Gene transfer for SCID-X1 using a self-inactivating (SIN) gammaretroviral vector

Protocol short title: Gene transfer for SCID-X1

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IMP: pSRS11.EFS.IL2RG.pre* retroviral vector

A multi-institutional phase I/II trial evaluating the treatment of SCID-X1 patients with retrovirus-mediated gene transfer

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The Principal Investigator at each collaborating clinical site should print, sign, and date at the indicated location below. A copy should be kept for your records and the original signature page sent to the Data Management Coordinating Center.

I confirm that I have read the above protocol and the corresponding consent form in the latest version. I understand them, and I will work according to the principles of Good Clinical Practice (GCP) as described in the United States Code of Federal Regulations (CFR) – 21 CFR Parts 45, 50, 56, and 312, and the International Conference on Harmonization (ICH) document "Guidance for Industry – E6 Good Clinical Practice: Consolidated Guidance", dated April 1996. Further, I will conduct the study in keeping with local, legal, and regulatory requirements.

As the Principal Investigator, I agree to conduct "A multi-institutional phase I/II trial evaluating the treatment of SCID-X1 patients with retrovirus-mediated gene transfer". I agree to carry out the study by the criteria written in the protocol and understand that no changes can be made to this protocol without written permission of the NIAID.

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Date: ----------

ABBREVIATIONS

BCG	Bacillus Calmette-Guerin
BMT	Bone Marrow Transplant
CBC	Complete Blood Count
CBER	Center for Biologics Evaluation and Research
CDR3	Complementarity Determining Region-3
CFCs	Colony Forming Cells
CFR	Code of Federal Regulations
CFU	Colony-Forming Unit
CGD	Chronic Granulomatous Disease
CIBMTR	The Center for International Blood and Marrow Transplant Research
CMCF	Cell Manipulation Core Facility
CNS	Central Nervous System
CpG	Cytosine Phosphate Guanine
CRF	Case Report Form
CSM	Committee for Safety of Medicines
CTC	Common Toxicity Criteria
CTIP	Clinical and Translational Investigator Program
DAIT	Division of Allergy, Immunology and Transplantation
DFCI	Dana-Farber Cancer Institute
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSMB	Data and Safety Monitoring Board
EBMT	European group for Blood and Marrow Transplantation
EBV	Epstein Barr Virus
EF-1α	Elongation factor-1α
EGFP	Enhanced Green Fluorescent Protein
ESID	European Society for Immune Deficiency
FACT	Foundation for the Accreditation of Cell Therapy
FDA	Food and Drug Administration
GALV	Gibbon Ape Leukemia Virus
GCP	Good Clinical Practice
GeMCRIS	Genetic Modification Clinical Research Information System
GMP	Good Manufacture ring Practice
GOSH	Great Ormond Street Hospital
GTAC	UK Gene Therapy Advisory Committee
GvHD	Graft-versus-host disease
HEK	Human Embryonic Kidney
HLA	Human leukocyte antigen
HSCs	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell transplantation
ICH	International Conference on Harmonization
IDE	Investigational Device Exception
IL-2RG	Interleukin-2 Receptor Gamma
IMP	Investigational Medicinal Product
IRB	Institutional Review Board
JAK	Janus Kinase
LAM	Linear Amplification Mediated
LSS	Lymphocyte Subsets
LTC-ICs	Long Term Culture-Initiating cells

ABBREVIATIONS

LTR	Long Terminal Repeat
MMRD	Mismatched Related Donor
MOI	Multiplicity of Infection
MUD	Matched Unrelated Donor
NCI	National Cancer Institute
NIAID	National Institute of Allergy and Infectious Diseases
NIH/OBA	National Institute of Health - Office of Biotechnology Activities
NK	Natural Killer
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
PI	Principal Investigator
PID	Primary Immune Deficiency
PTC	Points To Consider
pUC	Plasmid University of California
QACT	Quality Assurance for Clinical Trials Office
RCR	Replication Competent Retrovirus
SAE	Serious Adverse Event
SAS	Statistical Analysis Software
SCETIDE	Stem Cell Transplantation for Immunodeficiencies
SCID	Severe Combined Immunodeficiencies
SCID-X1	X-linked Severe Combined Immunodeficiency
SFFV	Spleen Focus Forming Virus
SIN	Self-inactivating
SOPs	Standard Operating Procedures
SRC	SCID-repopulating cells
SRCs	SCID-repopulating cells
STAT	Signal Tranducers and Activators of Transcription
T-ALL	T acute lymphoblastic leukemia
TCR	T-cell Receptor
TRECs	TCR Excision Circles
UCLA	University of California at Los Angeles
USP	United States Pharmacopeia
VPF	Vector Production Facility
WBC's	White Blood Cells
WPRE	Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element

Contents

1. Abstract

Severe combined immunodeficiencies (SCID) are a heterogeneous group of inherited disorders characterized by a profound reduction or absence of T lymphocyte function. They arise from a variety of molecular defects which affect lymphocyte development and function. The most common form of SCID is an X-linked form (SCID-X1) which accounts for 40-50% of all cases. SCID-X1 is caused by defects in the common cytokine receptor γ chain (γc), which was originally identified as a component of the high affinity interleukin-2 receptor (IL-2RG), but is now known to be an essential component of the IL-4, -7, -9 -15, and -21 cytokine receptor complexes. Classic SCID-X1 has an extremely poor prognosis without treatment. Death usually occurs in the first year of life from infectious complications unless definitive treatment can be administered. Until the recent advent of somatic gene therapy, hematopoietic stem cell transplantation (HSCT) offered the only curative option for patients with any form of SCID. If a genotypically matched sibling donor is available, HSCT is a highly successful procedure. However a genotypically matched family donor is only available for approximately 30% of patients. For the remaining individuals, alternative donor transplants, principally from matched unrelated (MUD) or haploidentical parental donors have been performed. These approaches are still problematic with toxicity from ablative therapy, graft-versus-host disease and incomplete lymphoid reconstitution. Recent gene transfer trials have documented the efficacy of gene transfer in this disease, albeit with toxicity related to insertional mutagenesis. A new generation of SIN vectors has been developed which lack all enhancer-promoter elements of the LTR U3 region and are also devoid of all gammaretroviral coding regions. A SIN vector expressing the IL-2RG gene, pSRS11.EFS.IL2RG.pre* has been developed and has shown a reduction in mutagenic potential compared to LTR configuration in non-clinical studies. The current study is a phase I/II trial of somatic gene therapy for patients with SCID-X1. Inclusion criteria include patients with a definitive diagnosis of SCIDX1 in whom HLA-matched family donors are unavailable and lack an HLA identical (A,B,C,DR,DQ) unrelated donor OR patients of any age with an active, therapy-resistant infection or other medical conditions that significantly increase the risk of allogeneic transplant. Primary endpoints include immunological reconstitution defined as absolute CD3 cells of >300/μl and PHA stimulation index >15 at 6 months post infusion and the incidence of life-threatening adverse reactions related to the gene transfer procedure.

2. Lay summary of proposal

X-linked severe combined immunodeficiency (SCID-X1) is a disease that runs in families, such that babies are born missing key parts of the immune system. While there are different types of SCID, SCID-X1 only affects boys because the genetic mistake is on the X-chromosome. In normal healthy people special cells in the blood called lymphocytes protect against many viruses and other infectious agents. In SCID-X1, the bone marrow cells which develop into lymphocytes do not grow, and as a result vital lymphocytes are either virtually absent from the blood (T lymphocytes and natural killer cells) or do not work (B lymphocytes). Therefore, affected boys are extremely susceptible to infection. Common viruses which have little effect on normal individuals can cause life-threatening illness. Although drugs such as antibiotics can offer partial protection, untreated boys typically die in the first year of life.

Bone marrow transplantation can cure SCID-X1. When an exact donor match from a brother or sister is available virtually all patients are cured. However, less than a third of affected boys have a fully matched brother or sister, and the chances of success from other donor sources, such as a parent or unrelated people, are not as good. In these situations 20-30% of patients will not survive. When a parent is the donor, about half of patients who do survive are not fully cured because the T lymphocytes are replaced but the B lymphocytes are still broken. When an unrelated person is the donor, the patient typically gets high dose chemotherapy, which kills off the bone marrow and allows full replacement of T and B cells. But these patients often have long term problems related to the chemotherapy. Both transplants from parents and especially from unrelated people can cause graft versus host disease (GvHD), a condition where donor lymphocytes in the transplant recognize the patient's own organs, such as skin and gut, as foreign and cause severe damage. Simply put, transplantation can cure SCID-

X1 much of the time but current therapy is not 100% effective and has short-term and long-term toxicities.

Over the past decade a new treatment has been developed based on our knowledge of the defective gene causing SCID-X1. We can now use genes as a type of medicine that corrects the problem in the patient's own bone marrow cells and allows the development of a new immune system, with or without chemotherapy. The use of gene transfer on the patient's own bone marrow cells results in normal numbers of functional T and B lymphocytes, and avoids the risk of GvHD entirely. Although still experimental in nature, this procedure has been carried out effectively in around 40 patients with different forms of SCID including 20 children with SCID-X1, and most of these children are living healthy lives. Unfortunately, in a few patients the gene therapy vector (the vehicle that carries the new gene into the bone marrow cells) has caused leukemia a few years after treatment because it has accidentally altered the way in which the growth of lymphocytes is normally controlled. Due to scientific advances, the technology now exists to reduce this risk by changing the design of the vector. In this trial we aim to evaluate the treatment of patients with SCID-X1 with a new and safer gene transfer vector.

3. Overall objectives

The objectives of this proposal are to initiate a trial of somatic gene transfer for patients with SCID-X1 in whom HLA-matched family or unrelated donors are unavailable in a timely fashion or for patients deemed not suitable for allogeneic stem cell transplantation due to infection. For this study, CD34+ cells will be purified from harvested patient bone marrow or mobilized peripheral blood, and transduced *ex vivo* using a novel gibbon ape leukaemia (GALV)-virus pseudotyped gammaretroviral vector encoding the human common cytokine receptor gamma chain (γc). The vector has been designed with a self inactivating (SIN) configuration in which expression of the therapeutic gene is regulated by an internal housekeeping gene promoter derived from human elongation factor-1α (EF-1α) gene. These technological features are anticipated to substantially reduce risks of insertional mutagenesis and to retain efficacy.

4. Background: X-linked severe combined immunodeficiency (SCID-X1)

Severe combined immunodeficiencies (SCID) are a heterogeneous group of inherited disorders characterized by a profound reduction or absence of T lymphocyte function. They arise from a variety of molecular defects which affect lymphocyte development and function(1). The most common form of SCID is an X-linked form (SCID-X1) which accounts for 40-50% of all cases. SCID-X1 is caused by defects in the common cytokine receptor γ chain (γc), which was originally identified as a component of the high affinity interleukin-2 receptor (IL-2RG(2), but is now known to be an essential component of the IL-4, -7, -9 -15, and -21 cytokine receptor complexes(3) (Figure 1).

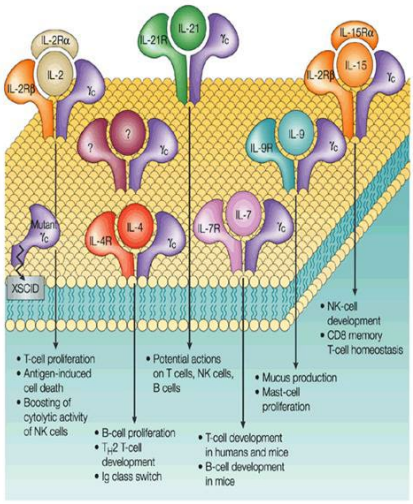


Figure 1. Cartoon depicting participation of γc in multiple cytokine receptor complexes.

The molecular defect in SCID-X1 results in the complete absence of T cell and natural killer (NK) cell development and an as yet uncharacterized defect of B cell function (T-B+NK- SCID). These profound abnormalities in cellular and humoral immune function leave patients extremely susceptible to recurrent and opportunistic infection. The prognosis without treatment is uniformly fatal and affected boys normally die in the first year of life.

4.1 Molecular Pathology of SCID-X1

The SCID-X1 gene locus was first mapped by linkage analysis to Xq12-13(4). In 1993, it was shown by two groups that the *IL2RG* gene was located precisely in the critical region where the SCID-X1 locus had been placed(2, 5, 6). Subsequent analysis of a number of unrelated patients with T-B+NK- SCID demonstrated disease-causing mutations in the *IL2RG* gene(2)*IL2RG* is organized into eight exons and spans 4.5kb of genomic DNA in Xq13.1(2). The coding sequence of 1,124 nucleotides gives rise to a 232 amino acid transmembrane glycoprotein which is expressed constitutively in all lymphoid cells(7, 8). The protein has a number of structural motifs characteristic of cytokine receptor superfamily members. Four conserved cysteine residues are found at the extracellular amino-terminal end; a juxtamembrane conserved extracellular motif, the WSXSW box, found in all cytokine receptors is encoded in exon 5; the highly hydrophobic transmembrane domain of 29 amino acids occupies most of exon 6 and the proximal intracellular domain in exon 7 has a signaling sequence called Box1/Box 2 which has homology to the SH2 domains of Src family tyrosine kinases.

Genetic analysis of boys with T-B+NK- SCID has now identified hundreds of patients with mutations in the *IL2RG* gene. Although mutations have been found in all the exons, they are not evenly distributed. Exon 5 is the site of over 25% of all mutations followed by exons 3 and 4 with 15% each. Only a relatively small number of mutations have been found in exon 8. A number of hotspots for mutation have also been noted within CpG dinucleotides, the most prominent being in exon 5 where 16 independent missense mutations have been reported(9). The most frequently encountered defects are missense mutations, resulting in non-conservative amino acid substitutions, followed by nonsense mutations and together these account for approximately two thirds of all mutations.

The consequences of the different mutations on γc protein expression and function are variable. Missense mutations in the extracellular domain of the protein can result in normal, trace or absent expression of γc but missense defects in the intracellular domains appear to result in intact cell surface protein expression(10), although the intensity of staining by flow cytometry is reduced compared to expression in normal lymphocytes (Filipovich et al, unpublished observations). γc was initially thought only to be a component of the IL-2R complex and thus did not offer a satisfactory explanation for the T-B+NK- immunophenotype of SCID-X1. Subsequent realization that it was also an essential component of the IL-4,-7,-9 –15 and -21 receptor complexes has since allowed a greater understanding of the lineage specific abnormalities. Murine 'knockout' models and *in vitro* cellular studies have shown that functional IL-7/IL-7R signaling is essential for normal T cell development at an early stage in thymopoiesis(11). NK cell development is unaffected in these mice but B cell development is significantly disrupted in contrast to the human phenotype(12). The essential and lineage specific role of IL-7/IL-7R function in human T cell development is further illustrated by the finding of mutations in the IL-7Rα gene in some patients with T-B+NK+ SCID(13). Similar *in vitro* and murine studies have identified the dominant role of IL-15 in NK cell development and survival. IL-15 efficiently promotes NK cell differentiation from bone marrow precursors in humans and mice and maintains the survival of mature NK cells even at low concentrations(14, 15). Mice deficient in IL-15Rα or IL-15 completely lack mature NK cells but show normal B and T cell development(16, 17).

B lymphopoiesis appears to proceed normally in most cases of SCID-X1, but a number of studies suggest that there are intrinsic functional B cell abnormalities. IL-2 and IL-15 fail to induce B cell responses *in vitro* and signaling molecules downstream of the IL-4R receptor (JAK3 and STAT6) are not activated after ligand binding(18, 19). Molecular detail of VDJ recombination in B cells from patients by analysis of complementarity determining region-3 (CDR3) sequences showed that the diversity of CDR3 lengths was characteristic of a normal primary repertoire, indicating that the ability to generate junction diversity is retained although there was little evidence for somatic hypermutation(20). It has also been shown using B cell spectratyping analysis that γc-deficient B cells are unable to class switch and undergo somatic hypermutation even in the presence of adequate T cell help after bone marrow transplantation(21).

The downstream consequences of γc activation have been defined (reviewed in(22, 23). Stimulation of the receptor complex by cytokine results in the heterodimerization of the receptor subunits and tyrosine phosphorylation of JAK3, a cytoplasmic tyrosine kinase which binds specifically to the γc subunit. Tyrosine phosphorylated JAK3 in turn phosphorylates one of the STAT (Signal Tranducers and Activators of Transcription) family of transcription factors which then dimerizes and translocates to the nucleus where it binds to specific sites to initiate transcriptional events. The specificity of γc binding to JAK3 rather than to other JAK molecules is demonstrated by the lack of JAK3 tyrosine phosphorylation in patients with SCID-X1 and also by the identification of mutations in JAK3 in patients with an autosomal recessive form of T-B+NK- SCID(24, 25). This latter finding implies that any disruption to the specific interaction between these two molecules can lead to the T-B+NK- immunophenotype. Further downstream, all γc cytokine stimulation pathways (except IL-4) activate the same members of the STAT family of molecules, STAT 3 and STAT 5, whereas IL-4 activates STAT6(26).

4.2 The pattern of clinical disease

4.2.1 Classical presentations

SCID-X1 is estimated to affect around 1 in 100,000 births although the frequency may be greater in certain geographical areas or ethnic groups. Clinically it is characterized by severe and recurrent infections and a high frequency of opportunistic infections. The clinical presentation in SCID-X1 is similar to patients with autosomal forms of SCID and it is difficult to distinguish between the different forms of SCID on the basis of clinical presentation alone. The mean age at diagnosis for all types of SCID is around 6 months(27) and this most likely reflects the time when the protective effect of

placentally transferred maternal immunoglobulin has diminished and children have been exposed to a range of microorganisms. The most common infective problems are oral candidiasis, respiratory infection due to *Pneumocytis jiroveci*, respiratory syncytial virus and parainfluenza 3, adenoviral infection, persistent diarrhea and failure to thrive. In countries which administer antituberculous vaccination to infants with bacillus Calmette-Guerin (BCG), disseminated infection with BCG has occurred. Live polio vaccine has also caused poliomyelitis and myocarditis but only rarely and this may be due to the continued presence of maternal immunoglobulin at the time of initial vaccination.

4.2.2 Atypical presentation

Atypical forms in terms of both immunological and clinical phenotype have been described. B cell numbers are normal in most, but pedigrees have been described in which B cell development is also affected thus presenting as a T-B- form of SCID(28). In the reported cases the clinical presentation was no different from the typical phenotypes. Certain individuals have also been reported who, despite a mutation in the γc gene, have some residual T cell function and thus present with a less severe clinical phenotype. In these cases, patients present later, have less severe infections and a prolonged clinical course with progressive loss of T and B cell function. In one pedigree, this was associated with a splice site mutation that generated two transcripts: one truncated and one normal sized, which accounted for 80% and 20% of the total γc mRNA respectively. IL-2 binding to high affinity receptor complexes was severely reduced and T cells from affected individuals showed impaired *in vitro* stimulation and a restricted TCR repertoire(29). A missense mutation (R222C) in the extracellular region of the γc was shown to result in preserved expression of the γc, and normal development of T cells, that however failed to respond to IL-2(30). Finally, a few patients have preserved NK cell development(31).

4.2.3 Spontaneous reversion mutations

SCID-X1 patients in whom somatic reversions have occurred provide important information for a gene transfer approach to the treatment of the condition One patient with a normal number of poorly functional T cells, high B cell count, hypogammaglobulinemia, absent NK cells and an X-linked family history has been described in detail(32). B cells from the patient showed absent γc expression and analysis of genomic DNA derived from a B cell line demonstrated a missense mutation in exon 3 of *IL-2RG*. Analysis of monocyte and neutrophil populations also showed the presence of the mutation. However, T cells displayed normal γc surface expression and sequencing revealed wild-type sequence. These results are explained by a reversion of the mutation in a committed T cell precursor giving rise to a pool of mature T cells. Similar findings have been observed in other patients (A. Thrasher, unpublished observations). These cases confirm previous experimental data that γc expression and signaling are essential for T lineage development and suggests that a significant growth advantage was conferred to the reverted T cell precursors(33). Such data also suggests that introduction of γc into lymphoid precursors by gene transfer even at relatively low frequency, could have significant therapeutic benefit.

4.3 Diagnosis of SCID-X1

Prior to the identification of the genetic defects, diagnosis was based on family history and clinical and immunological profile. Linkage analysis and examination of X-inactivation pattern in T cells of female relatives (a unilateral pattern is seen in T and B lymphocytes from female carriers) was used to guide diagnosis and carrier status but could only offer a degree of probability(34). These techniques have largely been replaced by direct analysis of the *IL2RG* gene(35). If a mutation is identified an unambiguous molecular diagnosis can be made. In addition carrier assessment for female relatives can be made with absolute certainty and accurate prenatal diagnosis can be offered. More rapid tests based on the expression patterns and function of the mutant γc are also now available for diagnosis of affected infants. Approximately 65–90% of children have abnormal expression of γc on the surface of

mononuclear cells, allowing confirmation of the molecular diagnosis by flow cytometric analysis of peripheral blood mononuclear cells(10). In infants affected by T-B+NK- SCID who have normal γc expression, further dissection of the signalling pathway can now be undertaken. IL-2 stimulation of mononuclear cells results in tyrosine phosphorylation of JAK3 at specific tyrosine based motifs. A monoclonal antibody directed against phosphotyrosine residues can be used to demonstrate JAK3 activation, so abnormalities in this signalling pathway can be detected at a protein level prior to genetic analysis(36). Similarly, STAT-5 phosphorylation can now also be determined by flow cytometric methodology. The variability in clinical presentation again underlines the need to identify the molecular defect so that earlier referral for definitive therapy by bone marrow transplantation or gene transfer can be made.

4.4 Prognosis and conventional treatment

Classic SCID-X1 has an extremely poor prognosis without treatment. Death usually occurs in the first year of life from infectious complications unless definitive treatment can be administered. Upon diagnosis, patients are started on bacterial, fungal and pneumocystis prophylaxis and immunoglobulin substitution therapy. In some cases fungal prophylaxis is also initiated. In a few atypical cases (see above), patients have been maintained on this regime for a number of years. However, it is generally accepted that in the vast majority of cases, prophylactic therapy is only a means of protecting the child until stem cell transplantation or gene transfer can be performed.

Due to the severity of the condition and the risks associated with infection in the untreated child , recommendations from the European group for Blood and Marrow Transplantation (EBMT)/European Society for Immune Deficiency (ESID) inborn errors working party state that a definitive procedure should be undertaken as soon as possible, and within 3 months of diagnosis (including donor search time).

4.4.1 Survival after hematopoietic stem cell transplantation

Until the recent advent of somatic gene transfer, hematopoietic stem cell transplantation (HSCT) offered the only curative option for patients with any form of SCID. If a genotypically matched sibling donor is available, HSCT is a highly successful procedure. Based on data accumulated from the European SCETIDE (Stem Cell Transplantation for Immunodeficiencies) database of 566 transplants (in 475 patients with all forms of SCID), the 3 year survival rate for HLA-matched related (matched sibling donor, MSD) procedures was over 80% (n=104) for patients treated from 1968 until 1999, and over 90% for patients treated since 1996(1, 37). The high survival rates are partly due to the fact that the absence of T and sometimes NK cells allows infusion of an HLA-identical donor graft without the need for prior myeloablative conditioning. However a genotypically matched family donor is only available for approximately 30% of patients. For the remaining individuals, alternative donor transplants, principally from matched unrelated (MUD) or haploidentical parental donors have been performed. In the same cohort noted above, 3 year survival rates following MUD transplants (n=28) were not significantly different from genotypically HLA-identical related grafts (n=104). The survival rates for patients treated by a haploidentical T-cell depleted HSCT were less good overall, although they have improved over time due to better GvHD prophylaxis and treatment of infective complications (from 35% 3 year survival before 1985, to 75% for those treated between 1996 and 1999)(38). For B+ SCID (which predominantly includes SCID-X1 patients), outcome has also improved over time and the 1 year survival for patients treated between 1996-1999 was 84%. While a significant improvement in 5-year survival rate had been observed between T-cell depleted mismatched related transplants performed prior to 1995 (survival rate: 47%) and the period 1995-1999 (65%), no further improvement has been observed for transplants performed in the period 2000-2005 (66% survival rate), as shown below for all forms of SCID (Figure 2).

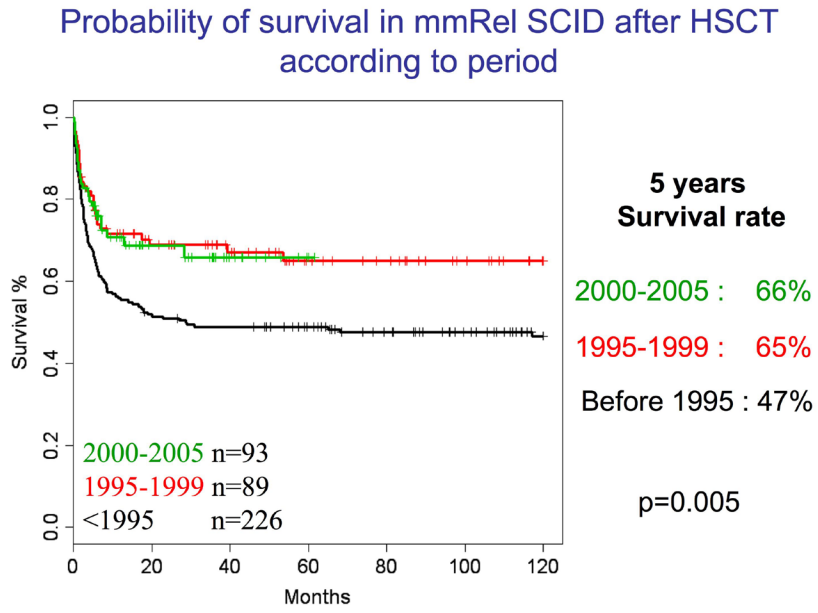


Figure 2. Data from SCETIDE Registry for hematopoietic cell transplantation for SCID

Moreover, the 10-year survival rate for 223 patients with B+ SCID (most of whom have SCIDX1) after T-cell depleted mismatched related transplantation is 63% (Figure 3), with no significant improvement in recent years.

