroviral vector map.



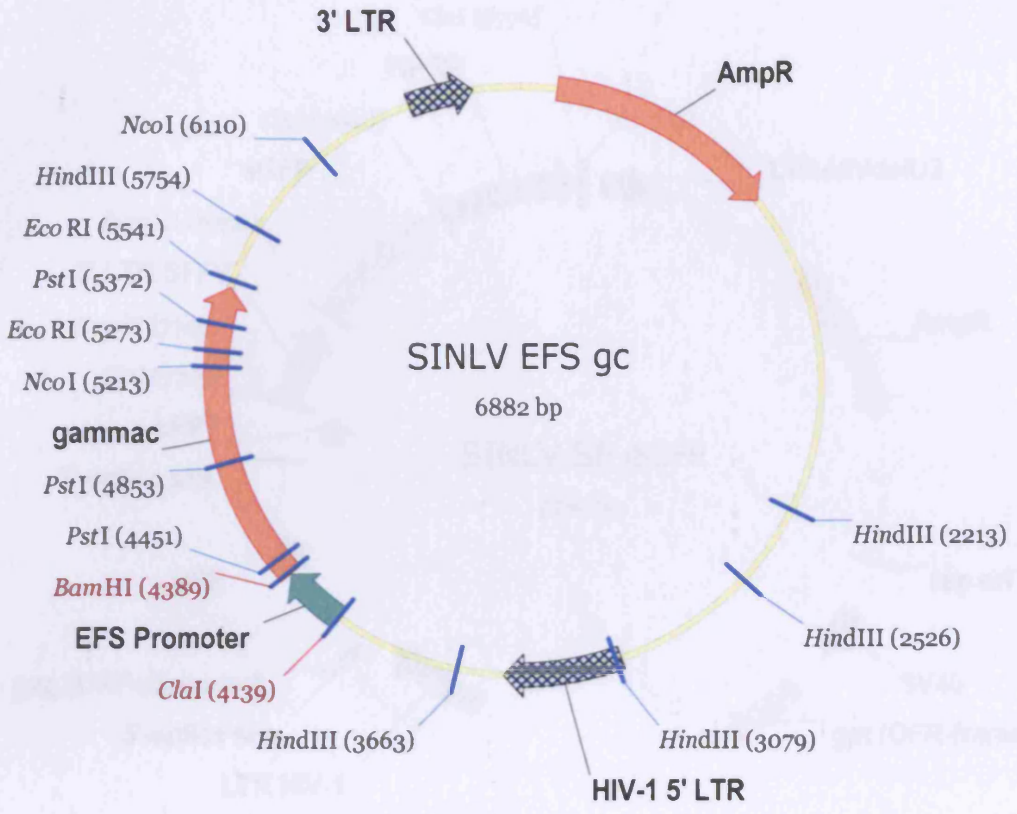
Appendix 3. SRS II SF γc gammaretroviral vector map.



