

Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector

H Bobby Gaspar, Kathryn L Parsley, Steven Howe, Doug King, Kimberly C Gilmour, Joanna Sinclair, Gaby Brouns, Manfred Schmidt, Christof Von Kalle, Torben Barington, Marianne A Jakobsen, Hans O Christensen, Abdulaziz Al Ghonaium, Harry N White, John L Smith, Roland J Levinsky, Robin R Ali, Christine Kinnon, Adrian J Thrasher

Summary

Background X-linked severe combined immunodeficiency (SCID-X1) is caused by mutations in the common cytokine-receptor γ chain (γ c), resulting in disruption of development of T lymphocytes and natural-killer cells. B-lymphocyte function is also intrinsically compromised. Allogeneic bone-marrow transplantation is successful if HLA-matched family donors are available, but HLA-mismatched procedures are associated with substantial morbidity and mortality. We investigated the application of somatic gene therapy by use of a gibbon-ape-leukaemia-virus pseudotyped gammaretroviral vector.

Methods Four children with SCID-X1 were enrolled. Autologous CD34-positive haemopoietic bone-marrow stem cells were transduced *ex vivo* and returned to the patients without preceding cytoreductive chemotherapy. The patients were monitored for integration and expression of the γ c vector and for functional immunological recovery.

Findings All patients have shown substantial improvements in clinical and immunological features, and prophylactic medication could be withdrawn in two. No serious adverse events have been recorded. T cells responded normally to mitogenic and antigenic stimuli, and the T-cell-receptor (TCR) repertoire was highly diverse. Where assessable, humoral immunity, in terms of antibody production, was also restored and associated with increasing rates of somatic mutation in immunoglobulin genes.

Interpretation Gene therapy for SCID-X1 is a highly effective strategy for restoration of functional cellular and humoral immunity.

Introduction

Severe combined immunodeficiencies (SCID) are a heterogeneous group of inherited disorders characterised by the abnormal development or function of T, B, and natural-killer cells.¹ Defects in the gene encoding the common cytokine-receptor γ chain (γ c) result in the X-linked form (SCID-X1), which accounts for about 50% of all cases of SCID.² γ c is an essential component of the high-affinity cytokine-receptor complexes for interleukins 2, 4, 7, 9, 15, and 21. Abnormal signalling through these receptors results in many immunological defects, the most apparent of which are severe disruption of development of T cells and natural-killer cells, and the intrinsic dysfunction of B cells, which are present in normal numbers in many cases.³ Survival depends on reconstitution of T-cell development and function by allogeneic bone-marrow transplantation.^{4,5} If a genotypically matched family donor is available, this procedure is highly successful. It has long-term survival of more than 90%, partly because the absence of T cells and natural-killer cells in patients with SCID-X1 allows engraftment of donor cells without myelosuppressive conditioning. For most individuals, well-matched family donors are not available. Survival after mismatched (mostly haploidentical parental donors) transplants is less good, partly owing to the predictable toxicity arising from the use of conditioning chemotherapy to increase the chance of effective engraftment and full restoration

of immunity.⁶ The consensus for standard care in this group, especially the use of conditioning, is also unclear because outcome is almost certainly influenced by age and clinical status at the time of treatment.

SCID disorders are good targets for the application of somatic gene therapy partly owing to the strong growth and survival advantage that is predicted for functionally corrected mutant haemopoietic precursors. For SCID-X1, there is evidence of correction in animal models⁷ and partial immunological reconstitution arising from a somatic reversion in a single T-lymphocyte precursor cell in a patient.⁸ In this phase I/II clinical trial, we treated four patients with SCID-X1 by somatic gene therapy with a gammaretroviral vector. We assessed patients for cellular and humoral immune recovery and the safety of the procedure.

Methods

Patients

The protocol was approved by the UK Gene Therapy Advisory Committee, the Medicines Control Agency (now Medicine and Healthcare products Regulatory Agency), and the local institutional research ethics committee. Criteria for entry to the study were molecular confirmation of SCID-X1 by identification of a mutation in γ c and lack of an HLA-identical sibling donor. All patients with SCID-X1 referred to Great Ormond Street Hospital, London, UK, between July,

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See [Comment](#) page 2155

Molecular Immunology Unit, Institute of Child Health, University College London, London, UK (H B Gaspar MRCP, K L Parsley MPhil, S Howe PhD, D King BSc, K C Gilmour PhD, J Sinclair BSc, G Brouns PhD, H N White PhD, Prof R J Levinsky FRCP, Prof R R Ali PhD, Prof C Kinnon PhD, Prof A J Thrasher FRCP); Department of Clinical Immunology, Great Ormond Street Hospital NHS Trust, London, UK (H B Gaspar, K C Gilmour, Prof A J Thrasher); Department of Internal Medicine and Institute of Molecular Medicine and Cell Research, University of Freiburg, Freiburg, Germany (M Schmidt PhD, C Von Kalle MD); Division of Experimental Hematology, Cincinnati Children's Research Foundation, Cincinnati, OH, USA (C Von Kalle); Departments of Clinical Immunology (T Barington MD, M A Jakobsen MSc) and Paediatrics (H O Christensen MD), Odense University Hospital, Odense, Denmark; Department of Paediatric Allergy/Immunology, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia (A Al Ghonaium MD); Department of Immunology, Southampton General Hospital, Southampton, UK (J L Smith FRCPath); and Department of Molecular Genetics, Institute of Ophthalmology, University College London, London, UK (Prof R R Ali)