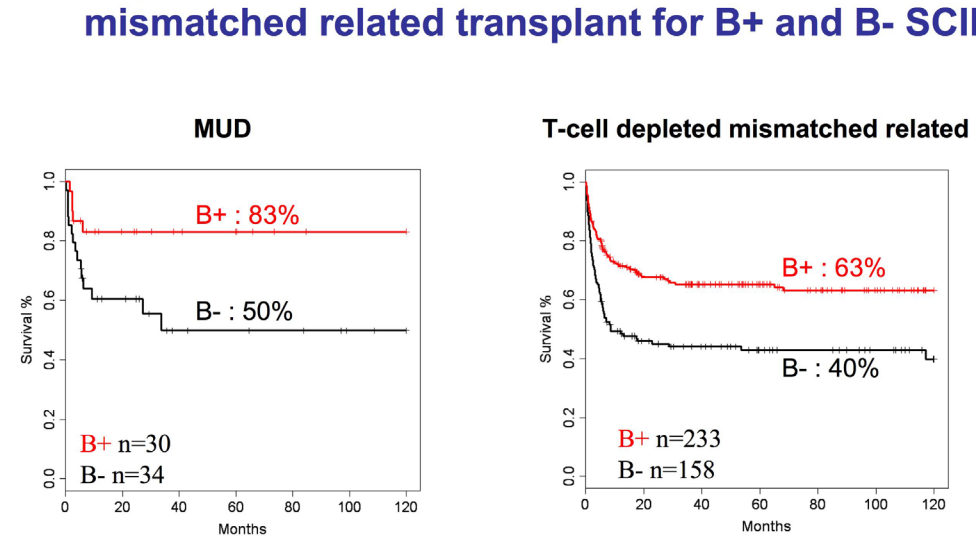


Figure 3. 10-year survival rate after MUD or T-cell depleted mismatched related transplantation for B+ and B- SCID. Data from the SCETIDE European Registry

A recent analysis of the outcome of HLA-mismatched related (MMRD) HSCT for SCIDX1 and JAK3, performed in Paris (France) and Brescia (Italy) after 1990, indicates that 30 such patients received MMRD-HSCT. Of these, 16 had severe infections at the time of transplantation. Only 8 of these 16 patients (50%) are currently alive. This contrasts with the fact that 12 out of 14 patients (85.7%) who received MMRD HCT and who did not have significant infections at the time of HSCT, are currently alive. Furthermore, among the 20 patients in this combined series who are currently alive after MMRD HCT for SCIDX1 and JAK3 deficiency, 7 have significant long-term problems (chronic/persistent infections, colitis requiring nutritional support). In addition, 8 patients have not attained humoral immune reconstitution and remain dependent on IVIG. Similar data on inadequate immune reconstitution after MMRD HCT for patients with SCID (and specifically for patients with SCIDX1) have been reported by other groups. In particular, 57% of the patients who have received unconditioned HSCT at Duke

University (NC) have inadequate B cell function and require intravenous immune globulin (IVIG) infusions monthly. The percentage requiring IVIG is highest in patients with γc, Jak 3 and RAG deficiencies (73%), and those with γc and Jak3 deficiencies also usually remain NK cell deficient despite adequate T cell reconstitution (39).

Finally, the Primary Immune Deficiency Treatment Consortium (PIDTC) has conducted a detailed retrospective study of 240 patients with SCID undergoing HSCT between 2000-2009 (100). This study confirms the inferior survival after alternative donor HSCT including MMRD, whether without conditioning (none) or with immunosuppression, reduced intensity or myeloablative conditioning (IS/RIC/MAC), adult volunteer unrelated donor or phenotypically matched related donors (OD) or unrelated umbilical cord blood (UCB). Additionally, the poor survival with these donor types is accentuated in patients with active infection at the time of HSCT (Figure 4). These data were published in 2014 (100).

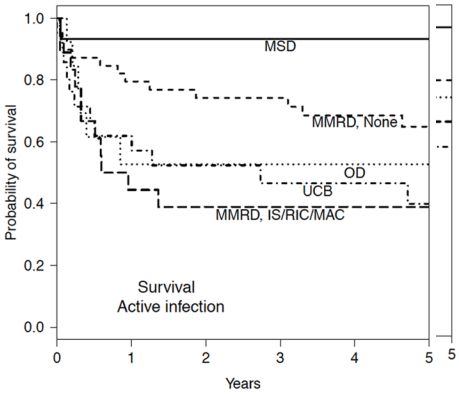


Figure 4. 5-year overall survival after matched sibling donor (MSD), mismatched unrelated donor (MMRD), other donor (OD), umbilical cord blood donor (UCB) in 2000-2009 at PIDTC centers of 240 SCID patients (left) or of 107 patients with active infection at time of HSCT (right).

Overall, these data reinforce the notion that MMRD HSCT may not provide rapid and sufficient immune reconstitution in SCIDX1 infants with severe infections. Also, while difficult to summarize the data from many centers, overall the survival rate after matched unrelated donor transplantation appears to be around 70-80%. Gene modification of autologous hematopoietic progenitor cells can be expected to provide a more rapid and robust immune reconstitution in these infants, and hence improve survival.

Information is also available from large individual centers experienced in the treatment of SCID by HSCT.

1. At Great Ormond Street Hospital (GOSH) in London, 8 haploidentical transplants were performed for SCID-X1 from1982 (6 with conditioning chemotherapy), of which 1 patient died. Of 7 survivors, the levels of immune reconstitution were similarly variable in the long term, and one received gene transfer as an adult in an attempt to alleviate severe lung and gut disease. 7 patients at GOSH received MUD transplants, of which 1 patient died and 1 (treated in 2005) suffered from chronic GvHD with persisting poor immune reconstitution. All MUD transplants received chemotherapy as pre-conditioning.
2. At Necker Hospital in Paris, 33 haploidentical transplants were performed specifically for SCID-X1 from 1971, of which 18 patients survived. 8 patients of these were treated after 1998, of which 6 are alive with variable levels of T cell reconstitution. Interestingly, long term morbidity and mortality in this cohort was also significant. Of those surviving past 2 years, 3 subsequently died from autoimmune and immunopathological disease. More than half of the others suffered from recurrent respiratory tract infections despite immunoglobulin prophylaxis. Analysis of patients treated after 1997 suggests that the clinical status of the patients at the time of transplantation has a significant influence on outcome. Five out of five patients who had no active infection at time of HSCT are alive (follow up 1 to 8 years, median 4). All have normal T cell counts and function, and 3 continue immunoglobulin substitution. In contrast, among 6 patients who had active infection or inflammation at time of HSCT (including disseminated BCG infection in 2, B cell lymphoproliferation in 1, protracted diarrhea requiring parenteral nutrition in 4, viral infection

of the lung in 2), only 3 are alive (1,2 years and 4 years follow up respectively), one with persistent BCG infection despite the presence of functional donor T cells.
3. In a joint Italian/Canadian study, 19 SCID-X1 patients treated between 1990-2004 were reported. The survival rates for HSCT from the different donor sources were: MSD 100% (3/3), MUD 89% (8/9), HLA-haploidentical 29% (2/7).
4. In a cohort of patients treated at Duke University Medical Centre (which does not use pre-conditioning), the long term survival of 132 SCID patients (of which 70 had B+ SCID) was 77% (40, 41). This included 117 HLA-haploidentical and 15 HLA-identical related transplants most of which were T cell depleted. More than a quarter of these patients required up to 3 additional transplants, mostly unconditioned, and many had residual immunological deficits (see below).
5. The Center for International Blood and Marrow Transplant Research (CIBMTR) data collected between 1990 and 2005 reports 248 cases of BMT for XSCID, the majority performed with haploidentical parental BM donors. Overall 5 year survival is 70%; however survival of the 35 boys treated under 6 months without conditioning was 95%.
6. Six patients with XSCID have received transplants from unrelated donors at Cincinnati Children's Hospital Medical Center during the past 6 years following conditioning with rituxan and thymoglobulin – all are alive and well, but none have fully recovered B and NK cell function.

4.4.2 Quality of immunological reconstitution after HSCT

The strategies for transplantation from alternative donors are variable. In the case of transplantation from HLA-mismatched related donors, T cell depletion of the graft is used, thus minimizing the risk of GvHD. In certain centers, unconditioned transplants are undertaken thus decreasing the short term and long term side effects associated with the cytotoxicity of conditioning agents used in young children (including infertility, endocrine abnormalities, and dental abnormalities). Alternatively a conditioning regimen may be used to facilitate engraftment of donor cells, which may have important implications for long term efficacy (42). Although an unconditioned transplant would appear to be a more favorable approach for both genotypically matched and alternative donor transplants, there are certain disadvantages. Under these circumstances, donor derived T cell function becomes established in patients with SCID-X1, rapidly in the case of full grafts from matched family donors and more slowly following T cell depleted grafts, and co-exists with the host derived γc-defective B cells. Following myeloablative conditioning regimens, the pattern of engraftment is difficult to predict but most commonly results in complete donor-derived T and B cell lineages presumably due to enhanced engraftment of donor stem cells. Residual humoral deficits have been described following both types of transplantation(43-45) but are more commonly seen following unconditioned transplants. In a single center report of 102 unconditioned transplants from both HLA and non-HLA identical donors, 62% of patients remained on immunoglobulin substitution(40) suggesting that the continued presence of host γc defective B cells might result in residual humoral defects. Studies of SCID-X1 patients following HSCT showed that there are intrinsic defects of isotype switching and somatic mutation in host γc-defective B cells and effective humoral function is presumably derived almost entirely from the engrafted donor B cell pool (21). Since donor B cells are derived from the engraftment of donor stem cells, this suggests that a degree of donor stem cell engraftment (or correction) is necessary for functional cellular and humoral reconstitution.

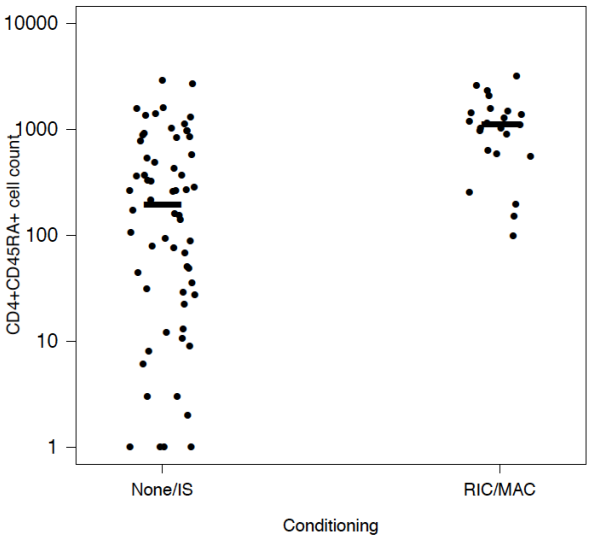


Figure 5. Naïve CD4+ CD45RA+ T cell counts are higher in SCID patients receiving busulfan based reduced intensity or myeloablative conditioning (RIC/MAC, median 1094 cells/ul) compared to those receiving no conditioning or immunosuppression. without busulfan (none/IS, median 192 cells/ul, p<0.001).

These findings of improved humoral immune reconstitution have been confirmed in a retrospective study of 240 SCID patients transplanted from 2000-2009 at PIDTC centers (100). In multivariate analysis of 149 2-5 year survivors, receipt of busulfan-based conditioning was associated with a higher likelihood of humoral immune reconstitution defined as freedom from immunoglobulin substitution (84% versus 41% without busulfan, OR 8.87, p<0.001). Additionally, busulfan based conditioning was associated with higher T cell counts in 2-year survivors in multivariate analysis, defined as CD3+ T cell count >1000 (89% versus 62% without busulfan, OR 8.84, p=0.007). The improvement in total T cell numbers may be due to improved thymic function, as shown by a dramatic increase in CD4+ T cells with a naïve, CD45RA+ phenotype (Figure 5). These data form the basis for inclusion of low dose busulfan-based chemotherapy in patients without active infection, to increase the likelihood of complete immune reconstitution in survivors of SCID-X1 patients following transplant.

5. Alternative therapy for SCID-X1 based on somatic gene transfer

As outlined above, allogeneic bone marrow transplantation for SCID-X1 remains problematic when HLA-matched donors are unavailable. A number of features make SCID-X1 an attractive candidate for treatment by somatic gene transfer.

1. It is a monogenic disease well characterized at the molecular level.
2. Introduction of γc into deficient T lymphoid precursor cells confers a powerful survival and developmental advantage to their progeny.
3. Introduction of γc into hematopoietic stem cells can restore the long term functionality of B lymphoid, T lymphoid, and NK cells.
4. The survival advantage of T cells obviates the absolute requirement for myeloablative conditioning.
5. Two recent clinical trials of somatic gene transfer for SCID-X1 have demonstrated good correction of the immunological defects in 17 patients, thus verifying the feasibility and efficacy of this approach in human subjects.

5.1 Target cell populations for somatic gene transfer of SCID-X1

Hematopoietic stem cells (HSCs) are capable of self-renewal and differentiation to all blood lineages. In mice, this activity is highly enriched in the Thy-1.1^{lo}lineage (Lin)$^{-/lo}$Sca-1^{+} population which represents about 0.05% of bone marrow cells(46, 47). This heterogeneous population of cells has been further sub-divided based on surface immunophenotype and the ability to self-renew, but all retain multipotency(48). The question of whether HSCs commit to specific lineages, and at that point lose their characteristic high proliferative potential or whether they give rise to lineage-restricted progenitors with significant proliferative and repopulating activity has been more difficult to answer. The study of radiation-induced chromosomal aberrations in the progeny of stem cells injected into mice at limiting dilution originally suggested that in addition to the multipotent population there existed myeloid and lymphoid restricted stem cells(49). These findings were later supported by retroviral marking studies(50). However, it has been difficult to define a clear phenotypic identity for these lineage-

restricted cells, in part because of contamination of sorted populations with multipotent HSCs, and their existence has therefore been questioned. For example, Thy-1^{lo}Mac-1^{+}B220^{-} and Thy-1^{lo}Mac-1^{-}B220^{+} populations which were originally identified as highly proliferative myeloid and B lymphoid-restricted progenitors respectively, were later shown to be unable to significantly repopulate lethally-irradiated recipients(51). Despite these reservations, good evidence has emerged for the existence of murine clonogenic common lymphoid stem cells which possess lymphoid-restricted (T,B and natural killer (NK)) repopulating activity, and which are defined phenotypically as a Lin^{-}IL-7R^{+}Thy^{-}1^{-}Sca-1^{lo}c-kit^{lo} population(52). An equivalent population of cells with restricted myeloid potential has also been isolated(53).

Less is known about the human HSC compartment predominantly because of the failure of *in vitro* assay systems to measure defining activities. However, studies have recently been facilitated by the development of xenogeneic transplantation assays for the ability of human hematopoietic cells to competitively repopulate immunodeficient mice. SCID-repopulating cells (SRCs) are a population of human hematopoietic cells that have been conventionally defined by their capacity for bone marrow engraftment, extensive proliferation and multilineage (lymphoid and myeloid) differentiation in SCID, NOD/SCID and most recently NOD/SCID/IL-2RG-deficient mice. This activity is highly enriched in CD34^{+}CD38^{-} fractions, and based on the kinetics of engraftment, defines a more primitive cell population than most long term culture-initiating cells (LTC-ICs) and colony forming cells (CFCs)(54-56). A small fraction of CD34^{-}CD38^{-}lin^{-} cells have been shown to possess multilineage repopulating activity *in vivo* (CD34^{-}SRC), and to have the capacity to mature into CD34^{+} cells both *in vitro* and *in vivo* (54). These studies are consistent with other experiments which have identified stem cell activity in CD34^{-} populations of mice, primates, and humans, and together indicate that as in mice, the human repopulating stem cell compartment is phenotypically heterogeneous(57-60). The phenotypic definition of murine clonogenic common lymphoid and myeloid progenitor populations goes some way to support these ideas, and may be paralleled by similar populations of human committed cells(52, 53, 61-63).

For SCID-X1, gene transfer to pluripotential hematopoietic stem cells *in vivo* would provide a continuing source of cells expressing γc, and is therefore predicted to result in complete reconstitution of the immunological deficit including B cell function. For reasons discussed previously, it is also likely that gene transfer to more committed populations, for example common lymphoid progenitors, will be sufficient to reconstitute complex T cell, B cell and NK cell immunity. Furthermore, conditioning with busulfan would promote engraftment of gene marked HSC that can give rise to multi-lineage gene marked cells, including B cells. Therefore, both HSCs and lymphoid precursor cells are the target for gene transfer.

5.2 Studies in γc-deficient mice generated by gene targeting

The existence of murine and canine models of γc deficiency offers the possibility to test gene transfer as an alternative treatment for SCID-X1. In contrast to the human disease, γc deficient mice are characterized by severe reductions in B cells, NK cells and gut associated intraepithelial cells(64, 65). Mature activated T cells do develop but are not able to proliferate in response to mitogens or in a mixed lymphocyte culture and lack the capacity to reject tumors. Thus, this murine model functionally represents many of the features of the human disease, but is not a phenocopy of the human disease. Several laboratories have reported the correction of SCID-X1 in mice by gammaretrovirus-mediated gene transfer(66, 67). In one study using a vector subsequently used for clinical study (see below), transduced γc- cells were transferred into an alymphoid recipient mice (a γc-/RAG- double knockout strain, which is deficient in both B and T cells and therefore a clearer indicator of the effect of *IL-2RG* gene transfer on B and T cell development)(68). Circulating B and T lymphocytes developed after 4 weeks, showing integration of the transgene and cell surface expression of γc. Humoral immunity was reconstituted as evidenced by normal production of all immunoglobulin subclasses and by normal antibody responses following vaccination. Cellular function was also restored with normal T cell numbers, CD4/CD8 ratio and proliferative responses to IL-2, -4 and –7. However, in more recent and

upublished studies studies in our own laboratories using the γc-/RAG- double knockout strain of mice, we have noted a high incidence of 'spontaneous' leukemia/lymphoma development when cells are 'mock' transduced, calling into question the validity of this model for safety studies.

5.3 Clinical trials of gene transfer for SCID-X1

5.3.1 Successful restoration of immunological function

Many incremental advances in gene transfer technology, several by investigators of this protocol, have contributed to the successful application of gene transfer for SCID-X1 (including the optimization of cell culture and gene transfer conditions *ex vivo*), which complement the intrinsic profound selective growth advantage imparted to successfully transduced cells. Two studies carried out in Hospital Necker, Paris and ICH/GOSH, London by Thrasher and colleagues and Fisher and colleagues in a total of 20 patients with classical presentation have demonstrated that gene transfer is highly effective in SCID-X1(69-71). Both studies utilized an MFG-based gammaretroviral vector encoding a *IL2RG* cDNA (regulated by intact Moloney murine leukemia virus long terminal repeat (LTR) sequences), to transduce autologous $CD34^+$ cells *ex vivo* which were re-infused into the patients in the absence of pre-conditioning. In nearly all patients NK cells appeared between 2 and 4 weeks after infusion of cells, followed by new thymic T lymphocyte emigrants at 10-12 weeks (see Figure 4 below showing Paris patients in black and London patients in red, normal range for age matched controls in blue, yellow stars represent lymphocyte recovery in 3 patients who developed abnormal lymphoproliferation).

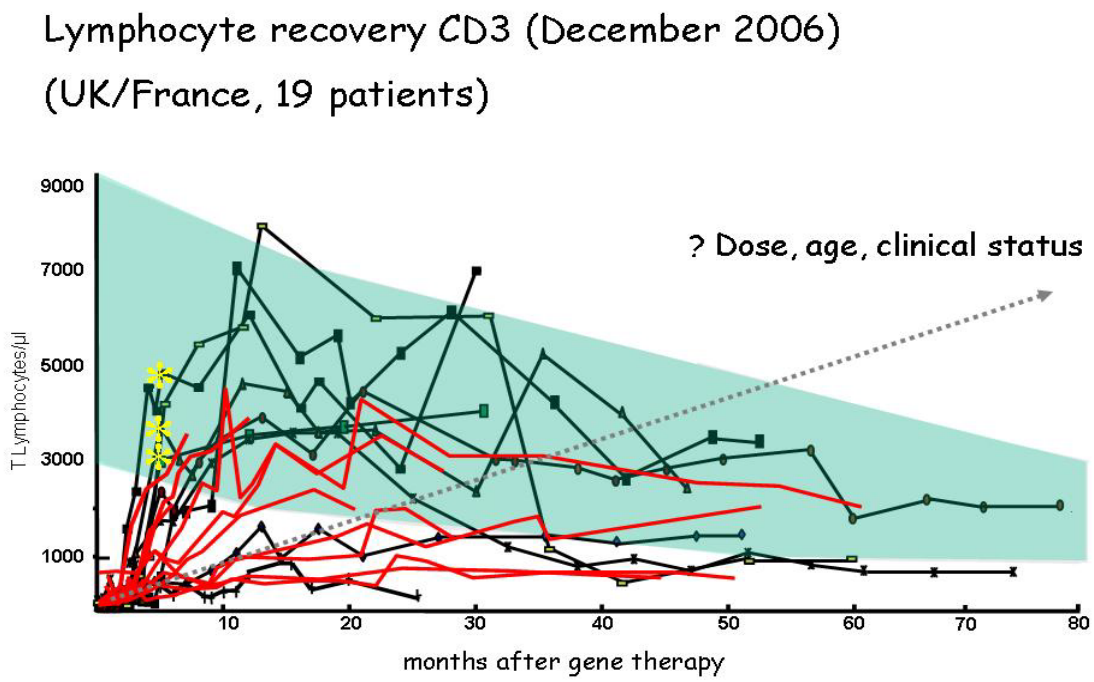


Figure 6. T cell recovery in previous SCID-X1 trials.

With some variation which may relate to the age of the patients treated, dosage of transduced cells, and clinical status, the number and distribution of these T cells increased rapidly (often more rapidly than observed following haploidentical transplantation), usually achieving normal numbers compared to age-matched control values. These T cells also functioned normally in terms of proliferative response to mitogens, T-cell receptor (TCR), and specific antigen stimulation, and were shown to have a complex phenotypic and molecular diversity of TCR. Functionality of the humoral system was also restored to a

sufficient degree that discontinuation of immunoglobulin therapy was possible in most patients with sufficient follow-up. In Paris, 8/10 patients recovered good levels of immunity up to 8 years after treatment, 1/10 failed to develop T cells after receiving a low dose of transduced CD34+ cells (31), 4/8 patients developed T-ALL like complications (2 of whom were successfully treated and recovered good immunity, 1 patient died of refractory lymphoproliferation, and one of which is currently under treatment (8.2.07) see below). In London, 9/10 patients recovered good levels of immunity up to 5 years after treatment, 1 patient developed T-ALL complications and was successfully treated and recovered good immunity. In both studies, evidence exists for long term engraftment of transduced HSCs at low levels (estimated at 0.1-2% based on marking in myeloid lineages), which may have significant implications for maintenance of thymopoiesis long term and for persisting B cell functionality. This is further supported by the observed recovery of thymopoiesis after chemotherapy in 4 patients (see below). Interestingly, NK cell reconstitution has been partial in all patients, but without obvious clinical sequelae, and follows an identical pattern to that observed after allogeneic transplantation. All 10 patients treated in London were able to resume normal social interactions, with good quality of life and normal psychosocial development. Most patients were treated with only minimal hospitalization at the time of bone marrow harvest and cell infusion. This is in marked contrast to patients receiving conditioning chemotherapy in the context of mismatched allogeneic transplantation in whom there are significant risks associated with GvHD and prolonged immunosuppression. In 2 older patients, immunological reconstitution failed despite effective gene transfer to bone marrow CD34+ cells(72). It is therefore likely that there are host-related restrictions to efficacy, primarily due to the inability to reinitiate an exhausted or failed program of thymopoiesis.

Long term follow up of patients also suggests that non-myeloablative regimens can have detrimental effects on T cell functionality many years after transplant, probably because there is insufficient HSC engraftment to sustain prolonged effective thymopoiesis(1). One probable explanation is that the initial unconditioned transplant only allowed the engraftment of committed T cell precursors or expanded populations of mature T cells. Therefore, the complexity of the T cell repertoire may diminish over time, and the engraftment of NK cells may not be durable. It therefore seems desirable to design protocols that augment long term engraftment of thymocyte precursor cells.