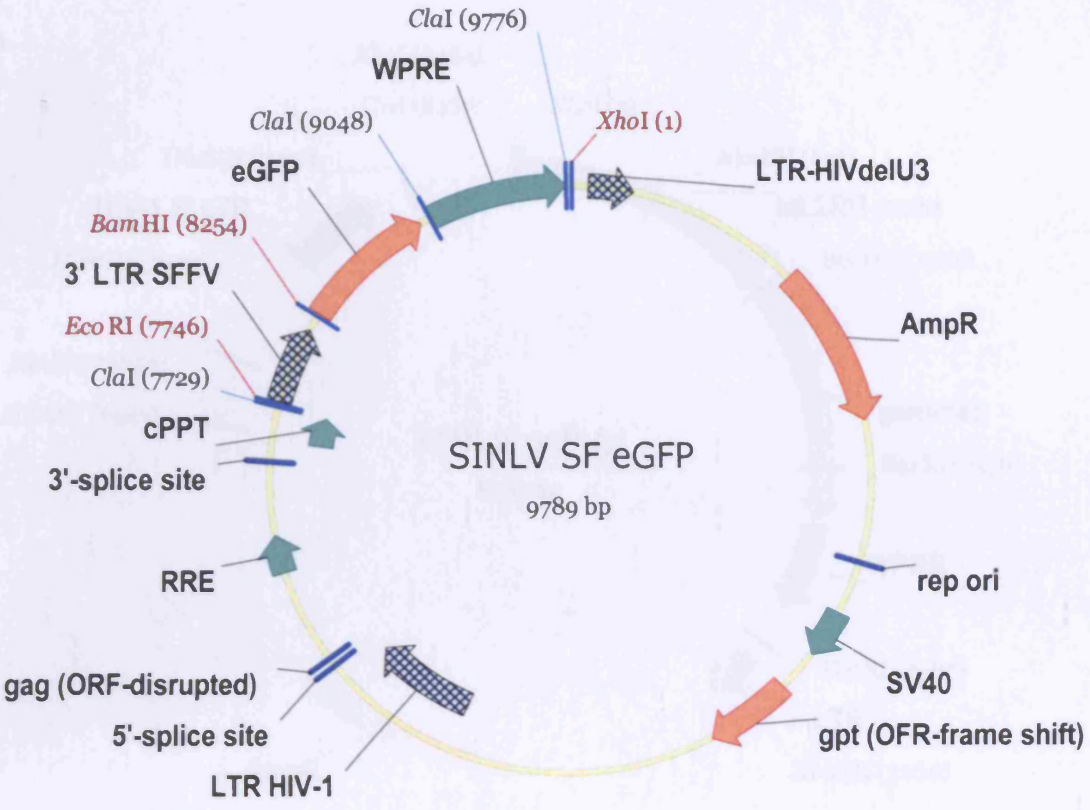
Appendix 4. SRS II EFS γc gammaretroviral vector map.



Appendix 5. SINLV SF γc lentiviral vector map.

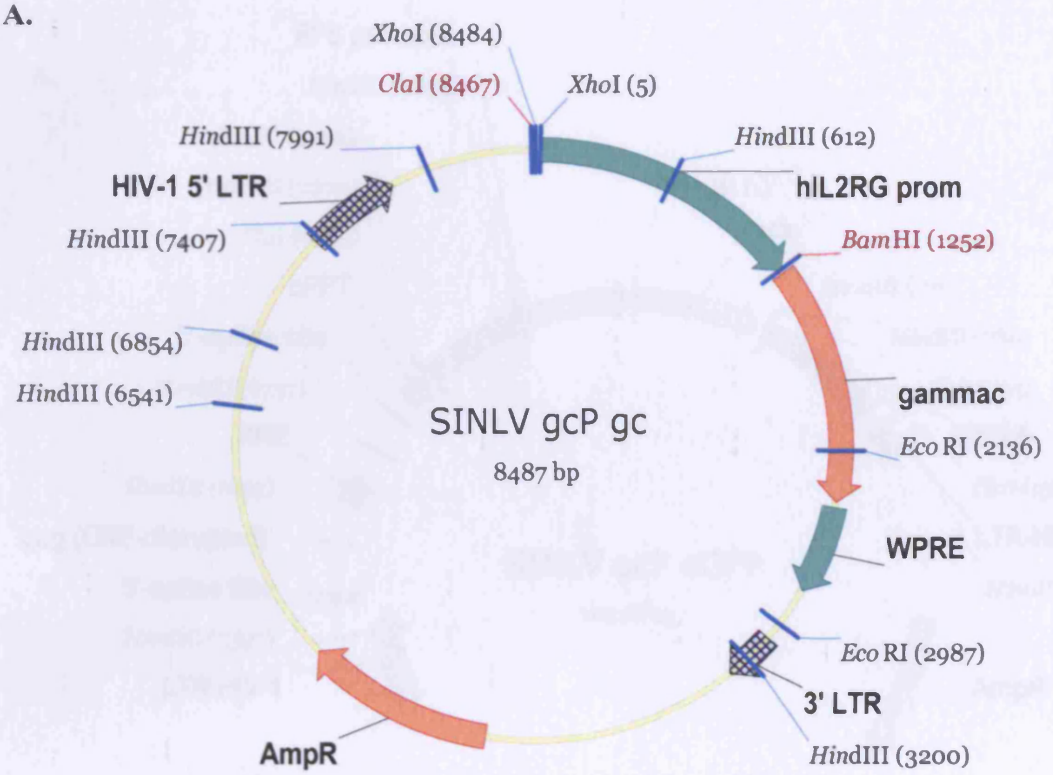


Appendix 6. SINLV EFS γc lentiviral vector map.