Correspondence to: Prof A J Thrasher, Molecular Immunology Unit, Institute of Child Health, University College London, London WC1N 1EH, UK a.thrasher@ich.ucl.ac.uk

SCID

A group of monogenic disorders arising from defects in cellular and humoral immune function or development.

Common cytokine-receptor γ chain (γ_c)
A subunit of the receptor complex for interleukins 2, 4, 7, 9, 15, and 21, which are important in many parts of immune-cell development and function.

Gene therapy
The introduction of genetic material (DNA or RNA) into cells or tissues for therapeutic effect.

Gammaretroviral vector
A replication-defective mammalian retrovirus, which has been disabled by removal of endogenous viral genes and engineered to express a therapeutic gene.

Pseudotypes
Production of viral particles with surface envelope proteins derived from a virus differing from the parent vector genome.

2001, and December, 2002, who met these criteria were offered gene therapy. All chose to join the study. Written informed consent was obtained from parents after discussion of other treatment options.

Procedures

The complete coding region of human γ_c was cloned into pMFG **gammaretroviral vector**. The integrity of the vector was confirmed by DNA sequencing. Vector genomes were packaged in PG13 cells, which **pseudotypes** viral particles with the gibbon-ape-leukaemia-virus envelope. The viral titre in the supernatant (clinical-grade vector supernatant; BioReliance, Stirling, UK) was 1×10^6 transducing units per mL.

Bone-marrow CD34-positive cells (CliniMACS, Miltenyi Biotec, Bisley, UK, were cultured in Lifecell X-Fold cell-culture containers (Nexell, Irvine, CA, USA), at a concentration of 0.5×10^6 per mL in serum-free X-VIVO 10 medium (Biowhittaker, Verviers, Belgium) supplemented with 1% human serum albumin (Baxter, Thetford, Norfolk, UK) and cytokines (300 μ g/L stem-cell factor, 100 μ g/L thrombopoietin, 20 μ g/L interleukin 3, and 300 μ g/L Flt3 ligand, R&D Systems, Abingdon, Oxfordshire, UK) for 40 h at 37°C in 5% carbon dioxide (preactivation). The culture containers were precoated with the CH296 human fragment of fibronectin (25 mg/L; donated by Takara Bio Inc, Shiga, Japan). Supernatant from the MFG- γ_c retroviral culture was added three times during a 56 h period. Cells were then harvested, washed three times, and infused into the patients.

For flow cytometry, whole blood was labelled with combinations of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin, and either peridinin chlorophyll protein (PerCP), cychrome,

or Tricolour. Isotype-matched fluorochrome-conjugated antibodies served as controls (BD Biosciences, Oxford, UK: CD3 FITC, CD45RO FITC, CD27 FITC, CD19 phycoerythrin, CD27 phycoerythrin, CD4 phycoerythrin, CD8 phycoerythrin, CD45 PerCP, CD4 PerCP, CD8 cychrome, CD45RO cychrome, CD16+CD56 FITC phycoerythrin Simultest. Caltag: IgM FITC, IgD FITC, CD19 Tricolour). The resulting three-colour cell staining was analysed with an Epics XL flow cytometer (Beckman Coulter, High Wycombe, Bucks, UK). Cells were sorted with a Beckman Coulter Epics Altra with Expo32 software.

To assess immunoglobulin superfamily antigen-receptor repertoires, we used direct immunofluorescence with fluorochrome-conjugated antibodies to T-cell receptor (TCR) family TCR β , TCR δ , and TCRV β (labelled with phycoerythrin and PerCP) obtained from BD, Beckman Coulter, and Serotec. For TCRV β analysis, samples were analysed on a FACS Calibur flow cytometer. TCRV β expression is represented as a percentage of CD3-positive cells for each family.