Potentially beneficial effects of busulfan based conditioning regimens on T cell number and thymic output are suggested by recent studies of allogeneic transplant outcome (Section 4.4.2). The likelihood of engrafting pluripotential hematopoietic stem cells is increased by complete or partial ablation of the recipient hematopoietic stem cell compartment with agents such as busulfan. This has been clearly demonstrated in gene transfer for adenosine deaminase deficient (ADA) SCID, where trials conducted using a total of 4 mg/kg busulfan dosed either every 8 hours over 2 days (101) or dosed daily once (102) resulted in multilineage engraftment with gene marked cells in T, B and myeloid compartments. The range of busulfan exposures measured as area-under-the-curve in these patients is 2377-7482 uM*minute or 9.7-30.7 mg*hour/liter, clearly submyeloablative and were well tolerated. As a reference, full myeloablative exposure is 20,740-19200 uM*minute or 85-95 mg*hour/liter. Metabolism of busulfan is highly variable according to age (103, 104). While the exposure required for significant hematopoietic stem cell engraftment in SCID-X1 is not known, exposures in higher end of the range effective in ADA SCID are likely to be effective.

Moreover, results of gene transfer for SCID-X1 using a self-inactivating lentiviral vector in 5 older patients who have failed allogeneic HSCT (NCT 01306019, 2011) have been recently reported (105). These patients received a total of 6 mg/kg busulfan given over 2 days resulting in exposure of 2519.6-4528.9 uM*min, a cumulative exposure of 5039.2-9057.8 uM*min or 20.7-37.1 mg*hour/liter (105). Preliminary data of immune reconstitution show evidence of gene marking in B and myeloid cells, and ability to maintain stable IgG trough levels without immunoglobulin replacement therapy (105). Thus a similar target exposure could be expected to enhance gene marking and function of B cells in SCID-X1 patients undergoing gene transfer in this protocol.

5.3.2 Insertional mutagenesis and lymphoproliferative disease

The dependence of retroviruses on chromosomal integration for stability of transduction brings with it the risk of insertional mutagenesis. Reproducible leukemogenesis and oncogenesis has now clearly been demonstrated in pre-clinical models, and may be directly associated with vector dose or cell copy number. Co-operating effects from expression of the transgene, or from other elements within the vector backbone may also be important, and are likely to be disease or context dependent(73). In human clinical trials, 5 patients with SCID-X1 having initially achieved successful immunological reconstitution, developed T cell lymphoproliferative disease 3-5 years after the gene transfer procedure(74, 75). Of 20 patients with SCIDX1 treated by gene transfer in these trials, 18 are currently alive and with good immune reconstitution but 5 have experienced a serious side effect. These 5 children developed leukemia related to the gene transfer itself. Of these children, 4 of the 5 have received chemotherapy are currently in clinical and molecular remission, whereas one died of therapy-related leukemia. One additional patient, who did not develop leukemia, died of complications of subsequent bone marrow transplant that was performed as a result of the gene transfer not working. This child had received a low dose of transduced CD34+ cells during the gene therapy protocol. In at least four of these patients, retroviral vector insertion into or near the *LMO-2* proto-oncogene resulted in high level expression of LMO-2 in the clones, as a result of retroviral enhancer-mediated activation of transcription (Figure 5). In another trial of gene transfer for Chronic Granulomatous Disease (CGD), non-malignant amplification of myeloid clones leading to acquisition of monosomy 7 and myelodysplasia occurred in 3 patients and contributed to the initial efficacy of the therapy, but occurred due to similar Spleen Focus Forming Virus (SFFV) LTR-mediated activation of *MDS1-EVI1*, *PRDM16* or *SETBP1* genes(76). All three CGD patients demonstrated signficant improvement in infections resistant to state-of-the-art medical management following gene therapeutic approach.

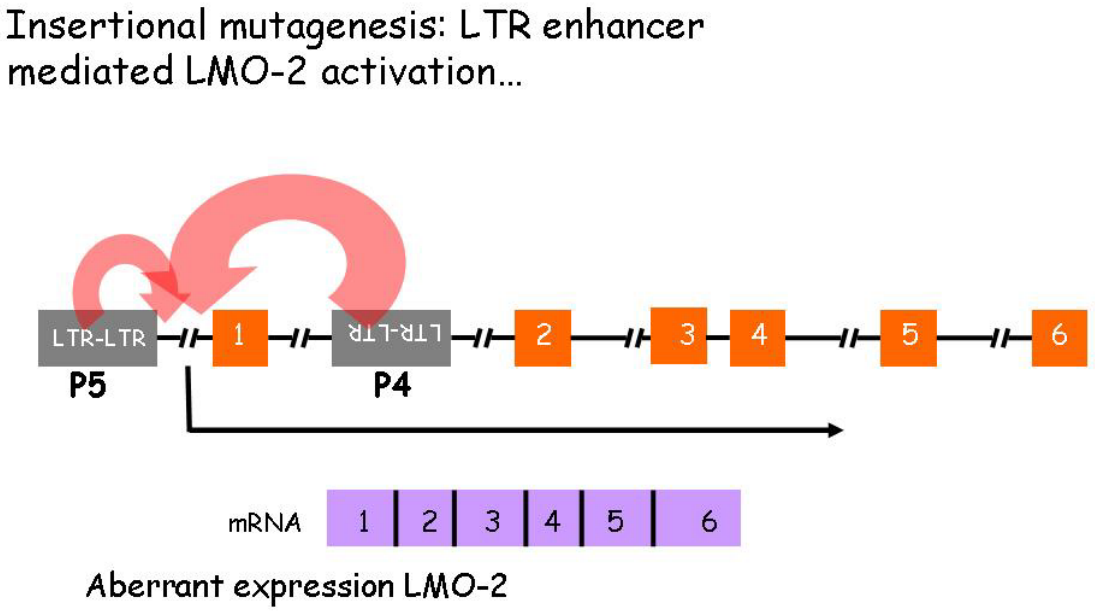


Figure 7. Insertional activation of LMO-2 from retrovirus insertion in SCID-X1 trial.

5.3.3 Mechanisms of leukemogenesis in SCID-X1 gene transfer

Activation of LMO-2 is known to participate in human leukemogenesis by chromosomal translocation, and results in the development of T cell lymphoproliferation and leukemia in mice, albeit with a long latency. It is therefore likely that other contributing factors are required for leukemia to evolve. Cells with high proliferative potential such as HSC and thymocytes are also likely to be more susceptible to

transformation following an insertional event than quiescent cells if they acquire additional adverse mutations unrelated to the gene transfer itself. One consideration is a contribution from the activity of the *IL-2RG* transgene. At least one tumor derived in susceptible mice following infection with replication gammaretroviruses has been shown to harbor separate but coincident integrations at the *Il2rg* and *lmo-2* gene loci, suggesting that there may be a significant synergistic interaction(77). However, this clone also contained insertions at least 2 other known oncogenes including *Bmi1* and *Rap1gds1*. Another study using forced high level lentiviral-vector-mediated expression of γc in mice claimed to demonstrate a pro-oncogenic effect of the transgene(78). However, the influence of insertional mutagenesis was not evaluated (which may be important given the high copy numbers of transgene), and other investigators using *in vitro* and *in vivo* murine models either in classical transgenic or retroviral gene transfer experiments have not observed this effect(79-81). In treated patients there is currently no evidence of dysregulated expression or signaling in lymphoid cells. The levels of expression of the IL-2RG transgene are generally normal or less than normal in primary lymphocytes (including the leukemic clones from patients). Furthermore, there has been no selection over time for more highly expressing cells. The accumulated evidence from murine studies (gene therapy and transgenic models) and expression data for human leukemias from the previous X-SCID trials have not shown constitutive activation of JAK/STAT pathways in presence of transgenic γc expression. In addition, in primary human cells, it has been shown that over-expression of γc does not alter T cell development *in vitro* (82). Therefore there is no good evidence at this time to support the suggestion that transgenic *IL-2RG* is potently oncogenic in its own right (see (80)**.**

5.3.4 Strategies to overcome insertional mutagenesis

It is now apparent from animal studies that mutagenesis can be reproducibly induced by gammaretrovirus-mediated gene transfer, at least using vectors with intact LTR sequences. Strategies for improvement of safety are therefore of general importance. According to updated recommendations from the UK Gene Therapy Advisory Committee (GTAC) and Committee for safety of Medicines (CSM), a number of advances in vector design should be pursued that may be advantageous in the longer term and for future generations of retroviral vectors:

Recommendation 6 states: 'Additional safety features should be considered for retroviral protocols, including the use of self-inactivating vectors, and non viral promoters to drive therapeutic genes. Ideally, new vectors should be selected on the basis of improved safety in pre-clinical testing models (in vitro and/or in vivo). However the current lack of validated systems remains a constraint to application of this principle.'
(http://www.advisorybodies.doh.gov.uk/genetics/gtac/).

6. Preliminary studies relevant to the proposed clinical trial

6.1 Development of a novel vector to enhance safety of gene transfer protocols for SCID-X1

The existing gene transfer study for SCID-X1 at ICH/GOSH (GTAC: 045) has been recently closed after successful treatment of 10 patients due to exhaustion of vector stocks. It was felt that significant advances in vector design should be incorporated into future studies to enhance the safety profile.

6.1.1 Self-inactivating (SIN) gammaretroviral vectors for expression of IL2RG

Several strategies have been suggested to improve efficiency and safety of current protocols. Patterns of integration into host chromosomes are to some degree vector dependent and could thereby contribute to the likelihood of inadvertent gene activation. However, both gammaretroviral vectors and lentiviral vectors have been shown to integrate preferentially within genes, and both are likely therefore to be susceptible to induction of mutagenic side effects. The overall design of vectors used for gene

delivery is probably most important and modifications may be possible that will limit the risks of mutagenesis, for example by the use of self-inactivating (SIN) configurations in which the powerful duplicated viral LTR enhancer sequences are deleted, and by the incorporation of regulatory domains with reduced transactivation potential.

6.1.2 SIN vector development

Dr. Christopher Baum and colleagues at Hannover Medical School have refined retroviral vectors for application to human gene transfer, and have been at the forefront of SIN gammaretroviral vector development. A new generation of SIN vectors has been developed which lack all enhancer-promoter elements of the LTR U3 region and are also devoid of all gammaretroviral coding regions, as previously described(83). In particular, modifications of the 5' promoter of the SIN vector plasmid (which is not present in the integrated provirus) have contributed to high titers after transient transfection in 293T-based packaging cells, thereby obviating some of the difficulties previously encountered with SIN vector production(84). On the basis of these studies, a series of gammaretroviral vectors have been constructed containing different internal promoters to express the IL2RGcDNA (Figure 6). All vectors contained the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), as this increases titers after transient transfection of 293T-based packaging cells (at present efficient stable packaging systems for production of SIN gammaretroviral vectors are not available). The WPRE sequence is devoid of the hepadnaviral X-protein open reading frame and contains a point mutation that destroys the largest residual open reading frame of this element (pre*)(84). In retrovirally transduced cells, SIN vectors carry an internal promoter immediately upstream of the IL2RG cDNA, without an additional element in the 5'UTR. After preliminary testing for expression in cell lines the following two internal promoters were selected for further evaluation: murine spleen focus-forming virus (SF promoter vector designation pSRS11.SF.IL2RG.pre* SF), human polypeptide chain elongation factor-1α (EFS promoter, vector designation pSRS11.EFS.IL2RG.pre*) promoter short version lacking the first intron. A gammaretroviral MFG-γc vector used in the French phaseI/II clinical SCID-X1 trial (kindly provided by Professor Marina Cavazzana-Calvo, Paris, France) was used as a positive control. Schematics of vectors are also shown below.

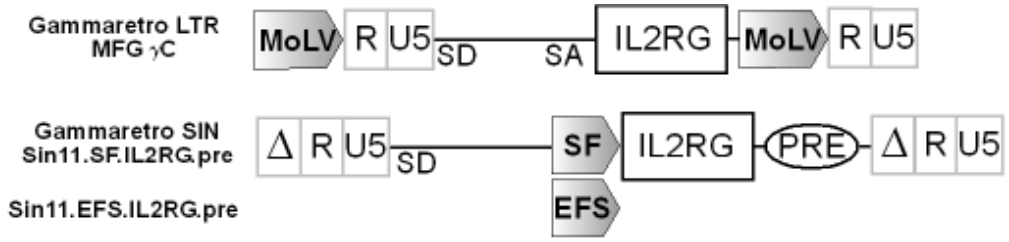


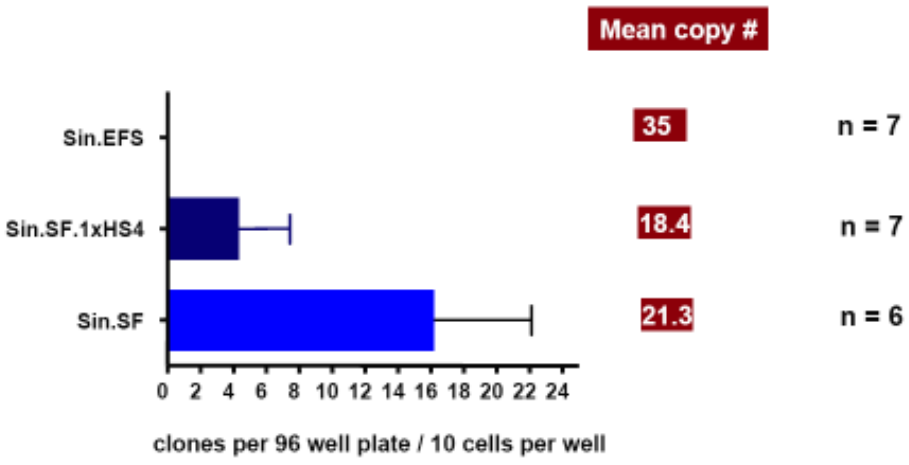
Figure 8. IL2RG vector design.

In mammalian cells, EF-1α is present in most tissues, and the associated CpG-rich gene regulatory domains have therefore been attractive for utilization in expression vectors, including those under development for gene transfer as an alternative to viral sequences(85-87). To address whether the internal EFS has a reduced potential to transactivate neighbouring genes, two reporter systems were designed and evaluated *in vitro*. (1) In a plasmid-based assay the enhancer interactions of different promoter/enhancer configurations on a minimal promoter driving a luciferase expression cassette were tested following transient transfection in human fibrosarcoma cells (293T) and murine hematopoietic progenitor cells (32D). (2) To test potential transactivating effects in retrovirally transduced cells, a novel design of a SIN vector was constructed with a minigene cassette placed in the residual U3 of the long terminal repeat (which would only be active if transactivated by the internal enhancer of the vector or a neighbouring cellular enhancer). Expression of the minigene cassette was examined in polyclonal cultures of murine SC1 fibroblasts and murine primary hematopoietic progenitor cells several days after

retroviral transduction, without previous selection. In both experimental settings, the cell-derived promoter EFS was a significantly weaker transactivator than viral enhancer-promoters such as the SFFV LTR. In the case of the minigene cassette, only the internal retroviral enhancer-promoter of SFFV was able to increase its expression above the background level observed in primary hematopoietic cells(88).

6.1.3 Platform studies addressing the genotoxic impact of gammaretroviral SIN vectors

In vivo murine studies in γc-deficient mice (and low dose transduction of normal bone marrow) have not previously been predictive of leukemogenesis as observed in human studies, and this remains a problem for pre-clinical evaluation as acknowledged in a recent GTAC/CSM report (see above). Thus, there is currently no uniformly accepted assay to predict mutagenic potential in humans. Surrogate assays are available. One of the more advanced assays is the cell based transformation assays established in the Baum laboratory (89, 90)In this assay, we have shown that gammaretroviral SIN vectors containing the SFFV promoter have a residual propensity to immortalize murine hematopoietic progenitor cells when examined in serial replating assay, though this was significantly reduced compared to vectors with intact SFFV LTRs(91). Recently, we further increased the sensitivity of the assay so that we can now introduce more than 20 vector copies per cell. Despite this high efficiency of retroviral transduction and very high mean copy number/cell (Figure 7), cell viability is maintained, so that the assay is sufficiently sensitive and reproducible to discriminate the genotoxic impact of different internal promoters in SIN vectors expressing a neutral reporter transgene (eGFP). Under equivalent conditions, a SIN vector with the internal EFS promoter (pSRS11.EFS.eGFP.pre* or 'Sin.EFS' in Figure 7) was significantly (P<0.01) less mutagenic than a SIN vector with the internal SF promoter (pSRS11.SF.eGFP.pre* or 'SinSF' in Figure 7). In these assays to date, the backbone containing EFS as the internal promoter to drive EGFP expression in the SIN configuration has not been associated with immortalization in MOI dose escalation leading to >30 copies/cell. Sin.EFS is the gammaretroviral SIN vector backbone identical to pSRS11.EFS.IL2RG.pre*, but with the cDNA of IL2RG replaced by EGFP. No induction of replating activity was observed with this backbone, although the copy number was escalated from 11 up to 95 copies per cell **(Table 1 in** (90)**.** Sin.SF is the same vector with the internal SFFV promoter instead of EFS. Replating activity was observed in all 6 assays with doses ranging from 16 to 27 copies per cell. Sin.SF.1xHS4 is Sin.SF carrying an insulator element in the Ur region of the SIN LTR. Of note, SIN vectors containing 'insulator' elements flanking the



internal SF promoter-driven cassette showed a significantly greater degree of transforming activity that the SIN vector with the EFS promoter (4/4 experiments; P< 0.01).The mean copy number of the vectors was determined by real-time PCR 4 days after transduction. Clones were counted under conditions of limiting dilution (10 cells plated per well and observed for 14 days), starting from polyclonal

Figure 9. Immortalization of primary mouse bone marrow after transduction.

cultures that were expanded for 14 days after transduction prior to replating in limiting dilution. Transformed clones were always observed with the SIN vector containing the internal SFFV promoter (3/3 cases), and with this vector, an average copy number of 1.7 was sufficient to induce transformation. This indicates that the transactivating potential of the SIN vector with the EFS promoter is at least substantially diminished(88). In summary, it has not been possible to induce transformation *in vitro* with a SIN vector harboring an internal EFS promoter(88). Finally, we have directly compared the potential for the SRS11.EFS.IL2RG.pre* vector to activate LMO2 in a model system developed by Nienhuis and colleagues that utilizes the Jurkat T cell line (92) Jurkat T cells express barely detectable LMO2 transcripts compared with K562 cells (3590-fold expression relative to Jurkat) (Figure 8). In this assay, a targeted insertion of the proviral genome is accomplished at the precise location of vector integration in one of the patients with X-SCID and LMO-2 mediated leukaemogenesis. Preliminary studies obtained from 2 clones indicate that SRS11.EFS.IL2RG.pre* (EFS#19,21) results in marked reduction in gene activation compared with a single LTR (G25-1-26), a double LTR provirus (656 #16), and an MFG-based LTR with a deletion of one enhancer repeat (MFG#43,5.2,54,92) (vectors used for both Paris and London clinical studies contained conventional dual enhancer LTRs). Furthermore the relative expression of LMO-2 was very similar in these two clones to an insulated SIN lentiviral vector in the same context. All of these values are derived by mRNA analysis using qRT-PCR, and are shown on a *log scale* relative to unmodified Jurkat cells.

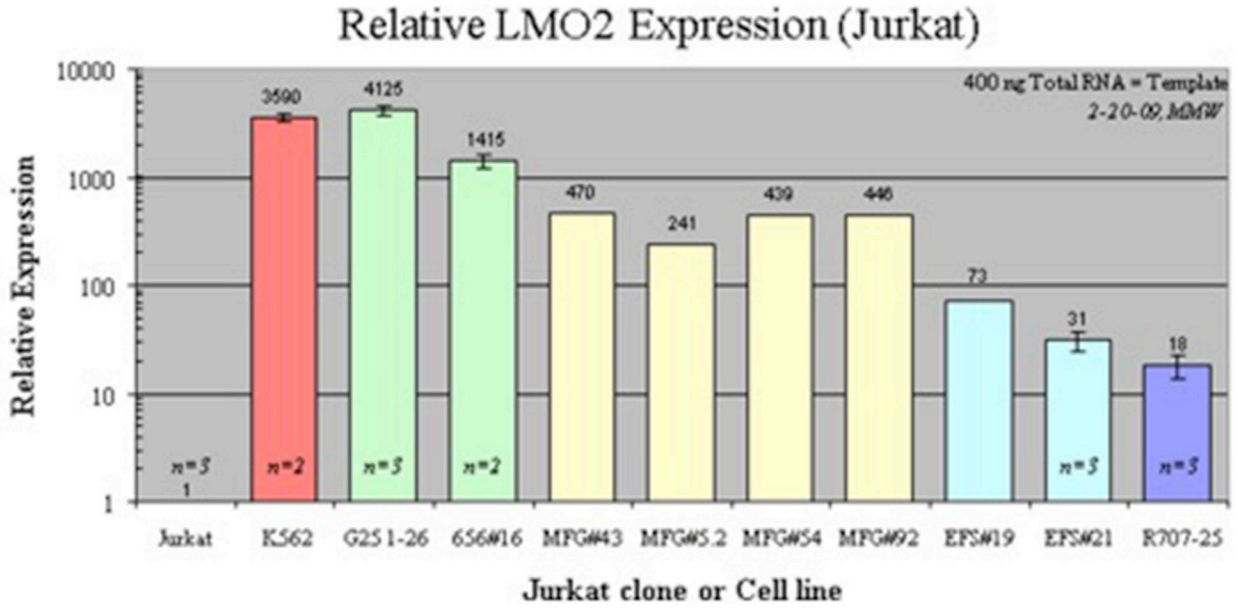


Figure 10. Expression of LMO2 in Jurkat Cells after Integration of Retrovirus Vectors *(note log scale)*.

Together these studies indicate that the transactivating potential of gammaretroviral vectors are substantially reduced by SIN configurations and further reduced by the utilization of EFS internal regulatory sequences.

6.1.4 Toxicological studies in murine models

Gammaretroviral vectors with intact LTRs (in the wild-type configuration) encoding reporter genes, or alternate transgenes induce leukemogenesis during experimental dose escalation through a process of combinatorial mutagenesis(93). This model is therefore being used to test for mutagenesis using the selected vectors to transduce lin- bone marrow cells from mice at variable multiplicities of infection(93).

Safety-enhanced gammaretroviral vectors have since emerged, that incorporate the self-inactivating (SIN) LTRs.

The potential of SIN gammaretroviral vectors encoding IL2RG (γc) driven by an internal promoter from the spleen focus forming virus (SF) to induce leukemia has been tested in a serial BMT model using C57BL/6J mice. Eight groups of 6 mice each received lin- bone marrow cells transduced with MFG-γc, a SIN gammaretroviral vector expressing IL2RG under control of an internal SF promoter (SRS11.SF.IL2RG.pre*), an equivalent SIN lentiviral vector (RRL.SF.IL2RG.pre*), and a control SIN gammaretroviral vector expressing the dsRedexpress fluorescent protein under control of an internal SF promoter (SRS11.SF.DsRedexpress.pre*). No vector-associated leukemias were observed in primary transplant recipients observed for 11 months. In these mice, average transgene copy numbers (defined by real-time PCR in peripheral blood leukocytes) reached up to 0.6 copies/cell for SRS11.SF.DsRedexpress.pre*, 1.1 copies/cell for SRS11.SF.IL2RG.pre*, 2.6 for RRL.SF.IL2RG.pre*, and <0.2 for MFG-γc (this vector had a low infectious titer). *Thus, in contrast to Woods et al.(78) gammaretroviral or lentiviral over-expression of IL2RG was not sufficient to induce leukemia in mice, despite a long observation time(80)*.

After termination of this study and detailed necropsy confirming the absence of leukemia, serial or secondary BMT was performed with bone marrow cells from the SRS11.SF.DsRedexpress.pre* or SRS11.SF.IL2RG.pre* groups of mice. The four donors of each group that showed the highest level of gene marking were chosen for these studies. Two recipients were transplanted from each donor, resulting in a total of 16 mice that were observed for another 7.5 months (18.5 months clone observation time since gene transfer). Two secondary recipients of SRS11.SF.DsRedexpress.pre* transduced cells showed signs of disease and were humanely sacrificed. Diseases were unrelated to vector insertion (one mouse suffering from leukopenia, another from a large renal cyst). No leukemia occurred in this group. However, two distinct leukemic clones were observed in secondary recipients of cells transduced with SRS11.SF.IL2RG.pre*. One clone showed a B cell precursor phenotype (latency 3-5 months in secondary recipients), the other a myeloid phenotype (latency 6 .5 months). The single vector insertion site of the B cell leukemia was found to map to the *Evi1* gene, encoding a transcription factor whose up-regulation by retroviruses may induce leukemia in mice(91, 94). Preliminary data showed that the leukemic cells did not require interleukins for growth *in vitro* and lost the B cell marker (B220) after culture in the presence of the cytokines mSCF and Flt3L, therefore questioning a significant role of the transgenic IL2RG in leukemia induction. The myeloid leukemia observed in the same transplant group had four vector insertion sites, and again one of these was found in *Evi1*. This study has been published by Baum's group(95).

These initial data suggest that leukemogenesis may be induced by random insertion of SRS11.SF.IL2RG.pre* (ie a viral LTR at an internal position) in the vicinity of a potent cellular proto-oncogene, in line with the predictions of the in vitro immortalization assay(91). These data raise caution regarding the therapeutic use of SIN vectors with the very strong internal viral promoters and enhancers such as the SF promoter, and supports for clinical use a vector that relies on an internal cellular gene promoter which may have reduced transactivation potential, such as the promoter of elongation factor-1α gene (EFS).