Appendix 7. SINLV SF eGFP lentiviral vector map.

A.



B.

***Xho*I**

ctcgagctatccccctctccatcttggtaaatttagttacttcctcttctgtgctcacatactttgtagtatctctacatttatgctata
ggacttgttacactatgttgtattacttgtttatgtcttccccacttttctgtgagtgtctagaaatatgaggatgtcttgttggtctattt
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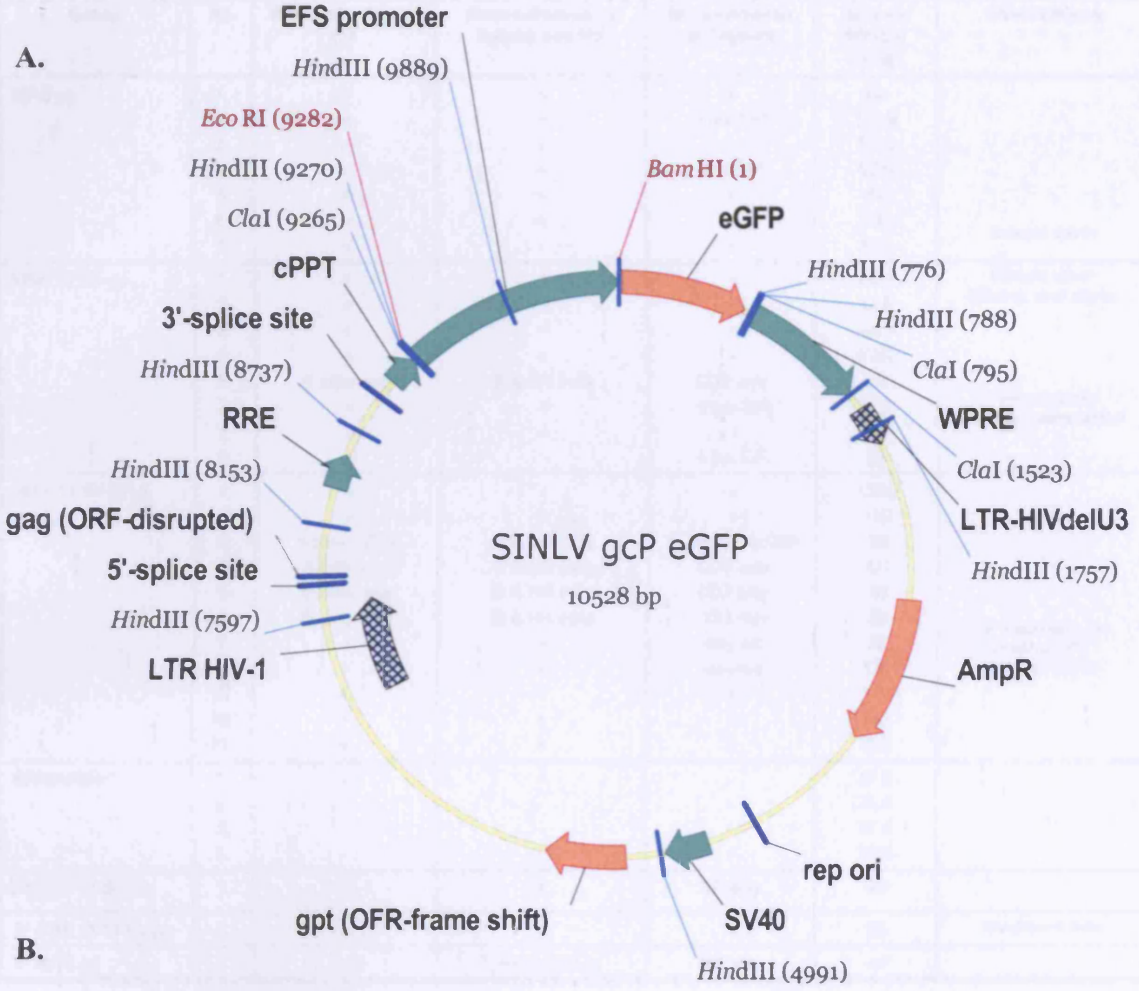
gggtagtgggtgagggacccaggttcctgacacagacagactacacccagggaatgaagagcaagcgcc**ggatcc**

***Bam*HI**

Appendix 8. (A) SINLV γcP γc lentiviral vector map. (B) Sequence of cloned *IL2RG* promoter.

A.



B.

***Eco*RI**

gaattcctatccccctctccatcttggtaaatttagttacttcctcttctgtgctcacatactttgtagtatctctacatttatgctatag
gacttgttacactatgttgtattacttgtttatgtcttccccactttctgtgagtgtctagaaatatgaggatgtcttgttggtctatttc
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ggtagtgggtgagggacccaggttcctgacacagacagactacacccagggaatgaagagcaagcgcc**ggatcc**

***Bam*HI**

Appendix 9. (A) SINLV γcP eGFP lentiviral vector map. (B) Sequence of cloned *IL2RG* promoter.

Group	No.	Reconstitution of PB	Reconstitution of Spleen and BM	Reconstitution of Thymus	Splenic Weight (mg)	Observations
MFG γc	1.	+	+	+	64	
	2.	+	+	+ (no DP)	141.8	
	3.	-	-	-	51.0	
	4.	+	+	+(no DP)	90.5	
	5.	+	+	+	160	
	6.	+	+	+	330	
	7.	+	+	+	120	Enlarged spleen
SRS 11 SF γc	1.	+	+	+	390.9	Enlarged spleen
	2.	+	+	+	42.6	Wasting, small spleen
	3.	+	+	+	108.4	
	4.	+	+	+	103.6	
	5.	B cells only	B & NK cells	CD3 only	nd	
	7.	+	+	+ (no DP)	280	Enlarged spleen
	8.	+	+	+	100	Wasting, anaemic spleen
	9.	+	+	+ (no DP)	80	
SRS 11 EF1α γc	1.	+	+	+	138.9	
	2.	+	+	+	150	
	3.	+ (few CD3s)	B & NK cells	v. few SP & CD3	50	
	4.	B cells only	B & NK cells	CD3 only	60	
	5.	B cells only	B & NK cells	CD3 only	40	
	6.	B cells only	B & NK cells	CD3 only	50	
	7.	+	+	athymic	70	NK expansion in BM
	8.	+	+	athymic	130	,Wasting, colitis?
	9.	+	+	+	110	Wasting, colitis?
	10.	+	+	+	160	
	11.	+	+	+	250	
SFFV-eGFP	1.	-	-	-	47.8	
	2.	-	-	-	25.3	
	3.	-	-	-	37.6	
	4.	-	-	-	30.9	
2° SRS 11 SF γc	1.	nd	+	SP only	40	
2° SRS 11 EF1α γc	1.	nd	+	+	40	Sacrificed at 8wks
2° MFG γc	1.	nd	T, NK & B220lo	SP only	nd	

Appendix 10. Summary of mice reconstituted with gammaretrovirally transduced cells.

Group	No.	Reconstitution of PB	Reconstitution of Spleen and BM	Reconstitution of Thymus	Splenic Weight (mg)	Observations
SINLV SF γc	1.	+	+	SP only	90	
	2.	+	+	SP only	60	Sick, anaemic spleen
	3.	B cells only	Bs & NKs in spleen	-	50	Sick, anaemic spleen
	4.	+	-	-	-	Died before analysis
SINLV γcP γc	1.	-	B220+	-	70	
	2.	-	B220+	-	100	
	3.	-	B220+	-	98	
SINLV γcP eGFP	1.	-	B220+, small eGFP+ in BM	-	40	
	2.	-	B220+	-	60	
	3.	-	B220+	-	60	
	4.	-	B220+	-	50	
MOCK	1.	-	B220+	-	40	
	2.	B220lo	B220+	-	70	

Appendix 11. Summary of mice reconstituted with lentivirally transduced cells.