Therefore, in additional studies the SRS11.EFS.IL2RG.pre* vector was tested in two different mouse models. In studies using γc/Rag2 double knockout (gc-/-Rag-/-) mice we found high frequencies of both host tumors and donor-derived leukemia and lymphoma formation even in the absence of vector insertions (including 'mock transduced' cells). Host cell derived malignancies are not unexpected in this mouse model, and the frequency is not higher than previously reported values by our group(96). More importantly, given that even donor derived cells without any vector insertions develop leukemia/lymphoma in these recipient mice, these data strongly suggest that gc-/-Rag-/- mice are not satisfactory for analysis of vector-induced leukemogenesis. Evaluation of vector-mediated toxicity was

therefore conducted in a standardinbred mouse strain. Safety studies were conducted using C57/BL6 Ly5.1/Ly5.2 congenic donors and recipients were undertaken in two experimental groups. ***To summarize data detailed below***, in a total of 31 mice transplanted with the clinical vector and followed for a minimum of 10 months, there were no donor derived tumors noted. An additional 8 animals received secondary transplants from a cohort of these primary mice and were observed for up to one additional year. No donor derived tumors were noted. Details of these cohorts are provided below.

Experimental group 1: In one set of studies, lineage negative (Lin-) cells from three experimental groups of Ly5.2 (CD45.1+) mice were concurrently transduced with 1) SRS11.EFS.IL2RG.pre* (IL2RG group), 2) control EGFP vector SERS11.EFS.EGFP.pre* (EGFP group), or 3) media alone (mock controls). Briefly, lineage negative bone marrow derived stem cells were isolated and pre-stimulated for 48 hours in Stemspan with 1% (v/v) Penicillin/Streptomycin, murine recombinant (m) SCF (100 ng/ml), mFlt3 (100 ng/ml), mIL-3 (20 ng/ml) and human recombinant IL-11 (100 ng/ml). The cells were then harvested and transduced on fibronectin (FN) CH296 fragment on two consecutive days with a multiplicity of infection (MOI) which was demonstrated in preliminary studies to yield approximately 40% transduced bone marrow clonogenic progenitors. Cells were harvested after the second transduction, washed and injected into recipient mice at doses of at least 4x10e5 cells per mouse. Twenty mice were transplanted for each experimental group, in 2 cohorts of 10 mice per cohort. The mice were observed for a period of 10-12 months. In this set of studies, evaluable mice were those that had >25% engraftment at 5 weeks and survived >10 months following transplant. Any mouse that died after 1 month of transplant was also autopsied to determine the cause of death, presence of donor/host derived tumor/s and vector status of the tumor. Peripheral blood was taken from the tail vein at 5 weeks, 2 months, 4 months, 6 months and 9 months post transplant. Complete blood counts were measured at each time point, and additional analysis by flow cytometry and real-time PCR was alternated. At sacrifice, all tissues were weighed and examined grossly. Liver, spleen, thymus, bone marrow, kidney, lung as well as any masses or enlarged organs were evaluated by a pathologist. Additionally, bone marrow, spleen, and thymus were examined by flow cytometry.

Details of the individual mice in all three cohorts (and data from the second experimental group) are summarized in **Table 1.**
There were 17 evaluable mice in the IL2RG group, 17 evaluable mice in the EGFP group and 18 evaluable mice in the mock group. On average, there was 60-80% engraftment in each group. The average vector copy number in peripheral blood mononuclear cells, determined by real-time PCR at 2 months post transplant was 1.25 copies/cell for the IL2RG test group and 2.2 copies/cell for the EGFP control group. Two host cell derived

Table 1: SRS11.EFS.IL2RG.pre* Animal Data Analysis

Recipient	Donor	Vector	Evaluable Mice^	Donor Derived Lymphomas	Percent
C57 (CD45.2)	BoyJ (CD45.1)	SRS11.EFS.IL2RG.pre*	31++	0	0
C57 (CD45.2)	BoyJ (CD45.1)	SERS11.EFS.GFP.pre*	17	0^^	0
C57 (CD45.2)	BoyJ (CD45.1)	Mock	18	0#	0

^ Evaluable mice in the C57 recipients were all mice followed for at least 10 months and with more than 30% engraftment at 5 weeks. On average, there was 60-80% engraftment in this group of mice. ++ includes 14 mice followed for 14 months in primary recipients. Four animals from this cohort with peripheral blood and spleen engraftment of 30-60% and 15-60% respectively (copy number 0.04-0.74) were sacrificed at 14 months and 1x10e6 cells transplanted into secondary recipient mice. Eight secondary mice were followed for 1 year.
^^in one mouse tumor (thymoma), the origin could not be determined; however, the thymoma mass was vector positive.
one mouse found dead with enlarged thymus

malignancies were noted in the test IL2RG group. One vector-negative thymoma and an ovarian tumor were noted in the GFP vector group. Host cell derived malignancies are not unexpected in this mouse model, and the frequency is not higher than previously reported values by our group (96)**.**There were no cases of vector positive donor cell derived leukemias in the IL2RG test group. There was one vector positive, donor cell derived T cell lymphoma noted in a C57BL/6J recipient mouse which received the EGFP control vector (mouse ID H.8). Of note, there was also an animal found dead with enlarged thymus in the mock control group.

Experimental group 2: In a second set of studies, the transduction protocol was identical to above except that the MOI was equal to 2. Cells were harvested after the second transduction and injected into recipient mice at doses of 5x10e5 cells per mouse. In this second group of mice, 13 out of 14 animals were followed for 14-16 months after reconstitution. The copy number in peripheral blood mononuclear cells determined by QT-PCR after 14 months was 0.008-0.74. These animals had a CD45.1 engraftment level of 4-60% in the peripheral blood and 4-60% in the spleen (at sacrifice). One host derived B220 leukemia was noted at 264 days. One mouse had a host derived liver mass at final sacrifice. Four healthy animals from this primary cohort with peripheral blood and spleen engraftment of 30-60% and 15-60% respectively (copy number 0.04-0.74) were sacrificed at 14 months and 1x10e6 cells transplanted into secondary recipient mice. Eight mice were followed for up to 1 year. Engraftment in the spleen was 4-33% and peripheral blood was 4-21% at sacrifice of these animals. The peripheral blood mononuclear cell copy number per CD45.1 cell 4 months post injection was 0.04-0.1. In the secondary animals, one host-derived B220 leukemia (10 weeks after engraftment) and one host-derived thymoma (7 months after engraftment) were noted. One animal was lost to evaluation. Five animals survived to one year and were normal at sacrifice. No donor derived tumors were noted in any primary or secondary mice.

In summary, the basic configuration of pSRS11.EFS.IL2RG.pre* has a clearly proven reduction in mutagenic potential compared to LTR configuration in *in vitro* assays and has not been associated with vector-positive tumors in a large number of mice followed over an extensive period, including after serial transplantation stress.

6.1.5 Evaluation of potency in murine model systems *in vitro* and *in vivo*

To investigate γc expression in hematopoietic progenitors, lin- cells from young adult donor C57Bl6 mice were transduced cells as described(97). Both SIN configuration vectors (incorporating SF or EFS promoters) mediated good surface expression of γc, as detected by flow cytometry, although at a somewhat lower level than that obtained with MFG-γc. More importantly, both were able to efficiently rescue the *in vitro* B and T cell differentiation capacity of lin- cells derived from Il2Rγ -/- mice in a surrogate lymphocyte differentiation system, and at equivalent levels to that achieved using the MFG-based vector. These results reveal that both SIN vectors are sufficiently potent to compensate the genetic deficiency in primary murine progenitor cells when examined in cell culture. pSRS11.EFS.IL2RG.pre* was further evaluated *in vivo* in preference to pSRS11.SF.IL2RG.pre to avoid use of potentially mutagenic viral LTR sequences (see above).

The γc-deficient mouse provides a convenient, though not perfect, model with which to compare gene transfer strategies for SCID-X1 *in vivo*. pSRS11.EFS.IL2RG.pre* was therefore compared to the vector currently used in the clinical trial, MFG γc, for efficacy of immune reconstitution following gene transfer *in vivo*. Briefly, lineage negative (lin^-) bone marrow cells from $\gamma c^{-/-}$ mice were transduced *ex vivo* using a low MOI with either the SIN vector, the LTR-regulated clinical vector or with a gammaretroviral vector encoding an eGFP transgene (SFFV-eGFP) under serum-free conditions in the presence of cytokines. Transduction levels of between 55-80% were achieved using this method. Transduced cells were subsequently injected into sublethally-irradiated alymphoid γc^-/$rag2^-$/$c5^-$ recipient mice. Transplanted

animals were maintained in pathogen-free environments for at least three months to allow engraftment and repopulation.

Fifteen to eighteen weeks post-transplant circulating T and B lymphocytes were detectable in the peripheral blood of all seven mice transplanted with pSRS11.EFS.IL2RG.pre* transduced cells and all six MFG γc mice. Four out of four mice repopulated with SFFV-eGFP transduced cells remained alymphoid. Experimental animals were sacrificed approximately five months post-transplant for analysis of immune reconstitution. Flow cytometric analysis of the spleens and bone marrow revealed restoration of mature $B220^{+}IgM^{+}$ B cells and NK cell populations in all pSRS11.EFS.IL2RG.pre and MFG γc transplanted mice. $CD4^{+}$ and $CD8^{+}$ T cells were also detected in both tissues and in thymi recovered from transplanted animals indicating restoration of thymopoiesis (Figure 9). The restored T lymphocyte populations in these mice appeared functional with splenocytes able to proliferate in response to mitogenic stimuli and at increased levels in the presence of a γc-dependent cytokine. Immunoglobulin subclasses IgG1 and IgG2a detected in the plasma from pSRS11.EFS.IL2RG.pre* reconstituted mice also indicate restored B cell function in these animals. Proviral copy number in splenocytes and bone marrow cells was detected by real-time PCR and found to be low (less than one copy per cell in all but one of the reconstituted mice). All control mice repopulated with cells transduced with the SFFV-eGFP vector remained athymic and had no detectable T, NK or mature IgM^{+} B cells in the spleens and bone marrow at the time of analysis. Further engrafted animals will be evaluated at later time points.

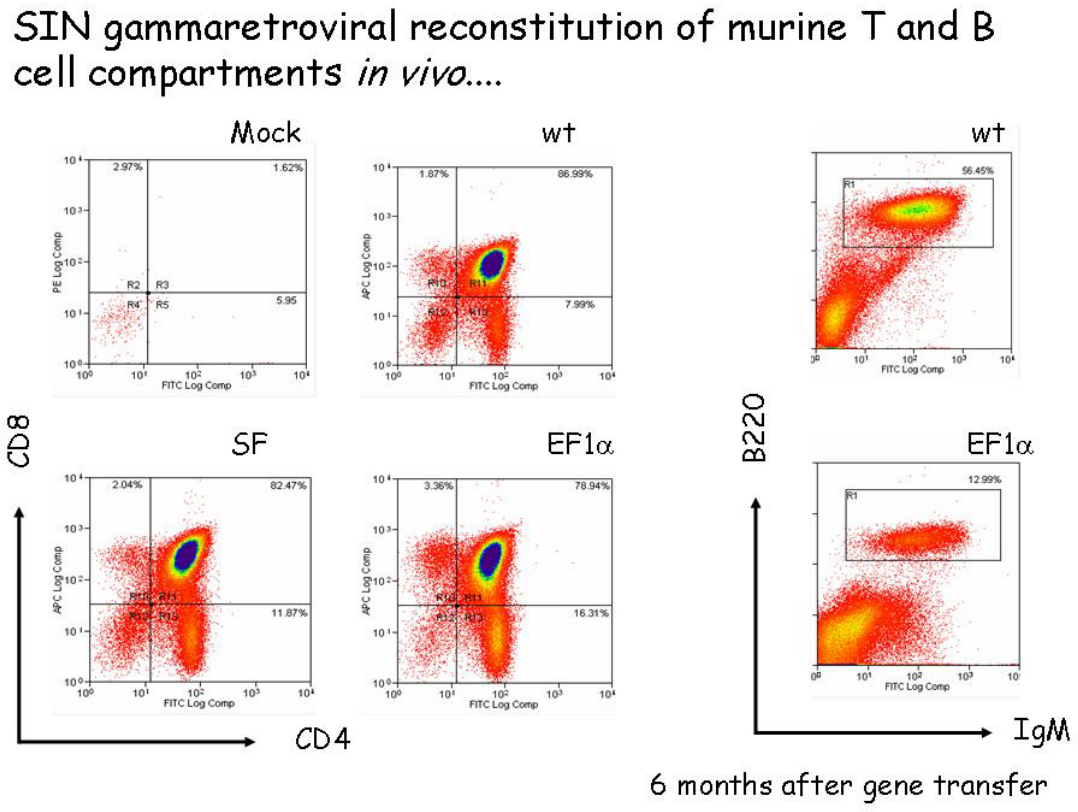


Figure 11. T and B cell compartments following gene therapy in a murine model. EF1αrepresents pSRS11.EFS.IL2RG.pre*, and SF represents pSRS11.SF.IL2RG.pre*.

6.1.6 Evaluation in human T cell lines and CD34+ cells

The ability of pSRS11.EFS.IL2RG.pre* to reconstitute signaling activity in human cells has been tested on a human γc-deficient T cell leukemia cell subline (ED7R) which retains expression of the IL2R α and β chains(98). Transduction of these cells *in vitro* using GLP-produced GALV-pseudotyped vector results in normal reconstitution of STAT5 phosphorylation following stimulation with γc-dependent cytokines such as IL-2 or IL-7 (Figure 10).

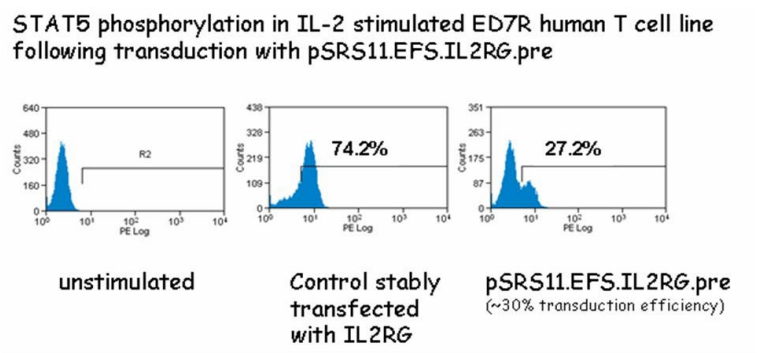


Figure 12. Restoration of Stat5 activation following gene transfer.

Preparations of GLP-produced GALV-pseudotyped vector stocks have also been tested on human CD34+ cells using standard transduction conditions (as used previously). The titer of the viral preparations was approximately $1x10^6$ transducing units per ml measured by expression of human γc in transduced HT1080 cells. In two independent experiments normal peripheral blood derived CD34+ cells were used as targets (Figure 11). In a further experiment, a limited number of CD34+ bone marrow cells were purified from a patient with classical SCID-X1. Overall transduction efficiency of CD34+ using pSRS11.EFS.IL2RG.pre* was between 20 and 60%. In one experiment, clinical grade MFG-γc vector was used to transduce cells in parallel. Transduction efficiency was in this case similar, although as expected the levels of expression were lower in cells transduced with pSRS11.EFS.IL2RG.pre*.

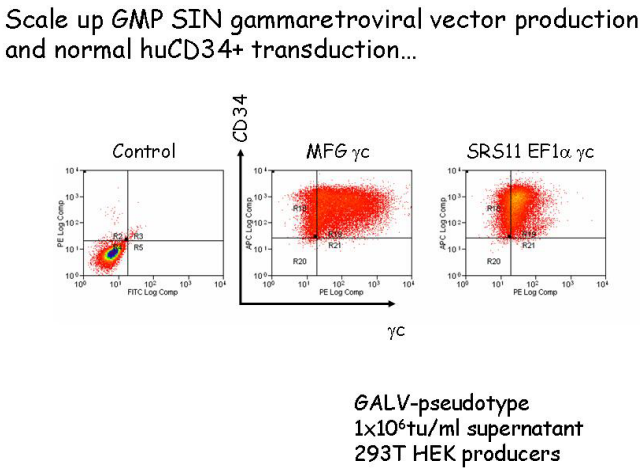


Figure 13. Expression of γc in transduced CD34+ cells.

Transduced cells derived from a SCID-X1 patient were also cultured under conditions favoring NK cell development *in vitro* (in the presence of IL-2 and IL-15 for 21 days). Due to the low number of cells

available for analysis, detailed confirmation of NK cell differentiation and function was not possible. However, untransduced cells cultured under the same conditions all died, whereas transduced SCID-X1 cells remained viable after 21 days suggesting that cell signaling had been successfully restored.

6.1.7 Details of final vector construction and derivation (Appendix 1)

The SIN gammaretroviral vector backbone used in this study has been described in an earlier section. A schematic map of the plasmid (backbone based on pUC) and detailed sequence data shown in Appendix 1.

6.1.8 Clinical grade vector production

This has been undertaken by the vector production facility (VPF) at Cincinnati Children's Research Foundation. Previously, production of SIN gammaretroviral vectors has been compromised by difficulties in achieving usable titers. However, advances in the design and configuration of these vectors, has resulted in greatly improved yields, albeit by transient production techniques in clinically validated cell lines such as 293 HEK, or following virus concentration. The vectors proposed for this study have been shown to yield effective titers of up to 10^7 transducing units (γc expression in cell lines) per ml of cell culture supernatant, which is readily applicable to existing clinical protocols. Consistent with the pre-existing vector, viral particles will be GALV-pseudotyped, as this may confer advantages with respect to transduction of early progenitor cells and HSC. Certification of clinical grade vector supernatant with a titer of >1 X 10^6 transducing units/ml is currently underway.

All clinical viral vector production open manipulations are performed in an ISO Class 5 Biosafety Cabinet located in an ISO Class 7 segregated tissue culture suite with ISO Class 8 support areas. All practices and documentation are consistent with GMP recommendations for early phase US FDA retroviral vector products to be administered under an IND. The retroviral vector is produced by transient transfection in a closed system disposable bioreactor. Cells from a 293T certified Master Cell Bank are expanded using standard tissue culture flasks and roller bottles in a fetal bovine supplemented cell culture medium. Cells are harvested, transfected with plasmids SRS11.EFS.IL2Rg.pre*, pCDNA3.MLV.g/p, and K83-GALV using Calcium Phosphate transfection reagents and seeded into Corning Cell Bind 10 Stacks. The 10 Stacks are placed in a temperature and CO_2 controlled incubator. Following overnight incubation tissue culture medium is replaced and virus is harvested with media re-feeds at three 12 hours intervals. The harvested virus is aliquoted into closed system cryobags and frozen at <-70° C., with appropriate samples aliquoted for certification and evaluation. Following the final harvest, end of production cells are removed for certification assays and appropriate reserves are cryopreserved in DMSO in vapor phase liquid nitrogen.

Vector and end of production cells are submitted for certification for purity, potency, and identity. These assays include sterility/bacteriostasis and fungistasis (21CFR610.12), mycoplasma (PTC), endotoxin (USP), in-vitro adventitious virus (3 cell lines), RCR (5% of supernatant and 10^8 post production cells by S+L- assay (using 293 HEK cells for amplification) performed at IU NGVL certification laboratory), residual DNA (PCR), identity (non-GMP LTR to LTR sequencing of proviral insert in permissive target cell), and potency (sponsor defined). All results and records are Quality Assurance reviewed prior to product release.

6.2 General Information related to the Investigational Medicinal Product (IMP)

6.2.1 Name description of the IMP:

pSRS11.EFS.IL2RG.pre* gammaretroviral vector (used to transduce autologous CD34+ cells, ex vivo), see **Appendix 1**. This vector is based on a Moloney murine leukemia virus packaged in a gibbon ape leukemia (GALV) pseudotype.

pSRS11.EFS.IL2RG.pre* is an unlicensed product (not applicable for licensing).

6.2.2 Production, Supply and Release of the IMP:

The Vector was produced, certified and will be supplied by the vector production facility of Cincinnati Children's Research Foundation, Division of Experimental Hematology, Cincinnati Children's Research Foundation, Cincinnati Children's Hospital Medical Center, Cincinnati OH, 45229, USA.

Transduction of autologous CD34+ cells with the retroviral vector pSRS11.EFS.IL2RG.pre* will be carried out in the GMP cell manipulation facilities at each participating institution.

The transduced cells will be packaged in sealed transfer bags prior to being transported according to local practice for cellular product distribution at room temperature to the clinical site of administration. Cells released from the GMP facility will be infused according to local SOPs and practice, with minimum requirements specified in the Manual of Operations.

6.2.3 Drug accountability

Drug accountability is ultimately the responsibility of the Study Sponsor. This responsibility however will be delegated to the Clinical Scientist responsible for the transduction procedure.

Detailed records will be kept to allow for accurate accountability of the vector & transduced CD34+ cells. These records will include details of shipping, receipt, storage, use & destruction of the vector. Transfer of CD34+ cells from the cell manipulation laboratory at each institution to the site of administration. Administration of the transduced CD34+ cells to patients will be recorded.

6.2.4 Description of and justification of the trial treatment & dosage

The retroviral vector pSRS11.EFS.IL2RG.pre* is used to transduce autologous CD34+ cells. These transduced cells are then infused into the patient. The autologous CD34+ cells undergo 2 or 3 rounds of retroviral transduction. The transduced cells are administered intravenously into the patient as one infusion over 15-30 minutes.The transduction protocol has been optimized to achieve a gene transfer efficiency of 30-40% in starting cells, which will produce a mean copy number of ~1 for engrafted T-cells.

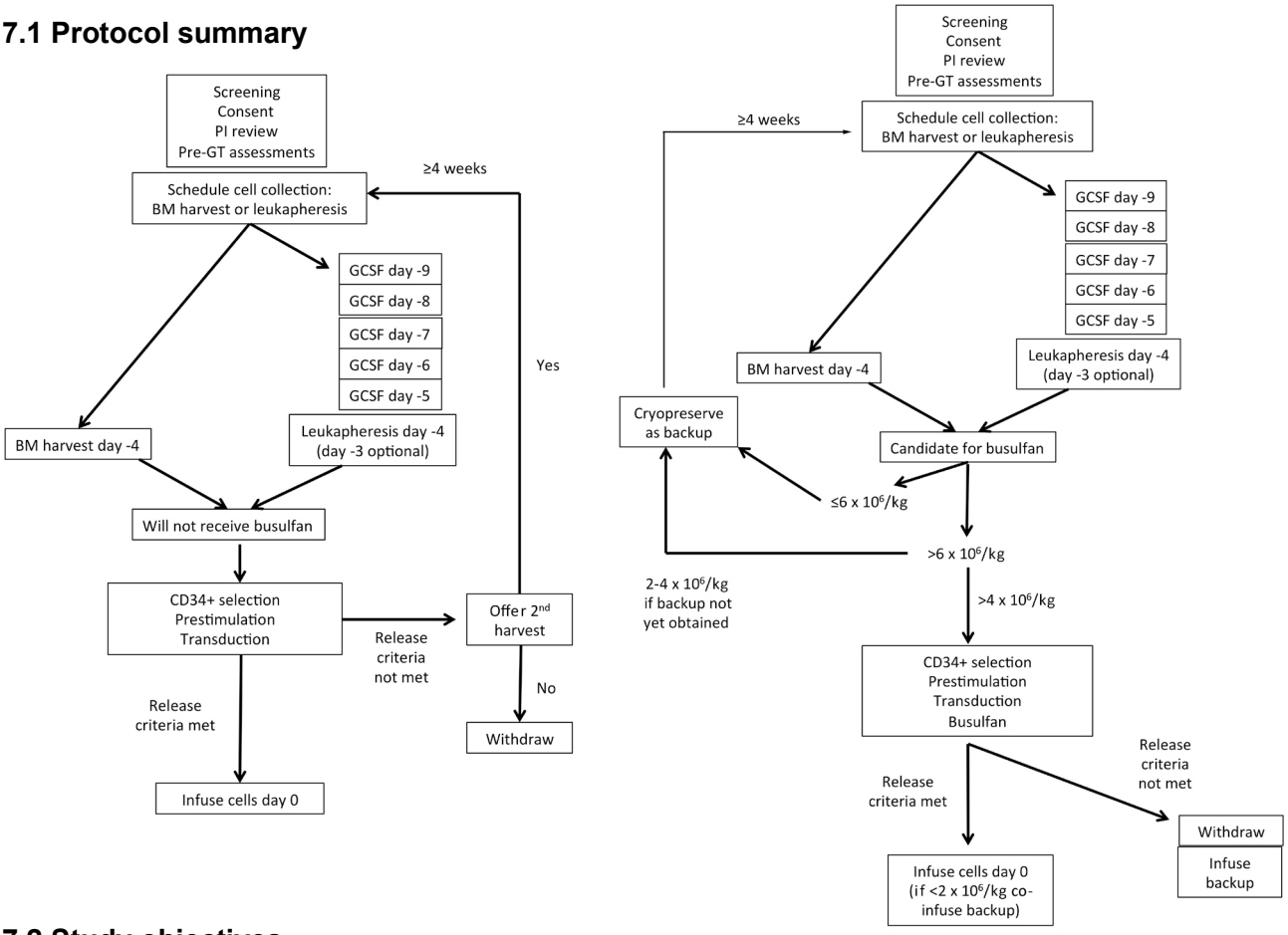
7. Study Design

An open labeled, non-randomized, multi-center, phase I/II, cohort study involving a single infusion of autologous CD34+ cells transduced with the self-inactivating (SIN) gammaretroviral vector pSRS11.EFS.IL2RG.pre* in up to 9 patients with SCID-X1 at Children's Hospital Boston, Mattel Children's Hospital UCLA, Cincinnati Children's Hospital Medical Center. These three sites are funded by NIAID/DAIT and fall under the IND 14067.

Adequate vector has been produced by Cincinnati Children's Medical Center to treat a minimum of 20 patients. In addition to the 9 patients in this trial, 11 patients will be enrolled in parallel independent trials under separate protocols at two European Centers (Great Ormond Street Hospital and Hopital Necker). We will collect data only on the 9 patients enrolled on this trial in the United States. We will review limited safety and efficacy data provided to us by the European sites for statistical purposes and reporting to the DSMB as detailed in section 10.5.

We detailed evidence in section 5.3.1 regarding improved humoral immune reconstitution in older SCID-X1 patients undergoing gene transfer after low dose busulfan.Thus, this protocol envisages that of 20 patients total, 5 who meet criteria for receiving chemotherapy conditioning will undergo treatment with low dose busulfan prior to receiving transduced cells. The remaining 15 will receive transduced cells without conditioning. The primary endpoint for all patients remains the same, T cell reconstitution and safety.

7.1 Protocol summary



7.2 Study objectives

The objectives of this proposal are to initiate a trial of somatic gene transfer for patients with SCID-X1 in whom HLA-matched family donors are unavailable or in whom underlying clinical problems would exclude chemotherapy conditioning. For this study, autologous CD34+ cells will be transduced *ex vivo* using a novel gibbon ape leukemia (GALV)-virus pseudotyped self-inactivating (SIN) gammaretroviral vector (pSRS11.EFS.IL2RG.pre*). The specific objectives are as follows:

1. Treatment of SCID-X1 patients by somatic gene transfer when HLA-matched family donors are unavailable.
2. Successful *ex vivo* transduction of CD34+ hematopoietic cells from SCID-X1 patients by *ex vivo* gammaretrovirus-mediated gene transfer.
3. Evaluation of immunological and functional reconstitution in progeny of engrafted cells.
4. Longitudinal evaluation of clinical effect in terms of augmented immunity.
5. Evaluation of the functional performance of novel SIN gammaretroviral configuration.
6. Evaluation of the molecular characteristics of vector integration.
7. Evaluation of safety.
8. In a descriptive graphical fashion, to present immune reconstitution data for the subgroup of patients treated with a low-dose conditioning regimen including busulfan.

7.3 Study endpoints

Primary endpoint

1) Immunological reconstitution defined as absolute CD3 cells of >300/μl and PHA stimulation index >15 at 6 months post infusion.
2) Incidence of life-threatening adverse reactions related to the gene therapy procedure.

Secondary Endpoints

1) Molecular characterization of gene transfer.
2) Ability to mount antibody responses to vaccination.
3) Normalization of nutritional status, growth, and development.

8. Patient selection and recruitment

Up to 9 patients will be recruited at Children's Hospital Boston, UCLA Mattel Children's Hospital and Cincinnati Children's Hospital Medical Center and will be selected for inclusion on the basis of the following defined criteria (see Appendix 2 for decision tree):

8.1 Inclusion and exclusion criteria

Inclusion criteria

1. *Diagnosis of SCID-X1 based on immunophenotype (<200 CD3+ autologous T cells,, and confirmed by DNA sequencing)*

AND

2. *Lack an HLA identical (A, B, C, DR, DQ) related donor*

AND either one of the following

3a. *Patients in good clinical condition who do not have a readily available HLA identical (A,B,C,DR,DQ) unrelated donor (readily available defined as: a donor confirmed within 6 weeks of searching, with ability to transplant within 3 months of diagnosis). Such a patient would be eligible to receive busulfan conditioning.*
3b. *Patients with an active, therapy-resistant infection or other medical conditions that significantly increase the risk of allogeneic transplant. Such a patient is NOT eligible to receive busulfan conditioning. Examples of "therapy-resistant infections that significantly increase the risk of allogeneic transplant" include but are not limited to:*

a) interstitial pneumonia due to adenovirus or parainfluenzae virus 3
b) protracted diarrhea requiring total parenteral nutrition
c) disseminated BCG infection
d) virus-induced lymphoproliferative disease
e) any active opportunistic infection (eg, due to Pneumocystis jiroveci, cytomegalovirus, cryptosporidium) that does not improve on medical management
f) active and progressive pulmonary disease requiring mechanic ventilation.

Inclusion of patients with disease-related organ dysfunction is justified by the known poor outcome for patients with active infection undergoing standard treatment and the potential life-saving nature of the

treatment proposed. Patients who are on high-dose steroids or other immunosuppressive agents will also be considered eligible, because use of these drugs is common in patients with SCID and maternal T cell engraftment or who present with severe interstitial lung disease. Use of immunosuppressive drugs does not affect efficacy of hematopoietic cell transplantation, and therefore should not affect efficacy of gene transfer.

Exclusion criteria

1. No available molecular diagnosis confirming SCID-X1.
2. Patients who have an available HLA-identical related donor.
3. Diagnosis of active malignant disease other than EBV-associated lymphoproliferative disease
4. Patients with evidence of infection with HIV-1
5. Major (life-threatening) congenital anomalies. Examples of "major (life-threatening) congenital anomalies" include, but are not limited to: unrepaired cyanotic heart disease, hypoplastic lungs, anencephaly or other major CNS malformations, other severe non-repairable malformations of the gastrointestinal or genitourinary tracts that significantly impair organ function.
6. Other conditions which in the opinion of the P.I. or co-investigators, contra-indicate collection and/or infusion of transduced cells or indicate patient's inability to follow the protocol. These may include for example clinical ineligibility to receive anesthesia, severe deterioriation of clinical condition of the patient after collection of bone marrow but before infusion of transduced cells, or documented refusal or inability of the family to return for scheduled visits. There may be other unforeseen rare circumstances that would result in exclusion of the patient, such as sudden loss of legal guardianship.

Although the presentation of the disease may be variable in type, the severity of the immunodeficiency is uniform. The gene transfer protocol will be instituted in the place of haploidentical transplant for those patients who do not have a matched family donor or in whom an unrelated donor transplant is not indicated for the reasons specified above. Apart from the gene transfer protocol, the patients will not undergo additional procedures that would not form part of an equivalent haploidentical transplantation regimen.

8.2 Enrollment of subjects

Patients will be enrolled following diagnosis and referral to the Immunology/BMT Services at each institution. SCID-X1 is an inherited disease and therefore most patients with this inherited disease are diagnosed while they are still infants and are therefore incapable of giving informed consent. Informed consent will be obtained from parents/guardians.

The Principal Investigator at each site will discuss the study at length with the parent/guardian of a potential new subject, and provide as much time as possible for their review and consideration. The parent/guardian will be encouraged to ask further questions about the study to the Investigator or designees. Consent procedures at each site will be in accordance with the site's IRB requirements for obtaining informed consent, and reviewed by the sponsor prior to site initiation.

After consent is obtained, the eligibility of each patient will be discussed by the site PIs, the study sponsor/grant PI, and grant co-PI by conference call. Study procedures will only commence if all PIs agree that the patient is eligible.

Because of the life-threatening nature of the underlying disease, and the potential life-saving benefits of the procedure proposed, no staggering of patients is considered necessary. However, because of the rarity of the disorder and the planned recruitment of 3 patients per U.S. site in 5 years, we anticipate simultaneous enrollment of two or more patients to be very unlikely.

9. Treatment protocol

9.1 Harvest of CD34+ cells

9.1.1 Bone marrow harvest or mobilized peripheral blood leukapheresis

For patients weighing less than 10 kg, bone marrow harvest will generally be used as the source of $CD34^+$ cells. For patients weighing 10 kg or more, mobilized peripheral blood cells will generally be used as the source of $CD34^+$ cells. In situations where one method fails or is not preferred, the other method will be considered as an alternative. Cryopreserved umbilical cord blood cells may also be used if available.

Bone marrow will be harvested from the patient under general anesthesia from the posterior iliac crests on both sides by multiple punctures. If necessary the anterior iliac crests may also be used. The amount of marrow collected will be up to 20 ml/kg of body weight.

For mobilized peripheral blood cell collection, filgrastim will be administered by subcutaneous injection for 4 days at 10 micrograms per kilogram of body weight per day. The morning after the 4th dose, leukapheresis will be performed according to standard clinical procedures. Plerixafor (Mobozil), administered by subcutaneous injection at 0.24 milligrams per kilogram of body weight 10 hours prior to the day of collection, can be given daily for up to 4 days in consultation with the clinical apheresis team. The number of CD34+ cells from 2 consecutive days of apheresis may be added together for consideration.

9.1.2 Back-up product collection in patients receiving busulfan

A minimum of bone marrow or mobilized peripheral blood cells containing 2 x 10^6 CD34+ cells/kg and optimally 4 x 10^6 CD34+ cells/kg will be cryopreserved unmanipulated, according to standard institutional procedure, for infusion in situations described in Sectios 9.3.4 and 10.3.

9.2 Low dose busulfan conditioning for selected subjects

Patients who meet inclusion criteria 1, 2, and 3a will receive low dose busulfan conditioning on days -3 and -2 with an optional dose on day -1. Conditioning may also be given starting day -4 to adjust for individual institutional or patient circumstances, and doses may be administered on non-consecutive days (e.g., -4, -2). Busulfan will be administered only after verifying an adequate estimated $CD34^+$ cell dose of at least 4 x 10^6 CD34+ cells for transduction (pre-manipulation). The starting dose of busulfan will be 3 mg/kg (see Appendix 5). Busulfan will be infused over 3 hours, and levels will be measured according to institutional norms starting at the end of infusion until 6-8 hours post infusion to calculate the area under the curve (AUC). The AUC will be calculated after each dose, and the dose will be adapted to reach a cumulative exposure of 30 mg*h/L. AUC exposure that falls +/- 5 mg*h/L will be accepted. If the total exposure falls short of the target after 2 doses, a 3rd dose may be given to achieve the goal. For further details see Appendix C. If the total exposure is achieved, subsequent doses should be omitted. At least 24 hours must elapse between the end of infusion of the last dose of busulfan and infusion of transduced cells.

Prophylaxis for seizures with levetiracetam or lorazepam should be given per institutional standards.

9.3 CD34+ cell culture, transduction and infusion

9.3.1 CD34+ cell culture and transduction

CD34+ cells will be purified, cultured and transduced in a dedicated cell manipulation facility at each institution. CD34+ cells will be purified using standard protocols for stem cell transplantation (CliniMACs, see 9.1.2). Purified CD34+ cells will be seeded into gas permeable flexible plastic

containers in serum free medium, and cytokines; IL-3 (20ng/ml), SCF (300ng/ml), FL (300ng/ml), TPO (100ng/ml). After pre-stimulation and 2 repeats of bag transduction in serum free medium using vector preloaded on clinical grade RetroNectin (Takara Bio) and treated with Pulmozyme (Genentech) cells will be formulated and samples collected as described in section 9.2.2.

The transduced cells that meet release criteria will be transported immediately to the patient for re-infusion. Infusion will occur less than or up to 4 days following initial vector exposure as described in section 9.3.

9.3.2 Testing of product prior to patient re-infusion

Samples are collected at approximately 0, 40 hours and at the end of incubation for cell viability (trypan blue stain, PI-annexin) and at 0 hr and end of incubation for proliferation and sterility (for bacteria, endotoxin and mycoplasma). Samples are taken at 0, 40 hours and at the end of incubation for immunophenotyping (CD45+ & CD34+), in addition to samples taken at 0 hours and the end of incubation to monitor the gene transduction procedure (PCR for transgene, flow cytometric determination of γc expression). Similarly, at completion of the transduction process, a sample will be analyzed for Gram stain (the result of which will be known prior to re-infusion) and endotoxin measurement. The endotoxin result in EU/kg/hour will be calculated based on the final infusion volume and patient weight (EU per ml x infusion volume / patient weight), this will be available prior to infusion. A further minimum of 5% of the used culture medium, and 1% of the total cell number (or 10^8 cells, whichever is less) will also be archived to test for replication-competent retrovirus (RCR). The result of this investigation will not be known prior to reinfusion, although the viral stocks will have been previously tested, and the chances that RCR will be generated during the transduction process are extremely small. Some cells will also be frozen for later analysis if required. A minimum of >1 x 10^6/kg CD34+ cells after transduction are required for infusion into the patient. Transduced cells must be endotoxin negative and Gram stain negative for release.

<u>Failure to meet release criteria</u>

If transduced cells fail to meet release criteria for viability, sterility or endotoxin testing, they will not be infused. Therapeutic options, including a second attempt to perform gene transfer or allogeneic hematopoietic stem cell transplantation, will be discussed with the subject or legal guardian. If consent is given to perform a second attempt of gene transfer, the bone marrow harvest will be performed not earlier than 4 and not later than 8 weeks after the first harvest. If the product from the second harvest fails to meet release criteria, the subject will come off study and proceed to alternative treatment, i.e. allogeneic hematopoietic stem cell transplantation.

Pre-infusion Sample Archive and testing.

A pre infusion sample of recipient blood will be sent to for mononuclear cell preparation to be cryopreserved and stored within a designated repository.

Pre-infusion RCR Screening:

Pre-infusion samples of the transduced product and patient peripheral blood will be archived. to test for replication competent retrovirus and S+L- assays.

<u>Post-Infusion Patient Positive PCR Screening Results</u>

PCR for GALV-env (Gibbon Ape Leukemia Virus envelope) will be performed on patient peripheral blood samples. In the event of a positive GALV-env PCR testing on patient peripheral blood samples, a repeat PCR and S+L- assays will be performed and the archived sample of pre-infusion peripheral

blood will be thawed, tested by PCR and S+L-assays.
A subject with a persistently positive PCR assay but negative S+L- assay may continue on the study but only after consultation with the DSMB and the monitoring plan post infusion will require RCR monitoring by S+L- assay.

9.3.3 Infusion of transduced cells

Cells will be washed and infused in a volume of 20-50 mls intravenously over 15-30 minutes on day 0. Routine observations will be performed throughout the infusion period, and hourly thereafter for 6 hours.

9.3.4 Criteria for co-infusion of back-up product in patients receiving busulfan

$CD34^+$ cells ≥1 x 10^6/kg after transduction is required to infuse transduced cells. If $CD34^+$ cell count after transduction is <1 x 10^6/kg, transduced cells will not infused and back-up product will be thawed and infused. If $CD34^+$ cell count after transduction is ≥1 x 10^6/kg but <2 x 10^6/kg, transduced cells will be co-infused with back-up product. If $CD34^+$ cell count after transduction is ≥2 x 10^6/kg, transduced cells will be infused alone.

10. Evaluation and Follow-up

10.1 Assessment of safety and efficacy (Appendix 4)

10.1.1 Immunological reconstitution

Detailed analysis of immune recovery following gene transfer for primary immune deficiency (PID) is essential to evaluate the effectiveness of the procedure, and is largely beyond the scope of our routine clinical immunology resources. We have therefore established dedicated resources and methodologies that enable us to 1) measure the diversity and complexity of T and B reconstitution 2) analyze thymic education and output following gene modification of lymphocyte precursors and 3) measure cellular antigen specific responses:

- The lymphocyte subsets (LSS) immunophenotyping panel will be carried out to show the distribution of cells and is used to detect an increase in naïve CD3+ T lymphocyte cell numbers and assess the development of normal distribution of CD4,CD8, TCRαβ, TCRγδ, CD16+CD56+ NK & γc surface expression. CD45RA, CD45RO, and CD31 expression is used to monitor naive and activated/memory T cells. TCR excision circles (TRECs) may be enumerated as a surrogate marker for new thymic emigrants following gene transfer.
- Whole blood lymphocyte proliferation assays will be carried out to test function of T cells.
- Representation of TCR families by flow cytometric analysis (Vβ phenotyping), combined with CDR3 PCR spectratyping (Vβ spectratyping) also forms an important part of monitoring for both physiological and potentially pathological clonal expansions.
- Restoration of antibody production (IgA, IgM, IgG), and serological responses to vaccinations and natural infections (such as varicella) will be assessed.

For the purpose of assessing success of gene transfer, we define successful reconstitution as a peripheral blood CD3 count of >300/µl and in vitro PHA stimulation index of >15 at 6 months post-infusion of gene modified cells.

Failure will be defined as patients who do not meet these reconstitution criteria at 6 months, OR are described as an early failure by 10.2.3.

Weight and linear growth will be followed and recorded during each evaluation.

10.1.2 Molecular characterization of gene transfer

Molecular characterization of gene transfer in patient cells is also an important parameter for assessment of efficiency, and potentially for assessment of safety:

-Percentage of vector positive T cells

- Quantification of transgene copy numbers is determined on sorted cell populations by real-time PCR methodology. Detailed integration analysis maybe used to investigate specific clonal expansions.

For the purposes of this protocol, subjects who have lack of gene marking (<0.1% of vector positive T cells detected by quantitative PCR i.e. <0.001 copies per cell) on two consecutive determinations will be considered to have failure of gene transfer. If insufficient T cells are available for gene marking, peripheral blood mononuclear cells will be tested instead by the same criteria. Such subjects will generally be taken off study (see section 10.3.1). If the subject is eligible for and proceeds to a 2nd gene transfer procedure (see Section 10.2.4), he will remain on study.

Peripheral Blood Clonal Analysis

Peripheral blood with detectable proviral sequences of > 0.01 copies per cell by 6 months post infusion of gene modified cells will be further analyzed for clonality by LAM PCR. A copy number of 0.01 copies per cell is presumed equal to 1% of circulating white blood cells (WBC's) containing the proviral sequence, based on a copy number of one proviral insertion site per transduced cell. Clonal analysis will be monitored at a minimum of every 6 months for the first 5 years and then yearly for up to 15 years post infusion if > 1% of the WBC's shows the presence of the corrective gene. Peripheral blood with < 1% of the WBC's showing detectable proviral sequences during the first year post infusion will be analyzed for clonality by LAM-PCR at 6 months and 1 year. Peripheral blood with no detectable proviral sequences will not undergo clonal analysis.

Clinical laboratory abnormalities may trigger further clinical work-up and insertional analysis, and conversely, insertional analysis suspicious for clonal expansion may trigger further clinical work-up. Laboratory abnormalities that may trigger clinical evaluation will include WBC >30,000/microliter, absolute lymphocyte count >10,000/microliter, or expansion of Vβ or Vδ TCR families suggesting proliferation of a homogenous population on two consecutive evaluations that are 1 week apart. If clonal analysis of peripheral blood with gene marking in > 1% of the WBC's reveals that greater than 1/5th (>20%) of the gene modified population is derived from a single clone in conjunction with leukocytosis, the site investigator, Sponsor and Project Manager will be notified. This will also trigger additional clinical and laboratory investigation.

Work-up will include detailed physical examination, complete blood count and differential, lymphocyte subsets, infectious studies of blood, stool, sputum, urine and/or tissue cultures, and imaging studies if deemed appropriate. If an infectious cause is found or is considered likely, monitoring of clinical lab abnormalities at regular intervals is recommended, every 1-4 weeks at the discretion of the site investigator, with regular updates to the Sponsor and Project Manager. If imaging studies are suggestive of a possible proliferative disorder, biopsies and appropriate infectious, pathological and molecular tests will be performed. If laboratory abnormalities resolve or improve significantly, the patient will be monitored at the next scheduled study point.

If more than 20% of the gene modified population is derived from a single clone, and yet no other clinical or laboratory abnormalities are identified, close clinical monitoring (with visits at least every 2 weeks) and careful repeat of peripheral blood clonal analysis (at least once a month) will be performed to document possible further expansion of the clonal population.

If laboratory or pathology studies are suggestive of hematologic malignancy, a bone marrow aspirate will be performed to assess the possibility of myelodysplasia or leukemia, including morphology and cytogenetics and other clinically indicated molecular diagnostics. Insertion site analysis and immunophenotypic analysis will be performed on both blood and bone marrow. If a subject undergoes this evaluation, the DSMB, NIH and FDA will be informed for consideration of a temporary suspension of inclusion of new subjects in the study until diagnostic evaluation is complete.

If more than 20% of the gene modified population is confirmed to be derived from a single clone for 3 consecutive times, in the absence of clinical or laboratory signs indicative of infections or autoimmunity, a bone marrow aspirate and appropriate morphology, cytogenetics and molecular tests will be performed as indicated above. Insertion site analysis and immunophenotypic analysis will be performed on both blood and bone marrow. If a subject undergoes this evaluation, the DSMB will be informed.

If there is no evidence of myelodysplasia, leukemia or any other tumors that might be caused by clonal expansion of gene modified cells, proviral copy number, clonal analysis, immunophenotyping, and TCR repertoire from peripheral blood will be performed a minimum of every 3 months as long as a predominant clone is detected and > 1% gene marking of circulating WBC's persists. If the predominant clone falls consistently below 20% of the gene modified population, the patient will be monitored at the next scheduled time point. If the predominant clone is expanding further or if other lab abnormalities are detected, bone marrow aspirate will be repeated.

If the copy number decreases to < 1% of the circulating WBC's, the frequency of monitoring after the first year will be decreased to every 6 months for up to 5 years post infusion. If proviral sequences become undetectable, clonal analysis will not be performed. Proviral copy number will continue to be evaluated on a yearly basis for up to 15 years post infusion. Insertion site specific PCR may be utilized if available for early screening for re-emergence of specific clones.

10.1.3 Safety assessments

Efficacy and safety of the gene transfer procedure will be further assessed though clinical examinations, clinical laboratory assessments. Adverse reactions observed by the investigator or reported by the patient/parent/guardian during the study period will be recorded for evaluation (see section 11.0 Recording and Reporting of Adverse Events). Patient samples will be analysed for replication competent retrovirus (RCR), an essential safety test to detect the potentially pathogenic wildtype strains of the virus at 3 months, 6 months, 12 months and then yearly for 15 years. Discarded samples at any time point when blood is scheduled to be drawn will be archived for further studies relevant to this protocol.

10.1.4 Additional assessments in patients receiving busulfan
Complete blood counts will be monitored per institutional norm, at minimum twice weekly from date of infusion until documentation of neutrophil recovery, defined as the first day of absolute neutrophil count >500/microliter for 3 consecutive days after neutrophil nadir. Need for platelet transfusions and packed red blood cell transfusions will be recorded. Monitoring for veno-occlusive disease will be performed per institutional norm, including weight, liver function tests and physical examination, at minimum twice weekly from date of infusion until 21 days after infusion.

10.2 Investigations & Monitoring schedule

Trial Coordination and Monitoring Structure

The Clinical and Translational Investigator Program (CTIP), Quality Assurance for Clinical Trials Office (QACT, Dana-Farber Cancer Institute), Project Manager will provide important services necessary for

this multi-institutional clinical trial. CTIP will provide the methodological and technical support needed to ensure excellence and quality in the proposed studies. More specifically CTIP will support 1) development of study forms and training for data entry; 2) statistical analysis and reporting of results to promote the rapid dissemination of the findings; 3) preparation of study data and adverse events to provide to the DSMB. QACT will be responsible for coordination of enrollment, with documentation of agreement of enrollment by the PI's of participating institutions and the study sponsor. The Project Manager will be responsible for 1) development and maintenance of manuals of operation; 2) study coordination and standard operating procedures and monitoring; 3) coordination of the DSMP across all institutions; 4) coordination and dissemination of adverse event reports including expedited reports C.R. Matthew Hodgson will be responsible for trial monitoring at Children's Hospital Boston and coordination of monitoring of other participating sites.

10.2.1 Pre-treatment Investigations

For all investigations requiring phlebotomy (pre-treatment and post-treatment) associated with this protocol, the volume of blood draws will be limited to 5ml/kg at one time. If maximum volume is reached, not all studies may be possible at each time point and a prioritization of tests would be utilized. The priority for testing would be 1) safety endpoints (CBC, chemistries, RCR, integration sites; 2) clinical immunology end-points, gene marking; and 3) more research related analysis such as TCR and TREC.

Routine gene transfer pre-treatment investigations will be carried out according to **Appendix 4**.

Pre-treatment monitoring investigations will be carried out within 3 months of the start of gene transfer treatment according to **Appendix 4**. In case a second bone marrow harvest is planned, results of pre-treatment investigations will be considered valid if performed within 3 months of the date of the repeat harvest.

10.2.2 Post-infusion monitoring

Monitoring of patients will follow the patient monitoring protocol (see **Appendix 4**). Modifications to this protocol will be adopted as necessary to improve sample-processing capability, and a degree of flexibility regarding the actual dates of assessment will be maintained. Additional tests may be carried out in the event of a significant adverse effect to ensure optimal clinical care.

CBC and blood chemistries will be performed prior to infusion and then daily for three consecutive days to detect post-infusion acute adverse events. For patients with evidence of pre-infusion organ dysfunction, CBC and blood chemistry data will be made available to the DSMB to help discriminate between disease-related and procedure-related adverse events. In case of serious acute adverse events that are possibly- or probably related to the procedure, enrollment of new patients will be temporarily suspended until full review by the DSMB and the FDA.

Samples for RCR analysis after the first year will be archived for future testing should it be required (See section 10.3.5). Serum samples and cells stored in liquid nitrogen will be kept indefinitely for future analysis should it be required.

Bone marrow samples will be obtained if required as part of the clinical care of an unwell patient or in a situation where the patient is being administered a general anesthetic (a bone marrow sample would then be requested at the same time) or if clonal hematopoiesis is documented by LAM-PCR analysis. Routine morphological and cytogenetic analysis, analysis of transgene copies in CD34+ cell populations, and also if sufficient cells are available progenitor cell (CFU), and xenograft repopulating cell assays (SRC) will be carried out on the bone marrow. Common integration sequences will be sought between myeloid cells, CFU and T cell populations.

10.2.3 Early failure of immune reconstitution

Early failure of immune reconstitution is determined by:

1) lack of an increase in autologous T cell counts over baseline in at least two of three assessments at days 60, 90, and 120

AND

2.) lack of gene marking

Patients who are deemed to be an early failure have the option of retreatment as described in 10.2.4 or of referral for allogeneic hematopoietic stem cell transplantation.

Patients that undergo a allogeneic transplant will continue on with the monitoring protocol and data collection according to standard post BMT procedures. Subjects that give consent for retreatment with gene transfer will be monitored on study according to the monitoring protocol and data collection according to this protocol, however will be considered a failure under the criteria outlined in 10.1.1 with regards to analysis of the primary endpoint.

10.2.4 Retreatment Option for failure of immune reconstitution

Patients who are deemed to be a failure of immune reconstitution as detailed in Protocol section 10.1.1 or 10.2.3, will be offered the option of a repeat gene transfer procedure if the following criteria are met:

1.) Subject continues to meet the original protocol eligibility criteria per 8.1 including *do not have a readily available HLA identical (A,B,C,DR,DQ) unrelated donor.* Lack of a readily available HLA identical unrelated donor at the time that consent is obtained for the repeat procedure will be sufficient to meet this criterion.
2.) Agreement and consensus of all study PIs of the eligiblitiy of the patient for a 2nd gene transfer procedure.

The repeat gene transfer procedure will follow the study procedures outlined in Section 9 – Treatment Procedures and Section 10 – Evaluation and Follow – up for the 1st Gene transfer procedures. For purposes of study evaluation, patients would follow the evaluation and follow – up procedures with Day 0 being the day of the 2nd Gene transfer infusion.

10.3 Procedure for failure of hematologic reconstitution in patients receiving busulfan

If absolute neutrophil count >500/microliter for 3 consecutive days after neutrophil nadir is not achieved by day +35, clinical evaluation which may include bone marrow aspirate and peripheral blood analysis for gene marking will be performed in consultation with the protocol PI/co-I. Persistent failure of hematopoietic recovery will be treated with infusion of thawed back-up cells and/or by proceeding immediately to allogeneic transplantation with the most suitable donor available.

10.3 Withdrawal of individual subjects

A patient can withdraw or be withdrawn from protocol treatment in the study at any time from enrollment until the transduced CD34+ cells have been administered. Once a patient has been administered the transduced CD34+ cells study treatment is complete.

Subjects who wish to discontinue from the study at any time are free to do so. However the reasons for discontinuation should be documented by the investigator if possible.

Patients who withdraw from the study after administration of the transduced CD34+ cells will be encouraged to have follow-up investigations so that the consequences of the administration can be documented and analyzed.

Any patient withdrawn prior to administration of transduced CD34+ cells will be replaced in the study.

10.3.1 Off study criteria

A patient will be considered off study under the following circumstances:

a) The patient is withdrawn from the study prior to administration of transduced CD34+ cells.

b) The patients/parent/guardian withdraws consent for study procedures and data collection.

c) The patient is lost to follow-up.

d) The patient who has failure of gene transfer (defined as <0.1% of vector positive T cells or peripheral blood mononuclear cells on two consecutive determinations, see 10.1.2).

e) Completion of protocol specified therapy and observation.

Study procedures and assessments will not be performed once the patient is taken off study.
Patient who meet the definition of early failure of immune reconstitution at day 120, however, will have assessment of gene marking performed according to the study schedule until the definition of failure of gene transfer above has been met.

10.3.2 Follow-up of withdrawn subjects

Patients withdrawn from the study prior to administration of transduced CD34+ cells and patients, for whom consent has been withdrawn for the study, will resume normal clinical care. Efficacy and safety assessments will not be carried out from the point of withdrawal and data will not be recorded in the case report forms (CRFs).

10.4 Premature termination of the study

Enrollment of the study will be suspended under the following circumstances:

a) Expiration or exhaustion of vector stock may prevent further recruitment of patients.
b) Any leukemia potentially related to provirus insertion or treatment-related death. Planned infusion of a gene manipulated product in any subject already enrolled will be suspended; those who have already received a gene modified product will continue to be evaluated per protocol. The events will be reviewed as quickly as possible by the DSMB, IRB and FDA if one of these serious adverse events is observed.
c) Overall stopping of trial would be made in consultation with the DSMB due to other serious and related adverse events occurring on trial. We will notify the FDA and each site Institutional Review Board if the study stopping rules are triggered.
d) Lack of efficacy (see 10.5.1).

10.5 Statistical considerations

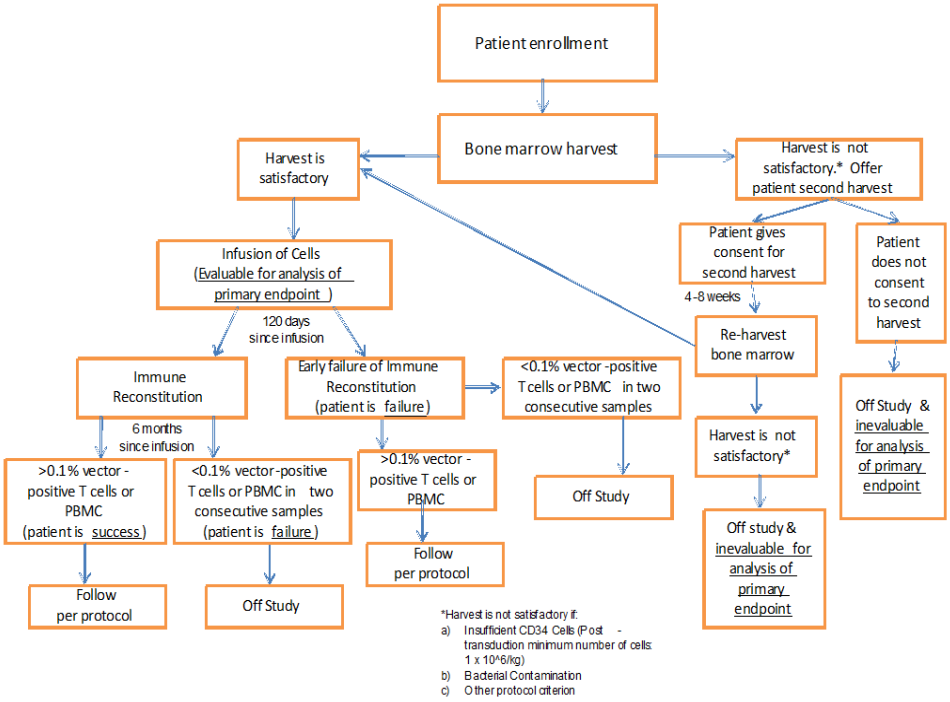
A maximum of 9 patients on this trial and an additional 11 patients in independent trials in Europe (total 20) will undergo gene transfer with this vector. A formal stopping rule for lack of efficacy will be applied to all 20 patients (see below), and additional data analyses will be mostly descriptive in nature.

"Success" in each individual patient will be determined by evidence of immune reconstitution defined by a peripheral blood CD3 lymphocyte count >300/µl and a PHA Stimulation Index >15 by 6 months post-infusion of gene modified cells.

"Failure" will be defined as patients who do not meet these reconstitution criteria at 6 months, OR are described as an early failure by 10.2.3.

Evaluability
All patients who receive an infusion of the transduced CD34+ cells are evaluable for inclusion in the stopping rule analysis. Patients for whom the bone marrow harvest is unsatisfactory after a maximum of two attempts will go off study, and will be inevaluable for (excluded from) the stopping rule analysis. See Figure 10.5.

Figure 10.5 Application of protocol definitions of evaluability, successes, failure, and off study



10.5.1 Stopping rule for lack of efficacy

This rule serves dual purposes of monitoring for safety and the determination of efficacy (assessment of primary objective 3, section 7.2). We designed a rule-based inferential procedure for this trial with the guidance of East clinical trial software (version 5, Cytel Inc., Cambridge, MA). The null hypothesis (H_0) is that the probability of success is at least P=0.65, a benchmark based on the historical success of haploidentical transplantation (37, 39, 99). Our design specifications included a maximum of 20 evaluable patients; decision points after 5, 10, and 20 evaluable patients; and a Type I error-spending budget proportional to the cumulative number of enrolled evaluable patients. We rounded the asymptotic software-generated stopping criteria to the next lower integral number of failures and obtained the following rule for the 3 decision points:

- If 4 of the first 5 evaluable patients fail immune reconstitution, then stop the trial and reject H_0; otherwise continue.
- If 7 of the first 10 evaluable patients fail, then stop the trial and reject H_0; otherwise continue.

- If 11 of the 20 evaluable patients fail, then reject H_0. Otherwise 'accept' H_0, which is to say, the results are consistent with H_0.

Each of the above criteria is to be interpreted as specifying that the trial will stop as soon as the criterion is reached; e.g., if the first 4 evaluable patients are failures, then trial stops immediately without going on to a fifth patient. Similarly, up to the point where 10 evaluable patients are enrolled and evaluated, the trial halts as soon as the seventh failure occurs. Finally, beyond the tenth patient, the trial halts as soon as 11 failures occur, only proceeding through to the full sample of 20 if the number of failures has never exceeded 10. If there are 12 or more patients who are a success, then we will conclude that there is sufficient evidence of benefit from gene transfer therapy. We will provide a descriptive analysis of the data if fewer than 20 evaluable patients are enrolled and the stopping rule was not triggered, including placement of a 95% exact confidence interval on the success rate.

We used SAS software (version 9, SAS Institute, Cary, NC) to simulate the performance of this rule under a range of assumed values for *P*. We conducted enough repetitions to determine the likelihood of rejecting or 'accepting' H_0 within 0.001. Results are shown in the accompanying table.

P (true success rate)	Power (probability of rejecting H_0: $P \geq 0.65$)	Expected evaluable patients
0.75	0.029	20
0.65	0.099	19
0.60	0.186	18
0.50	0.474	16
0.40	0.789	13
0.30	0.960	9

These figures indicate that our stopping rule is a conservative strategy, in that 'acceptance' of H_0 is virtually guaranteed only if the true rate somewhat higher than the hypothetical minimum for success (*P*=0.65). For example (first line), if the true rate is *P*=0.75 then the trial has a 98% chance of running through the full 20 evaluable patients and H_0 being 'accepted.' If the true rate is exactly *P*=0.65, the lower boundary of hypothesized success (second line), then the trial has only 90% probability of completing 20 evaluable patients and 'accepting' H_0; a 10% chance remains that the trial will be stopped early and H_0 rejected. The expectation in this case, because of occasional early stopping, is an average of 19 rather than 20 evaluable patients.

The table shows that smaller success rates carry higher probabilities of correctly rejecting H_0, with smaller expected patient numbers due to early stopping. At the extreme of the rates tabulated above, where the true value is only *P*=0.30 (bottom line), the trial has a 96% chance of stopping after an average of only 9 evaluable patients.

The Type I error rate for this inferential procedure may be defined as the average power for all values of *P* greater than 0.65, weighted according to their *a priori* likelihood. A conservative estimate is obtained by assuming equal likelihood for all values between 0.65 and 0.75. We conducted simulations for this range, using close spacing (not shown) and obtained average power of 0.051. Thus the chance of rejecting H_0 under a conservative version of the null hypothesis, which is to say concluding that the success rate is lower than *P*=0.65 when in fact it is somewhere between 0.65 and 0.75, is 5.1%, virtually identical to the convention Type I error rate used in routine statistical inference.

10.5.2 Data analysis and reporting

Data to be reported, in addition to the outcome of the inferential procedure, will include detailed analysis of the immunological reconstitution of each patient, (detailed in Appendix 3), presence and copy number of the vector in specific myeloid and lymphoid cell populations with time after infusion of gene modified CD34+ cells, expression levels of the transgene by flow analysis in lymphoid cell populations and frequency of side effects. Analysis of data for the subgroup of patients undergoing busulfan conditioning, beyond data contributing to the primary endpoint and safety stopping rules, will be performed separately and limited to descriptive graphical presentation.

11. Safety issues related to gene transfer protocol

11.1 Venipuncture

Hazards associated with blood drawing are those associated with venipuncture and, those associated with the loss of red blood cells as a result of removal of blood from the procedures described in this protocol. Risks of venipuncture are pain, the potential for formation of a hematoma at the site of the needle stick and a small potential for infection at the site of needle stick. Occasionally, individuals having venipuncture may faint as a result of a vasovagal reaction to the procedure. These risks will be minimized by carefully preparing the site of needle puncture with alcohol swab, by using the smallest size needle required for the procedure, and by having the patient lie down or sit as appropriate for 5 or 10 minutes after venipuncture. The major risk of repeated blood drawing is anemia from removal of red blood cells. To minimize this risk the amount of blood being drawn for all purposes will be tracked to ensure that minimal volumes are collected, and to anticipate the need for blood transfusion.

11.2 Bone marrow harvest

Bone marrow will be harvested from the patient under general anesthesia from the posterior iliac crests on both sides by multiple punctures. The amount of marrow collected will be equivalent to 15% of total blood volume. The risks of the procedure include the standard risks of general anesthesia and complications associated with the bone marrow harvest procedure. The latter include pain at the site of puncture following the procedure, excessive blood loss resulting in anemia and the potential for infection at the puncture site. The anesthetic risks will be minimized by the anesthetic being administered by a pediatric anesthetist. The patient will be treated with appropriate pain medication after the procedure and will be monitored carefully to check hemodynamic status. The amount of marrow to be collected has been calculated such that the desired cell count will be obtained without the need for further transfusion. However, transfusion of red blood cells may be required. A CBC should be drawn no later than one day prior to marrow harvest. If anemia (Hb <9 g/dl) is observed prior to bone marrow harvest, the use of red blood cell transfusion will be recommended before the harvest is performed. At all other times, institutional guidelines should be followed to prevent and/or treat symptomatic anemia.

Infection risk will be minimized by careful cleansing of the site prior to needle insertion and strict adherence to sterile technique during the procedure. A second bone marrow harvest may be performed if the product fails to meet release criteria. The second harvest would be performed no sooner than 4 weeks and no later than 8 weeks from the first harvest. The risks of the second harvest remain the same as for the first harvest.

11.3 Retrovirus mediated gene transfer

11.3.1 Insertional mutagenesis

Insertional mutagenesis remains a finite risk of gene transfer using integrating vectors. However, modifications to the vector design including removal of LTR enhancer sequences, and utilization of a less potent promoter sequence for transgene expression, have been shown to significantly diminish

mutagenic risk in experimental systems. It is therefore probable that the risk of clinically significant mutagenesis will be substantially reduced.

11.3.2 Germline transmission of vector sequences

Hematopoietic cells are manipulated and transduced *ex vivo*, and extensively washed prior to re-infusion. In addition, retroviral particles are inactivated by human complement *in vivo*. Therefore the risk of gene transfer to other tissues, including gonads, is extremely small.

11.3.3 Quality control of harvest and transduction process

Manipulation of cells *ex vivo* is potentially associated with contamination. However, all manipulations will be undertaken in a dedicated cell manipulation laboratory at each site, following clinically acceptable guidelines and to GMP standards. To minimize contamination, all procedures are conducted within closed culture bag systems. In addition, cells will be tested for microbial contamination and endotoxin prior to re-infusion. A minimum of 1 x 10^6/kg CD34+ cells after transduction are required for infusion into the patient.

11.3.4 Infusion of transduced cells

Based on results from other gene transfer studies and our own experience, the infusion of cultured and gene altered autologous blood progenitors does not appear to be associated with any significant reactions. In this study, the medium used to culture the CD34+ cells is free of animal serum. The virus producer cell cultures must be maintained in medium with fetal calf serum and therefore the virus supernatant will contain animal serum. Infusion of any type of blood cell product can be associated with reactions resulting from clumping of these cells or other immediate reactions related to sticking of these cells to blood vessels in the lungs. Reactions are treated by stopping the infusion and providing oxygen, antihistamines, steroids and medications or fluids to increase blood pressure. Because infused cells in this stud are autologous CD34+ cells, the possibility that this type of agglutination or vascular reaction will occur is very low. It is theoretically possible for the CD34+ cell cultures to become contaminated with microrganisms. One of the important safety features of this gene transfer protocol is that CD34+ cells will be cultured in a sealed bag culture system. Gram stains and routine cultures will be performed on cell samples the day before infusion and on the day of infusion. If significant growth of pathogenic microrganisms is detected, the cells will not be returned to the patient. Endotoxin levels will be measured prior to infusion and cells will not be infused if levels exceed 5 EU/kg per hour of infusion.

Excess cells remaining after testing required for the protocol will be frozen in DMSO and archived at each participating site. Serum samples and cells stored in liquid nitrogen will be kept indefinitely for future analysis should it be required.

11.4 Conditioning Regimen (Busulfan)

Immediate side effects relating to the use of alkylating agent chemotherapy are mainly limited to the gastrointestinal tract with transient nausea and vomiting followed by stomatitis, esophagitis and diarrhea, which resolve with neutrophil recovery. All subjects will receive standard antimicrobial prophylaxis according to Children's Hospital Boston Hematopoietic Stem Cell Transplantation Unit standard guidelines. With these measures, the immediate risk to the subject as a direct result of the conditioning and period of neutropenia will be no more than that associated with standard allogeneic HSCT. If there is a failure of bone marrow recovery after infusion of transduced cells, back up bone marrow harvested prior to therapy will be administered. The risk of secondary malignancy associated with the use of this regimen for HSCT in non-malignant disease is very low, however fertility may be compromised. As is standard prior to HSCT, sperm or testicular tissue preservation when appropriate and available will be offered prior to the procedure.

12. Reporting Adverse Events

Adverse Event Reporting

A. Definitions of adverse events, serious events, and unexpected AEs

For purposes of this study, an adverse event will be defined as any unfavorable and unintended diagnosis, symptom, sign (including any abnormal laboratory finding deemed by the PI to be clinically significant), syndrome or disease which either occurs during the study, having been absent at baseline, or, if present at baseline, appears to worsen.

Unexpected adverse event will be defined as any adverse event that is not listed as a risk in the current version of the Protocol or Investigator Brochure.

Adverse reaction will be defined as any adverse event caused by the Investigational Product. Suspected adverse reaction will be defined as any adverse event for which there is a reasonable possibility that the Investigational Product caused the event.

Adverse Event recording and reporting begins once the subject starts the bone marrow harvest, which is the first protocol procedure.

All adverse events will be graded by the site investigator according to the criteria specified by the National Cancer Institute's (NCI) Common Toxicity Criteria (CTC) V 3.0.

It is the responsibility of the treating physician to document all adverse experiences in the patient chart. At each assessment, adverse experiences will be evaluated and a detailed description of the event will be documented on an adverse event case report form that will include:

- Description of event
- Onset date/Start date
- Resolution date/Stop date
- Severity of event (Grade I-IV based on NCI common toxicity criteria)
- Relationship to study:
    - Unrelated
    - Unlikely
    - Possible
    - Probable
    - Definite
- Classification of event:
    - Expected
    - Unexpected
- Action taken:
    - None
    - Medication Therapy
    - Procedure
    - Hospitalization
    - Other

- Patient outcome to date:
    - Recovered
    - Under Treatment/Observation
    - Alive with sequelae
    - Death
    - Unknown

All adverse events will be entered into the InForm database in the manner and timeframe specified in the Manual of Procedures.

Serious Adverse Events:

For purposes of this study, a serious adverse event will be defined as any untoward medical occurrence that:

- Results in death: this is defined as death from any cause while the patient is on study.
- Is life-threatening. This is defined as any Grade IV event that is possibly, probably or definitely related to the investigational agent, gene modified autologous CD34+ cells
- Results in persistent or significant disability/incapacity
- Is a new malignancy
- Any clonal changes found on gene transfer safety testing analysis or routine bone marrow (defined as greater than 1/5 or 20% of clones derived from a single clone, as discussed in section 10.1.2)
- Requires unanticipated in-patient hospitalization or prolongation of existing hospitalization
- Leads to a congenital anomaly or birth defect
- Is an event that required intervention to prevent permanent impairment or damage

All serious adverse events that occur during the time the patient is on this study, whether or not related to the study, must be reported by the Principal Investigator to the Study Sponsor and Project Manager within 24 business hours of knowledge of the occurrence.

Investigator Reporting Requirements to Sponsor:

Expedited Reporting:

The following adverse events must be reported by the Principal Investigator to the Study Sponsor and Project Manager, within 24 business hours of knowledge of the occurrence. These events will be captured for up to 5 years post infusion. Sites will be provided with a Serious Adverse Event Form for expedited reporting purposes.

- Any Adverse Event that meets the criteria for a Serious Adverse Event.
- Grade IV events that are considered directly attributed to this study (related events: possible, probable or definite) and that are not listed as an expected event in the current version of the Investigator Brochure or Protocol.

Adverse events:

All adverse events that do not meet criteria for expedited reporting to the Sponsor will be reported at a minimum, monthly (first of the month) for the first year post infusion, on a quarterly basis (March 1st, June 1st, September 1st, and December 1st) up to 5 years post infusion. After 5 years post infusion, grade 3 and higher adverse events that do not meet criteria for expedited reporting to the Sponsor will be reported at a minimum annually (March 1st) to 15 years post infusion. Each site will be provided with

an Adverse Event Log which should be reviewed, signed and dated by the Principal Investigator and submitted to the Study Sponsor and the Project Manager at the following email addresses noted below:

1. Sponsor: David Williams, MD
Chief, Hematology/Oncology
Children's Hospital Boston
Karp 8
300 Longwood Ave.
Boston, MA 02115
Phone 617-919-2697
FAX: 617-730-4697
E-mail : DAWilliams@childrens.harvard.edu

2. Project Manager:
Colleen H. Dansereau, MSN,RN, CPN
Program Manager – Gene Therapy Program, Division of Hematology/Oncology
Boston Children's Hospital
Boston, MA 02115
Phone: 617-919-7008
Email: colleen.dansereau@childrens.harvard.edu

The CTIP will compile and report safety data information to the DSMB. The Study Sponsor verifies grading and determines relatedness. The DSMB will verify these classifications at the next regularly scheduled meeting.

Sponsor Reporting Requirements to Federal Regulatory Bodies

Expedited reporting requirements:
Serious adverse events, life threatening events or deaths are to be reported to the FDA and the Data Safety Monitoring Board by the Project Manager by telephone or fax as soon as possible but no later than 7 days after the IND's sponsor's receipt of the information. Report to the NIH/OBA (via GeMCRIS reporting mechanism) will occur concurrently.

Reports of serious, unexpected adverse events associated with the use of the gene transfer agent, will be reported within 15 days after the sponsor's receipt of information.

A copy of expedited reports to the FDA will also be concurrently forwarded to all site PIs, IRBs and IBCs, and NIAID Medical Monitor.

Non–expedited reporting requirements:
Other adverse events occurring under this IND not meeting the criteria for expedited reporting will be documented during the course of the trial and will be included in the report to the FDA and OBA/RAC annually.

Contact Information for Reporting Purposes:

CBER, Office of Therapeutic Research and Review
Food and Drug Administration
Document Mail Center HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 201052
301-1027-5101, phone
301-1027-5397, fax

Office of Biotechnology Activities
National Institutes of Health,
6705 Rockledge Drive,
Suite 750, MSC 7985
Bethesda, MD 20892-7985
(All non-USPS mail should use zip code 20817)
301-496-9838, phone
301-496-9839, fax
oba@nih.gov

Data Safety Monitoring Board (DSMB):

The DSMB has been assembled, and has been approved by the National Institutes of Health (NIH), NIAID/DAIT. The Medical Monitor from NIAID/DAIT is invited to participate in the open sessions of the DSMB meetings. Arrangements will be made for DSMB members to meet with the Study Sponsor and other members of the study to review any interim SAEs and otherwise on no less than a biannual basis. The DSMB findings will be provided to the Sponsor, Dr. Williams. The Project Manager will ensure that the FDA, NIH/OBA, NIAID/DAIT Medical Monitor, and Site Investigators all receive copies of the report.

European Data Consideration:

Data from patients enrolled in the trials in Paris and London will be collected by the investigators at those sites. Serious adverse events occurring in Paris or London will be made available to the Sponsor (Dr. David Williams) for distribution to the DSMB, the FDA and NIH/OBA for evaluation and possible actions. While data collection forms will be harmonized for the purpose of data analysis and ultimate publication, United States sites will not monitor European sites with respect to data collection. NIAID/DAIT funds will be used solely for data collection at US sites, not at European sites.

Table 2a: Years 0 – 5 Post Infusion (Subjects not receiving busulfan)~

Relation to Gene Transfer		Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Life-threatening)	Grade 5 (Fatal)
	Unrelated Unlikely	Required Report on the 1st of every month (-/+ 7 days) until 12 months post-infusion then quarterly* up to 5 years post infusion			Required Reported to the Sponsor on the 1st of every month (-/+ 7 days) until 12 months post-infusion then quarterly* up to 5 years post infusion	Required Report within 24 business hours to Sponsor
	Possible Probable Definite	Required Report within 24 business hours to Sponsor			Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor
Serious Adverse Event		Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor

* Adverse events not meeting criteria for expedited reporting should be submitted to the sponsor on the first of the month in year 1, then on a quarterly basis year 2 -5 (March 1st, June 1st, September 1st, and December 1st).
~ Events required to be reported within 24 hours to the Sponsor that meet criteria after Sponsor review will be reported as soon as possible but no later than 7 days after Sponsor receipt to DSMB, FDA and NIH/OBA. Concurrent copies of the FDA report will be reported to NIAID, to sites (PI, IRB, IBC). DSMB recommendations will be forwarded to sites (PI, IRB, IBC).

Table 2b: Years 0-5 Post Infusion (Subjects receiving busulfan)~

Relation to Gene Transfer		Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Life-threatening)	Grade 5 (Fatal)
	Unrelated Unlikely	Not required to be collected		Required – excluding abnormal hematologic laboratory results related to chemotherapy conditioning Reported to the Sponsor on the 1st of every month (-/+ 7 days) until 12 months post-infusion then quarterly* up to 5 years post infusion	Required - excluding abnormal hematologic laboratory results related to chemotherapy conditioning Report within 24 hours to Sponsor	Required Report within 24 hours to Sponsor
	Possible Probable Definite	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor
	Serious Adverse Event	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor

* Adverse events not meeting criteria for expedited reporting should be submitted to the sponsor on the first of the month in year 1, then on a quarterly basis year 2 -5 (March 1st, June 1st, September 1st, and December 1st).
~ Events required to be reported within 24 hours to the Sponsor that meet criteria after Sponsor review will be reported as soon as possible but no later than 7 days after Sponsor receipt to DSMB, FDA and NIH/OBA. Concurrent copies of the FDA report will be reported to NIAID, to sites (PI, IRB, IBC). DSMB recommendations will be forwarded to sites (PI, IRB, IBC).

Table 2c: Years 6-15 Post Infusion (all subjects)~

Relation to Gene Transfer		Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Life-threatening)	Grade 5 (Fatal)
	Unrelated Unlikely	Not required to be collected		Report annually	Report annually	Required Report within 24 business hours to Sponsor
	Possible Probable Definite	Required Report within 24 business hours to Sponsor			Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor
Serious Adverse Event		Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor	Required Report within 24 business hours to Sponsor

~ Events required to be reported within 24 hours to the Sponsor that meet criteria after Sponsor review will be reported as soon as possible but no later than 7 days after Sponsor receipt to DSMB, FDA and NIH/OBA. Concurrent copies of the FDA report will be reported to NIAID, to sites (PI, IRB, IBC). DSMB recommendations will be forwarded to sites (PI, IRB, IBC).

13. Public Health Considerations

Hematopoietic progenitors are transduced *ex vivo* in a closed culture system. The vector does not contain replication competent viruses, and will not be shed from transduced cells. The potential for transmission of vector sequences to other persons is therefore extremely small.

14. Ethical considerations

Good Clinical Practice

The study will be conducted in accordance with the International Conference on Harmonization (ICH) for Good Clinical Practice (GCP) and the appropriate regulatory requirement(s). Essential clinical documents will be maintained to demonstrate the validity of the study and the integrity of the data collected. Master files will be established at the beginning of the study, maintained for the duration of the study and retained according to the appropriate regulations.

Ethical Considerations

The study will be conducted in accordance with ethical principles founded in the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects (found at http://www.wma.net/e/). The DSMB and local IRB will review all appropriate study documentation in order to safeguard the rights, safety and well-being of the patients. The protocol, Investigator's Brochure(s), informed consent, written information given to the patients, safety updates, annual progress reports, and any revisions to these documents will be provided to the FDA by the Study Sponsor.

Data Safety and Monitoring Plan (See also Manual of Procedures)

Data and safety will be reviewed for each patient entered into this protocol by an independent Data Safety and Monitoring Board on at least a biannual basis. The Data Safety Monitoring Board is an independent group of experts who will advise the study investigators. The members of the DSMB serve in an individual capacity and provide their expertise and recommendations. The primary responsibilities of the DSMB are to 1) periodically review and evaluate the accumulated study data for participant safety, study conduct and progress, and when appropriate, efficacy and 2) make recommendations to the study investigators and regulatory agencies (IRB, IBC, FDA, etc.) concerning the continuation, modification, or termination of the trial. The review of data from each patient will occur by phone conference or in person on at least a biannual basis for all patients enrolled in the study once a subject is enrolled, either in the companion European trial or in the United States. Any SAE will be reported to the DSMB as outlined above.

Method to be Used in Procuring Consent of Subjects

All prospective patients will have the study explained by the PI of the research team at each site. The nature of the tests and procedures to be done will also be explained along with the potential hazards, possible adverse reactions and financial costs. The parent/guardian will be encouraged to ask further questions about the study to the Investigator or designee. Should a parent/guardian decide that the patient will participate they will be invited to sign the study consent form. Enrollment of each patient will be discussed with all site PIs, the study sponsor/grant PI, and grant co-PI after this consent is obtained; study sponsor will schedule the conference call to discuss eligibility within 48 hours of notification.

Prior to the initiation of the study, defined as initiating any procedure for purposes of evaluating patient eligibility not otherwise a part of routine patient care, acknowledgement of the receipt of this information and the subject's freely tendered offer to participate will be obtained in writing from each subject in the study. Those patients under the age of consent will voluntarily assent to the study under the same circumstances as their legal guardian and will sign the assent form.

This protocol, informed consent, assent form, and any amendments to the protocol will be reviewed by the IRB prior to initiation. The study will not be initiated without the approval of the IRB, whose operations must be in compliance with CFR 56; Title 21.

Written notice that the protocol and informed consent/assent forms have been reviewed and approved by the IRB will be submitted to the Investigator and the Sponsor prior to study initiation.

15. Administrative aspects

15.1 Data Handling, Record Keeping, Sample Storage

The Clinical and Translational Investigator Program (CTIP) will handle the data management for this trial. All data will be collated, analyzed and stored by CTIP. CTIP will also analyze data shared from the European sites as specified including data for purpose of joint publication (Section 12, European data considerations). Data shared from European sites will be obtained according to local approved practices and transferred in a coded fashion. Sample storage will be accomplished at each site. Specimens will be held indefinitely at each institution. If for any reasons samples cannot be maintained at the local institution, they will be shipped to the Study Sponsor for continued storage.

15.1.1 CRF completion

Selected data will be recorded on study specific case report forms (CRF's). Any paper CRFs will be completed in ink only, by personnel authorized to enter or change data on the CRF. Corrections can be made by striking out errors, with a single stroke, and not using correction fluid or obscuring the original entry. The correct entry must be entered by the side and initialled and dated by the person authorized to make the correction. Electronic CRFs will be completed in the InForm database.

Clinical and Research laboratory results will be held electronically in the study files of each subject. The CRF will document when the patient samples have been taken and when tests have been carried out.

15.1.2 Sample storage

A record of retained body fluids / tissue samples will be completed every time a sample is stored. This includes the patient trial identification number initials, date sample was stored, and storage location as well as the date the sample was moved or destroyed.

Samples (Cells, DNA & RNA) will be stored pre gene transfer and at regular intervals post gene transfer, and kept indefinitely at each site. Should it be required, samples will be disposed of in the appropriate manner according to local IRB guidelines, this will be detailed in the parent/guardian information sheet and consent obtained.

15.1.3 Record retention

Essential documents will be retained for a **minimum** of five years after completion of the trial. These documents will be retained for longer if required by the applicable regulatory requirements.

15.2 Patient confidentiality

In order to maintain patient privacy, all CRFs, IMP accountability records, study reports and communications will identify the patient by the assigned unique patient trial number.

Direct access to the patient's original medical records for verification of data gathered on the CRFs and to audit the data collection process will be permitted for trial-related monitoring & audits by the sponsor, IRB review, and regulatory inspection(s).

15.3 Monitoring & Audit

The trial will be monitored and audited according to the Standard Operating Procedures of the sponsor.

15.4 Amendments to study documents
Amendments are changes made to the research after approval by the IRB has been given.

A 'substantial amendment' is defined as an amendment to the terms of the IRB or FDA application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All amendments, whether considered substantial or not, must be approved by the sites IRB prior to initiating the amended protocol and/or consent. All amendments will be distributed to all sites for approval of local IRBs.

15.5 Definition of end of study & end of study report
The sponsor will notify the IRB and FDA of the end of the study within a period of 90 days.

The end of the study is defined as the last patient's last scheduled visit according to the protocol, which will be the 15 year follow-up of the last patient entered into the trial.

In case the study is ended prematurely, the Study Sponsor will notify the IRB and FDA within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the Study Sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study to the FDA and IRBs.

15.6 Insurance & indemnity
Children's Hospital Boston shall maintain the self-insurance program as approved by the Risk Management Foundation. Children's Hospital Boston is self-insured under CRICO, which has a rating equal to Best's A-.

15.7. Dissemination of results
Results of this study will be disseminated by publication, oral presentation at scientific meetings, and by direct communication with regulatory agencies.

16. Clinical facilities and arrangements
Children's Hospital Boston is a leading pediatric institution in the United States. CHB has an active Immunodeficiency Program within the Division of Immunology and an active Bone Marrow Transplant Program within the Division of Hematology/Oncology. Immunodeficiency patients are admitted to the Hematology/Oncology service at CHB and jointly managed by both services. Patients are cared for the Stem Cell Transplantation Unit that specializes in the care of immunocompromised patients.

A GMP facility (managed by Dr. Jerome Ritz) is located in the Jimmy Fund Building of the Dana-Farber Cancer Institute and is connected to CHB and managed by the Joint Program in Transfusion Medicine, which supports CHB, Dana-Farber Cancer Institute and the Brigham and Women's Hospital. The Connell and O'Reilly Families Cell Manipulation Core Facility (CMCF) at the DFCI is located in the Jimmy Fund Building-3 (JF-3). Standard operating procedures (SOPs) for CMCF have been approved by the Laboratory Director and Assistant Medical Director and are reviewed at least annually. Specific procedures have been implemented to ensure the integrity of this product. The laboratory has adequate space for the orderly placement of equipment and materials within the facility and to ensure that only

one product is processed in a given work space at a time. The manufacturing facility is classified as an ISO 7 cleanroom. Appropriate process and environmental controls are in place to ensure the facility operated well within the guidelines for an ISO Class 7 clean room. The CMCF laboratory at Dana-Farber is fully equipped for processing and cryopreservation of hematopoietic progenitor and other therapeutic cells as well as providing processing for other cellular products. The CMCF laboratory at the DFCI operates under the direction of Jerome Ritz, MD (Laboratory Director) and Grace Kao, MD (Assistant Medical Director). The CMCF and their processes have been designed to meet the current good manufacturing practice (cGMP) and current Good Tissue Practice (cGTP) for hematopoietic cell and other cell processing as required by the Food and Drug Administration's Center for Biologics Evaluation and Research (CBER), The Joint Commission and the Foundation for the Accreditation of Cell Therapy (FACT).

17. Qualifications of responsible clinicians and investigators

Dr. David Williams, Sponsor, is Chief of Hematology/Oncology, CHB and Professor of Pediatrics at Harvard Medical School and actively cares for children with non-malignant hematology diagnosis. He has served as PI, co-PI or sponsor of five human gene transfer trials in the past 15 years and has been active in gene transfer research since 1982. He currently serves as Editor-in-Chief of Molecular Therapy, the leading journal of gene transfer and serves on the NIH Recombinant DNA Advisory Committee.

Dr. Sung-Yun Pai, Principal Investigator, is Associate Professor of Pediatrics, Harvard Medical School and a member of the Hematology/Oncology faculty at CHB and DFCI. Dr. Pai has extensive training in immunodeficiency, attends on the Bone Marrow Transplant service at CHB and coordinates the care of children with immunodeficiency with the Immunology and Hematology/Oncology programs.

Dr. Wendy B. London, Co-Investigator, is the Director of Biostatistics and the Co-Director of the Clinical Translational Investigation Program. Dr. London has extensive experience in the design, conduct, interim monitoring, and analysis of clinical trials. In addition to being a faculty member in Hematology/Oncology at CHB and DFCI, she is a Senior Statistician in the Children's Oncology Group (COG) which conducts biologic studies and Phase I, II, and III therapeutic trials for children with cancer.

Dr. Jerome Ritz is Professor of Medicine, Harvard Medical School and Director of the Connell and O'Reilly Families Cell Manipulation Core, a GMP facility at the DFCI. Dr. Ritz has long-standing expertise in cell purification and cell manipulation and will oversee the purification and transduction of CD34+ cells.

Ms. Colleen H. Dansereau, MSN, RN, CPN, has extensive experience in gene therapy clinical research trial coordination and clinical research related activities. She is a board certified pediatric hematology/oncology registered nurse. She is responsible for the overall project management of this and other gene therapy trials at Boston Childrens Hospital.

18. References cited

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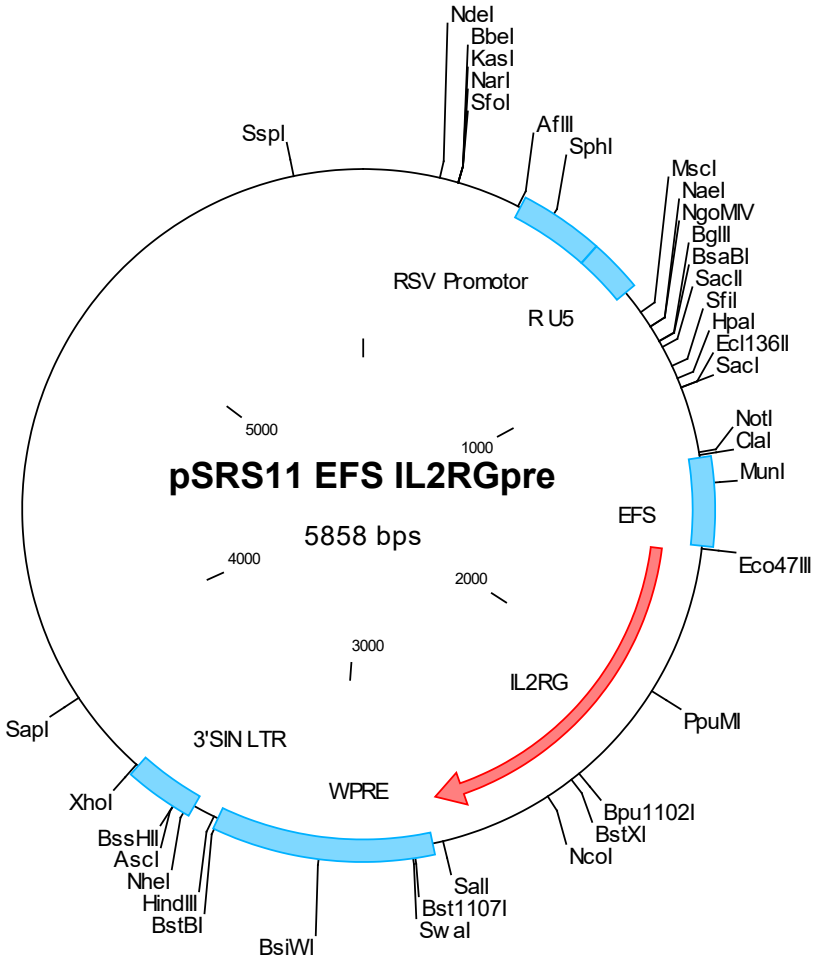
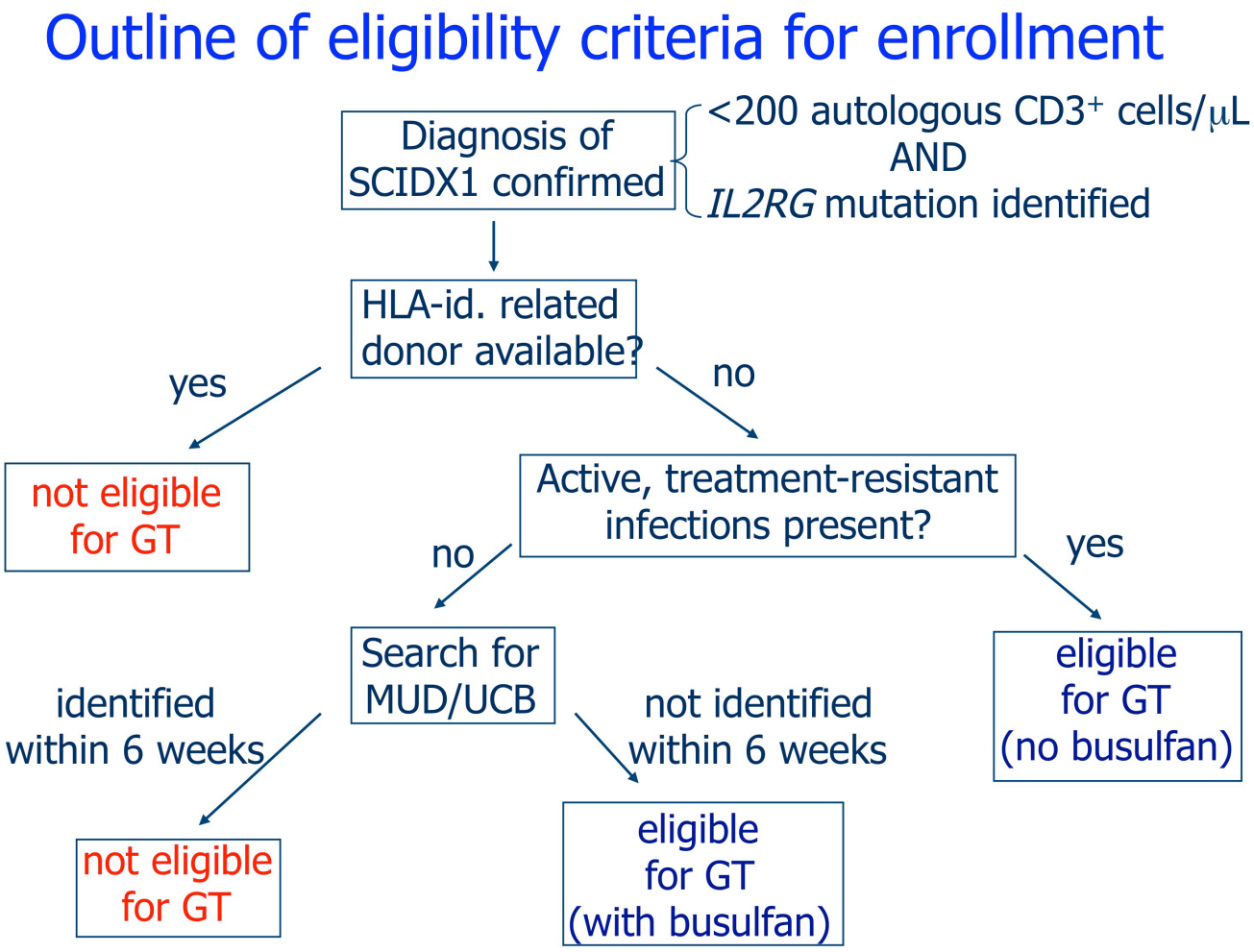
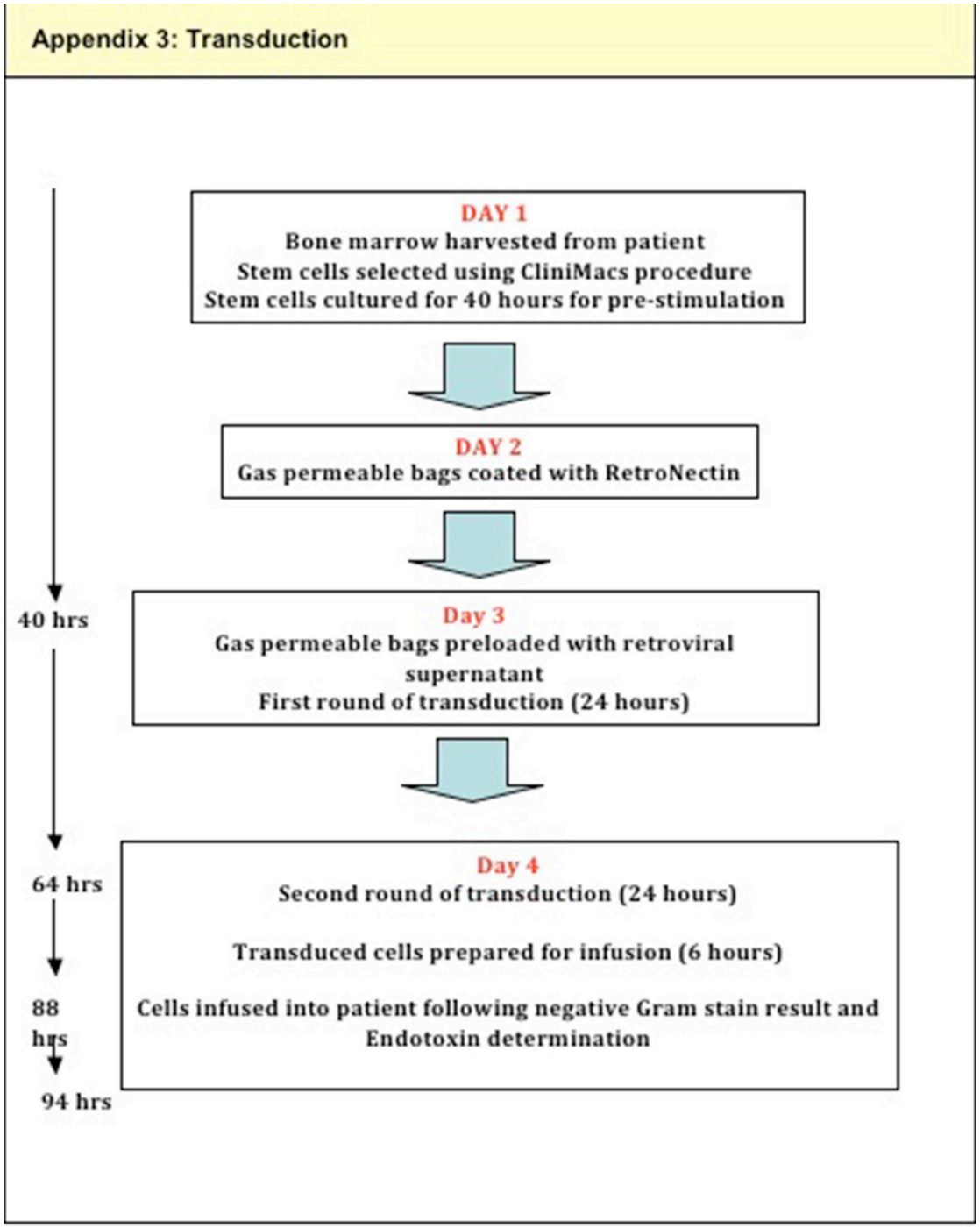
Appendix 1: Construction and sequence of SIN gammaretroviral vector pSRS11.EFS.IL2RG.pre*



Appendix 2: Outline of proposed criteria for enrollment



Appendix 3: Transduction



Appendix 4: Patient Monitoring Plan

	Time Points																
Lab category	**pre gene transfer**	**30 days**	**60 days**	**90 days**	**120 days**	**180 days**	**9 months**	**12 months**	**18 months**	**2 years**	**2 ½ years**	**3 years**	**3 ½ years**	**4 years**	**4 ½ years**	**5 years**	**5 ½ years**	**Yearly 6 to 15 years**
CBC and differential	X	X[7]	X	X	X	X	X	X	X	X		X		X		X		X
lymphocyte subsets	X	X	X	X	X	X	X	X	X	X		X		X		X		X
proliferation to PHA	X			X		X		X		X		X		X		X		X
Chemistries[5]	X	X	X	X	X	X	X	X	X	X		X		X		X		X
proliferation to tetanus									X[1]	X[1]		X[1]		X[1]		X[1]		X[1]
IgG, IgA, IgM	X					X[2]		X	X	X		X		X		X		X
tetanus IgG									X[3]	X[3]		X[3]		X[3]		X[3]		X[3]
Isohemagglutinins	X							X		X		X		X		X		X
γc expression and other research flow	X			X	X	X	X	X	X	X		X		X		X		X
lineage specific gene marking					X[6]	X		X	X	X		X		X		X		X
lineage specific transgene integration and clonality analysis						X		X		X[4]	X[4]	X[4]	X[4]	X[4]	X[4]	X[4]	X[4]	X
TREC	X					X		X		X		X		X		X		X
TCR Vβpanel						X		X		X		X		X		X		X
TCR spectratyping								X		X		X		X		X		X
replication competent virus	X			X		X		X	X	X		X		X		X		X
save serum	X			X		X		X	X	X		X		X		X		X
save cells	X			X		X		X	X	X		X		X		X		X

Study visits should occur at 30 days (+/- 3 days), 60 days (+/-7 days), 90 days (+/- 10 days), 120 days (+/- 14 days), 180 days (+/- 20 days), 9 months (+/- 30 days), 12 months (+/- 30 days), 18 months (+/- 30 days), 2 Years (+/- 30 days) and thereafter +/- 90 days.

1) T cell proliferation to tetanus will be checked at least 4 weeks after completing 3 vaccinations.

2) IgG infusions will be given according to institutional standard, and in general are expected to be maintain trough IgG at least >500 mg/dL. IgA in all subjects and isohemagglutinins in subjects with blood type O, blood type A or blood type B will be checked at visits indicated above. If IgA is > 12 mg/dL, and trough IgG is at least 500 mg/dL, IgG infusion may be stopped. IgG levels will be checked to document independence from IgG infusion according to standard clinical care. If criteria to stay off of IgG infusion are not met, the subject should go back on replacement. If IgG levels at least 2 months later are maintained or rising, the subject may stay off of IgG and vaccination including 3 doses of tetanus may be given according to published clinical standards (Griffith LM, Journal of Allergy and Clinical Immunology, 2009 vol. 124 (6) pp. 1152-60.e12). Prevaccination tetanus and other titers should be drawn as part of clinical care. Post-vaccination titer to tetanus will be drawn at the next study visit if it has not been drawn already as part of clinical care. Until protective responses to vaccines have been documented, the subject should remain on antibiotic prophylaxis. If the subject fails to mount a protective antibody response to tetanus, as determined by a titer of anti-tetanus antibodies >0.15 IU/ml 4 weeks after the third immunization, replacement therapy with IVIG will be reinstituted. IVIG replacement therapy will also be reinstituted in the event that the subject develops infection requiring hospitalization while being off-IVIG and on antibiotic prophylaxis.

3) IgG antibody to tetanus will be measured at least 4 weeks after completing 3 vaccinations.

4) Assay run every six months in years 2-5 and then yearly until year 15.

5) Chemistries include sodium, potassium, chloride, bicarbonate, blood urea nitrogen, creatinine, glucose, calcium, magnesium, phosphorus, asparate aminotransferase, alanine aminotransferase, total bilirubin, direct bilirubin.

6) If CD3 count is deemed too low to perform lineage specific sorting, gene marking will be performed in peripheral blood mononuclear cell fraction.

7) For patients receiving busulfan, CBC and differential will be monitored at least twice weekly until documentation of 3 consecutive days of absolute neutrophil count >500 cells/microliter after neutrophil nadir.

Appendix 5: Busulfan dosing guidelines

Busulfex doses will be adjusted based upon the area-under-the-curve calculations. AUC measurements at Boston Children's Hospital are expressed as micromole-min/L, which can be converted into mg*h/L if divided by 244, a factor derived from the molecular weight of busulfan 246 g/mole. For full myeloablation a total AUC of 23,200 micromole-min/L or 95 mg*h/L (equivalent to 16 doses of every 6 hour busulfan with AUC of 1450 micromole-min/L) is the maximum tolerated with regard to incidence of veno-occlusive disease when busulfan is combined with fludarabine.

The goal busulfan area-under-the-curve (AUC) for this protocol will be a total target exposure of 30 mg*h/L). If goal busulfan is exceeded, less than 3 doses may be given to achieve the goal total.

Goal AUC for each of 2 doses is therefore 3050-4270 uM x minute.

Dose adjustments must be discussed with the protocol site PI.

References:

Bartelink et al. Body weight-dependent pharmacokinetics of busulfan in paediatric haematopoietic stem cell transplantation patients: towards individualized dosing. Clin pharmacokinet (2012) vol. 51 (5) pp.
331-45.

EBMT/ESID Guidelines for Haematopoietic Stem Cell Transplantation for Primary Immunodeficiencies, 2011. Available upon request.

O'Donnell et al. Phase I study of dose-escalated busulfan with fludarabine and alemtuzumab as conditioning for allogeneic hematopoietic stem cell transplant: reduced clearance at high doses and occurrence of late sinusoidal obstruction syndrome/veno-occlusive disease. Leukemia & Lymphoma (2010) pp 879-885.

Appendix 6: Changes to protocol

*All dates are the IRB approval date from the Main site Children's Hospital Boston

Version 1 – Initial application (IRB approval date 07/13/2009)

Version 2 - (IRB approval date 10/06/2010):

Summary of Changes to Version 1

Since the most recent renewal of the protocol "A multi-institutional phase I/II trial evaluating the treatment of SCID-X1 patients with retrovirus-mediated gene transfer," (CHB 09-05-0254) the study has been extensively reviewed and critiqued by the NIAID/DAIT Clinical Research Committee. The majority of changes in the document were made in response to this review, and the study is now funded by NIAID (U01 AI087628). Additional changes were suggested by the DSMB and by the IRB at UCLA, a participating center. There are very few, if any, substantive changes. The changes are summarized below in order of occurrence.

Front pages:	Change in Co-PI at UCLA (De Oliveira) Clarification of roles on front page (per NIAID) Addition of version number (per NIAID) Addition of funding sponsor information and contacts (per NIAID) Listing of Steering Committee (per NIAID) Separate listing of European collaborators (per NIAID) Addition of Investigator signature page (per NIAID)
Section 5.2.1	Clarification of vector type (per NIAID)
Section 5.2.2	Addition that vector is certified (per NIAID) Clarification that local SOPs will be followed for cell infusion at different sites (per NIAID)
Section 6	Clarification of study design, this trial has 9 patients, parallel trials in Europe have 11 patients (per NIAID)
Section 7.1	Clarification, 9 patients (per NIAID)
Section 7.2	Informed consent will be in accordance to each site's IRB requirements (per UCLA IRB)
Section 7.2	Clarification that after consent, eligibility will be confirmed by all site PIs, study sponsor/grant PI and grant co-PI.
Section 8.2.1	Clarification of CD34 cell number to proceed to transduction (per NIAID)
Section 8.2.2	Clarification of release criteria, excluding fungus (per UCLA) Deletion of hanging phrase regarding RCR testing. Clarification of the Pre-Infusion PCR test requirement for GALV
Section 9.1.1	Clarification of failure for efficacy definition at 6 months versus early failure defined at 120 days (per DSMB)
Section 9.2	Modification of personnel and responsibilities for trial coordination.
Section 9.2.3	Clarification of early failure definition at 120 days (per DSMB).
Section 9.4	Premature termination of study defined as suspension of enrollment pending review by DSMB and other agencies (per NIAID).
Section 9.5	Clarification of 9 patients on US trial, but 20 included for stopping rules (per NIAID). Clarification of statistical definition of failure (per DSMB).
Section 9.5.1	Clarification of stopping rule (per NIAID).
Section 11	Adverse event reporting modified for change in personnel (CTIP and Project Manager to subsume responsibilities of DARCC). Clarification of events to be recorded and reported with regards to relatedness and expectedness. Clarification of the definition of clonal

changes. Addition of Table for clarification. Clarification that NIAID funds will not be used for European data collection (per NIAID).
Clarification of reporting protocol deviations and exceptions (per NIAID)

Section 13 DSMP has been deleted from Appendix 5 and is included instead in MOP.
Detailed plan of verification of eligibility by PI committee after consent is obtained.
Clarification that co-investigator may also consent patient.

Section 14 Modification to reflect changes in personnel from DARCC to CTIP.

Section 16 Addition of study staff qualifications.

Appendix 4 Modification of criteria for revaccination and modification of study test windows.
Modification of lab tests, removing some tests from pre-gene transfer time point.

Version 3 (IRB Deferred 11/8/2010):

Summary of Changes to Version 2

1) We propose a modification to add the possibility of a repeat bone marrow harvest and gene transfer procedure for the possible circumstance when the first bone marrow harvest is not successful due to contamination or other failure to meet release criteria. If cells fail to meet release criteria, they are not suitable for infusion. In this circumstance, the investigator will explain to the subject/guardian what happened, and the options open to the subject, including a) going through a second harvest 4-8 weeks after the first harvest and another attempt at gene transfer or b) coming off study and proceeding to allogeneic hematopoietic stem cell transplantation. (See page 21, 38, 45) We have reviewed past records and find that ~5-10% of bone marrow harvests are contaminated at our institution. The frequency at other institutions is not published, but it appears this level of contamination is compatible with what other programs experience. We will initiate at the same time a quality improvement process to reduce this incidence in our own program. The timing proposed is compatible with National Marrow Donor Program guidelines which call for a minimum of 4 weeks between marrow harvests, and has been discussed with our DSMB. The maximum time of 8 weeks is proposed to limit the time during which the subject remains without a definitive therapeutic procedure, recognizing the time it would take to arrange alternative bone marrow transplant. The consent form has been modified to reflect this change.
2) We propose a change in the minimum number of cells to infuse, based on available literature. Previously the protocol specified that a minimum of 2 x 10e6 CD34+ cells/kg would be required for infusion. Based on publications from the previous French trial, we propose to lower the minimum to 1 x 10e6 CD34+ cells/kg (Hacein-Bey-Abina et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. N Engl J Med (2010) vol. 363 (4) pp. 355-64; Ginn et al. Treatment of an infant with X-linked severe combined immunodeficiency (SCID-X1) by gene therapy in Australia. Med J Aust (2005) vol. 182 (9) pp. 458-63). The protocol previously stated that a minimum of 4 x 10e6 CD34/kg must be available for transduction to proceed. Given the high variability in growth of CD34+ cells in culture, we propose to proceed with transduction regardless of the recovery. (See page 37, 38, 46).
3) We clarified the definition of failure of gene transfer, which would lead to the subject coming off study. Subjects who have <0.1% of T cells and/or peripheral blood mononuclear cells containing vector sequences on 2 consecutive determinations will be defined as having failure of gene transfer and come off study (page 39, 42). We have also clarified that subjects who come off study because of early failure of immune reconstitution at day 120 (T cell count <200/microliter and/or absence of gene marking) would nevertheless be

monitored for gene marking until failure of gene transfer with 2 consecutive measurements has been documented (page 42).

4) Risk of possible blood transfusion because of anemia after bone marrow harvest was added to the protocol (page 45). This risk was already in the consent form.
5) Given that subjects enrolled on this protocol may be ill with active infection and therefore require a high volume of blood to be drawn for clinical sampling, we propose to eliminate the blood drawing maximum of 7.5 ml/kg or 450 ml as it would be unrealistic to get the necessary safety studies within this limitation. We will however continue to track the amount of blood drawn for clinical and research purposes (page 45). The consent form has been modified to reflect this change.
6) The consent form has been updated in the section regarding the occurrence of myelodysplastic syndrome in subjects undergoing gene transfer for chronic granulomatous disease, at the request of another site's IRB.
7) A number of other minor spelling errors were corrected and clarifications made.

Version 3 (IRB Approval date 11/22/2010):

Summary of Changes to version 2

The proposed modification to offer a second gene transfer procedure in case the first harvest product fails to meet release criteria potentially exposes the subject to additional risk including risks of second harvest (same risks as first harvest) and risk of remaining without immune reconstitution during the 4-8 week period required for hematopoietic recovery between harvests. We believe it is appropriate to offer this option to already enrolled subjects because the subject has not received gene manipulated cells and therefore has not yet had the chance to benefit from the trial. The consent has been modified to inform subjects that second harvest might be a possibility. The subject would only proceed to second harvest after discussion of alternatives, and informed consent for the second procedure would be documented.
The proposed modification to remove the limit on blood drawing increases the risk of subjects needing a blood transfusion for anemia. We believe this is justified because the study population may require clinical blood sampling that would make the drawing of blood for research purposes impractical within the limit and thus make it impossible to conduct the necessary safety monitoring. Thus we believe the risk of blood transfusion is less than the risk of failure to conduct safety monitoring.

Version 4 (IRB Approval Date 01/26/2011):

Summary Changes to Version 3

1) Section 9.2.2 – Change the wording of the RCR testing in order to reduce confusion. Used the words “Analyzed” and “Used” instead of “Withheld” as requested by the Indiana University Lab.
2) Section 9.2.2 - Integration analysis frequency duration change to be in line with FDA guidance, this was a requested by FDA.
3) Section 9.2.2 - Modify RCR testing to archive specimens, this is consistent with FDA guidance and simplifies process using a transient supernatant.

4) Section 10.1.2 – Change clonal analysis monitoring 2X/year post-GT for the first 5 years. This change is now in line with the FDA guidance, and requested by FDA.

5) Section 10.1.3 – Clarified that discarded blood samples will be archived for further studies relevant to this protocol.

6) Section 10.2.1 – Clarification on what pre-treatment investigations need to be recollected in case of a repeat bone marrow harvest.

7) Section 10.2.3 – Removed the term “intent to treat”, this was requested by our DSMB.

8) Section 10.5 – Change wording as DSMB requested that we clarify the protocol definitions of evaluability, success, failure and off study.

9) Section 11.2 – Clarification on blood draws if the patient is deemed to be anemic.

10) Section 12 – Clarification on AE reporting for events that do not meet the criteria for expedited reporting, this was a request of the NIH – NIAID.

11) Appendix 4 – Updated to reflect the changes in the protocol (Points 2 and 3 of this list).

12) ICF – Because data management is via our combined CHB/DFCI Clinical and Translational Investigation Program, we now include DFCI in the HIPAA section of our consent form

13) Study Ad – The UCLA IRB requested some minor changes to make it clearer that the pre-clinical safety data do not guarantee better clinical safety.

Version 5 (IRB Approval Date 05/23/2011):

Summary of changes to Version 4:

Requested that the stopping rules for the study be changed because the current criteria is too restrictive and may lead to premature referral for allogenic transplant, which would be unsafe for the patients. The proposed revision was recommended to us by our DSMB, documentation is attached. With regard of the approved version in section 10.2.3 of the protocol (page 41), early failure of immune reconstitution is determine by the following.

Current Approved Version:

1) Poor recover of T lymphocyte number (CD3<200µl) and/or
2) Absence of gene marking by 120 days

Our Proposed Version:

1) Lack of an increase in autologous T cell counts over baseline in at least two of three assessments at days 60, 90, and 120
AND
2) Lack of gene marking

The Sponsor Dr. Williams, will no longer have a financial interest in the use of Retronectin. The protocol and consent have been updated for this change.

Version 6 (Approved 11/14/2011):

Summary of scientific review for this amendment

This amendment broadens the inclusion criteria for the study.

X-linked SCID is a fatal disease due to failure of T cell development and B cell function, which is usually treated by allogeneic hematopoietic cell transplantation (HCT), i.e. bone marrow transplantation (BMT). Outcome of BMT is best when an HLA matched sibling donor is available. For those who do not have a matched sibling donor, alternatives include T cell depleted BMT from a parent, often performed without conditioning (haplo-BMT) and matched unrelated donor BMT with conditioning (URD-BMT). Survival in single institution and large survey studies are similar for these two approaches, ranging from 66-77% [1, 2]. Younger infants, those less than 3.5 months in one single institution experience, have higher survival after unconditioned haplo-BMT of 94% [3].

While survival overall is good, and survival in young infants is excellent, outcomes after alternative donor BMT for SCID are still not ideal. URD-BMT generally results in full T and B cell reconstitution but exposes small infants to chemotherapy conditioning [4]. Haplo-BMT without conditioning is nontoxic but sometimes requires repeat transplantation, frequently results in lack of B cell reconstitution (requiring life-long IVIG administration), and may be associated with waning immunity over time, though this last is controversial [5-9]. Either approach can be complicated by acute and/or chronic GVHD, and long-term a percentage of children treated with BMT have ongoing complications [10]. Gene therapy would avoid GVHD entirely by using the patient as his own donor, and if performed without conditioning, would avoid exposure of infants to chemotherapy. Gene therapy trials in Paris and London enrolled 20 subjects of whom 18 had successful and prompt T cell immune reconstitution ([11, 12] and slide 3). Unfortunately 5 of the 20 subjects developed T cell acute lymphoblastic leukemia due to insertion of the gene therapy retroviral vector into an oncogene, and 1 of these 5 children died [13-16]. The long-term outcome of the remaining 17 patients has been reported and all are infection free with no evidence of SCID ([17, 18] and slide 3). B cell reconstitution and function appear to be similar to patients undergoing unconditioned haplo-BMT [17, 18].

To maintain the excellent efficacy and reduce or eliminate the risk of leukemia, the retroviral vector was extensively modified (slide 4). In vitro data and murine in vivo data (not comparing directly to old clinical vector) were presented at the first review of this protocol at RAC on December 4, 2008. In the letter received on December 16, 2008 the following statement was made:

Healthy infants with X-SCID who are less than 3.5 months old should be excluded because they should be treated with a haploidentical transplantation [i.e. parent] which offers proven efficacy at their age.

Therefore the protocol inclusion criteria, exclusion criteria and informed consent document were revised to reflect this recommendation. The concerns cited included: 1. the risk of insertional mutagenesis, 2. the lack of efficacy data with this vector in humans, and 3. the established efficacy of alternative treatments.

We continue to maintain that gene therapy has the potential to be equally efficacious as standard BMT while avoiding GVHD, and that with full informed consent after discussion of alternative therapies and risks, parents of infants of <3.5 months of age may be offered the choice to enroll. Having enrolled 1 subject in Boston and learned of early efficacy in 2 subjects enrolled in Paris, we requested review of a potential amendment to the protocol and gave public review at the RAC on September 13, 2011. We addressed the three concerns above.

1. The risk of insertional mutagenesis
The onset of leukemia in the previously enrolled subjects was late, 3-5 years post gene therapy; therefore it is too soon to comment on safety with the new vector in humans. However, we did present key portions of extensive new data testing the old clinical vector (MFC □c) versus new clinical vector head to head in both in vitro human Jurkat cell experiments (slide 5), and in vivo murine bone marrow transplant experiments (slide 6). In all experiments there was less evidence of oncogenesis using the new clinical vector.

2. The lack of efficacy data with this vector in humans
We showed that the first patient enrolled in Boston on this trial has expression of the transgene (□c, slide 15), immune reconstitution as good (T cell) or better (NK cells) than the previous trial (slide 16 and 17), and clearance of therapy-resistant infection (slide 18, 19, 20). We also showed that 2 patients enrolled in Paris have evidence of T cell reconstitution, NK cell reconstitution and improvement for 1 patient in clinical infections (slide 23, 24). Thus 3 enrolled subjects have evidence of early efficacy and 2 of 3 have clinical improvement.

3. The established efficacy of alternative treatments
We compared current survival of 23 patients who have had gene therapy combining both trials and show that the crude survival is comparable to published and unpublished data of young children treated for SCID, with groups of 46-60 children (slide 28). Additionally, we showed unpublished data from the European large multi-institutional SCETIDE registry showing that 22% of babies with T-B+ SCID (many of these are X-linked SCID) treated at age <3.5 months with haplo-BMT had acute GVHD and 9% of these babies had chronic GVHD (slide 31). Thus gene therapy a) currently has equivalent survival to young infants treated with BMT, and b) GVHD, a complication of BMT that is avoided in gene therapy, is prevalent in the SCID patients treated with haplo-BMT.

In summary we presented data to support our argument that infants <3.5 months who might otherwise be treated with a haplo-BMT may be offered the opportunity to enroll on this trial of gene therapy with appropriate informed consent.

<u>Summary for IRB of changes to protocol version 5:</u>
Changes to Protocol:
On pages 34, 35, and 36 the relevant changes to inclusion and exclusion criteria have been made.

Other minor changes include:
--removal of “time” from the required information for adverse event reporting on page 48
--clarification of laboratory studies to be sent for chemistries in Appendix 4
--correction of typographical errors on page 39 and in Appendix 4

<u>Changes to consent version 5:</u>

In the letter received on September 29, 2011 the RAC stated:

After reviewing your data, together with the data and opinions of two transplantation experts, the RAC concluded that it is reasonable to offer gene transfer to an infant less than 3 ½ months of age, even if that infant has the option of a related haploidentical transplant.

The RAC further gave specific comments on modifications to the informed consent document to reflect the current status of alternative treatments. The following is a list of changes made to the informed consent document with specific reference to the letter from the RAC, to demonstrate how we have sought to incorporate the RAC recommendations in our amended protocol and ICF. Note that the RAC made comments based on the ICF activation date October 6, 2010.

Version 7 (IRB approved 04/11/2012)

Summary of changes to protocol version 6

Section 1 – Removed the exclusion criteria patients less than 3.5 months old, the change was previously approved
Section 6.2.4 – Clarified that autologous CD34+ cells undergo 2 or 3 rounds of retroviral transduction
Section 10.1.2 – Clarified failure of gene transfer, i.e. <0.001 copies per cell
Section 12 – The entire Adverse Event Reporting criteria has been updated to clarify what is reportable to the Sponsor. Because of the scrutiny this trial is under, the Sponsor is requesting additional reporting above and beyond what the FDA requires.
Appendix 2 – A new outline of eligibility criteria for enrollment has been added to reflect previously approved changes
Appendix 5 – The new Appendix for the summary of protocol changes has been added in order to keep the study team informed on the various changes the protocol has undergone.

Version 8 (IRB approved 08/01/2012)
Summary of changes to protocol version 7

Appendix 4 – At the May 2012 DSMB meeting, members recommended that the criteria for both discontinuation of IVIg and possible reinstatement of this supportive care be updated and clarified in the study protocol.

Version 9 (IRB approved 03/11/2013))

Summary of changes to protocol version 8:

Front Page: Addition of Wendy B. London, PhD as Co Investigator

Changes throughout document to reflect Colleen Dansereau, RN as Project manager and Matthew Wladkowski as Regulatory Coordinator

Section 9.2.2 Testing of product prior to patient re-infusion
Clarified testing done at at 0 hr and end of incubation for proliferation and sterility (for bacteria, endotoxin and mycoplasma).

10.1.2 Molecular characterization of gene transfer

Based on discussions at the PI meeting in Versailles, France, we have decided to update the section of the protocol dealing with insertional mutagenesis. This is based on the following: 1) this section of the protocol was taken from previous protocols written shortly after the leukemia SAEs in the original SCID-X1 trial and thus are quite old; 2) the experience in the gene therapy trial for thalassemia in Paris of non-leukemia related clonal skewing indicates that clonal skewing is not always predictive of leukemogenesis; 3) clinical and/or laboratory abnormalities are more likely to indicate possible leukemia. Thus we feel the new wording more accurately reflects the situation that might evolve in the setting of leukemia. The language change in this section was approved and recommended by the DSMB at the December 18, 2012 meeting.

Section 10.1.3
Change in monitoring from EQUIP to Christine McNally – Dufort, RN, MBA

Section 17
Addition of qualifications of Colleen Dansereau, RN as Project Manager

Version 10.0 (IRB approval date 8/21/2013)

Section 8.1
Deletion of previous gene transfer as exclusion criteria

Section 10.2.3

Definition of 2nd gene transfer procedure for patients that are deemed an early failure.

Section 10.2.4

Description of eligibility criteria for 2nd gene transfer procedure for patients who fail immune reconstitution. The eligibility criteria will be the same as the initial enrollment.

Appendix 4

Inclusion of timepoints for 2.5, 3.5, 4.5, and 5.5 years for insertional analysis samples.

Version 11.0 (IRB 11/14/13)

Section 15.1 Data Handling, Record Keeping, Sample Storage

To clarify the plan for data analysis to include data transferred in a coded fashion from European sites, the following text was added.

CTIP will also analyze data shared from the European sites as specified including data for purpose of joint publication (Section 12, European data considerations). Data shared from European sites will be obtained according to local approved practices and transferred in a coded fashion.

The change does not require any modification of the consent form, per discussion with Susan Kornetsky—Director of Clinical Research Compliance, Boston Children's Hospital Office of Clinical Investigation.

Version 12.0 (IRB 8/22/16)

Front page: replace Dr Notarangelo with Dr Sung-Yun Pai as PI

Section 4.4.1 (p. 18) inclusion of data from PIDTC demonstrating effects of conditioning on HSCT outcomes in SCID patients.

Section 4.4.2 (p. 20) inclusion of data from PIDTC demonstrating effects of conditioning on CD4+ counts in SCID patients.

Section 5.3.1 (p. 23) inclusion of data from allogeneic transplants and previous gene transfer trials demonstrating effects of conditioning on engraftment.

Section 7 (p. 36) clarification of enrollment numbers; 5 patients will receive busulfan conditioning.

Section 8.1 (p. 37) clarification of eligibility for conditioning.

Section 9.1.1 (p. 39) inclusion of mobilized peripheral blood leukapheresis as cell collection option.

Section 9.1.2 (p. 39) inclusion of back-up collection for patients receiving busulfan.

Section 9.2 (p. 39) addition of low dose busulfan conditioning for selected subjects.

Section 9.3.3 and 9.3.4 (p. 41) infusion of transduced cells and criteria for infusion of back-up cells.

Section 10.1.14 (p. 43) inclusion of additional assessments for patients receiving busulfan.

Section 10.3 (p 45) procedure for failure of hematologic reconstitution in patients receiving busulfan.

Section 11.4 (p.50) addition of side effects for busulfan conditioning.

Tables 2a, 2b, 2c (p. 55-57): adverse event reporting requirements.

Appendix 2: updated eligibility diagram.

Appendix 5: busulfan dosing guidelines.