For the restriction-enzyme-based hot-spot mutation assay, mRNA was extracted from patients' peripheral-blood mononuclear cells by standard procedures. RT-PCR was done with primers specific for the signal peptide region and the third framework region (Applied Biosystems, Warrington, UK) of the κ light-chain gene V κ A27 (sequences and PCR conditions available from the corresponding author on request). The 271 bp PCR product was digested with *Fnu*4HI (Medinova Scientific, Hellerup, Denmark), which recognises two adjacent AGCAGCAGC sites in the unmutated gene product of the CDR1 variable region at codons 29–31. The degree of somatic hypermutation was expressed as the fraction of PCR products with mutations preventing *Fnu*4HI

Age at treatment, months	Clinical status before treatment	Maternal engraftment and sequelae	Mutation	γ_c expression before treatment*	Total number of cells infused, $\times 10^6$ per kg	% infused cells positive for CD34 and γ_c	Clinical status after treatment	Latest follow-up (months since procedure)
Patient 1								
10	PCP, rotavirus infection, failure to thrive	++	Arg289→stop	++	23	27	Alive and well, normal growth	29
Patient 2								
10	Parainfluenza III, rotavirus infection, failure to thrive, mGvHD	++	Ser238→Asp	-	26	37	Alive and well, normal growth	24
Patient 3								
4	RSV, eczema	-	Tyr125→Cys	+	15	58	Alive and well, normal growth	23
Patient 4								
33	PCP at 10 months; well at time of treatment	-	Arg289→stop	++	8	58	Alive and well, normal growth	12

PCP=*Pneumocystis carinii* pneumonia; mGvHD=maternal graft-versus-host disease; RSV=respiratory syncytial virus. *Expression of γ_c as shown by surface expression on gated lymphocytes: ++=normal, +=low, -=absent.

Table 1: Characteristics of participants

cleavage in codons 29–31, calculated as time-integrated fluorescence intensity.

To assess T-cell functionality, 5×10^5 peripheral-blood mononuclear cells were seeded into a 96-well plate in RPMI containing 5% human AB serum. Mitogens (CD3/CD28, phytohaemagglutinin), antigen (candida), or allogeneic irradiated peripheral-blood mononuclear cells (for mixed lymphocyte reactions) were added to the wells in a final volume of 200 μ L. At least three replicates were done for each sample, with a positive control and unstimulated control. 3 days later, samples treated with mitogens were pulsed with 3.7×10^4 Bq/mL of tritiated thymidine for 6 h. Plates with antigen and mixed lymphocyte reactions were pulsed after 6 days, and the cells were harvested and the thymidine incorporation measured on a scintillation counter.

Insertion-site analysis for visualisation of the clonal contribution to T lymphopoiesis and other lineages was done by linear-amplification-mediated PCR, as previously described.⁹

Role of the funding source

The funding sources had no input into study design; collection, analysis, or interpretation of data; the writing of the report; or in the decision to submit it for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit the paper for publication.

Results

Four patients without HLA-identical family donors were enrolled (table 1). Patients 1, 2, and 3 presented with classic SCID. Patient 4 presented at the age of 9 months with *Pneumocystis carinii* pneumonia but was unusual in that he had survived with prophylaxis until the age of 33 months without further infection, in the absence of functional T cells or natural-killer cells.

Autologous CD34-positive cells were harvested from bone marrow taken under general anaesthetic. Cells were transduced as detailed above. For patient 2, in whom no expression of γ C was present before gene transfer, transduction efficiency was estimated to be 68% by cell-surface expression (figure 1). The proportions of cells positive for both CD34 and γ C at the time of reinfusion were 27–58% (table 1). At the end of transduction (total time in culture 96 h), cells were washed and returned to the patients by intravenous infusion over 30–40 min. No preconditioning chemotherapy was given.

Recovery of immunological cell numbers and function was achieved in all patients (figure 2). The kinetics of lymphocyte recovery in patients 1, 2, and 3 were similar up to 16 weeks. Patient 4, who was the oldest patient treated and who received the lowest dose of cells per kg, showed slower recovery in general, though the recovery has continued during the year since treatment. Patient 1 had substantial maternal T-cell engraftment (>90% CD3-

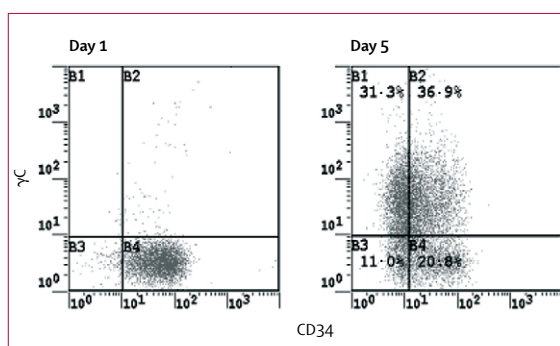


Figure 1: Efficiency of transduction of CD34-positive cells for patient 2 over the 5-day culture period

positive cells) at the time of treatment, but these cells were gradually replaced by new autologous cells and were undetectable by 20 weeks (as shown by fluorescence in-situ hybridisation). Similarly, there was transient expansion of non-transduced autologous mutant CD8-positive T cells in patient 4 at the time of engraftment; these cells were replaced by new cells. Patients 1 and 3 have achieved normal numbers of cells expressing CD3, CD4, and CD8. Immune recovery in patient 2 has remained incomplete. In all patients, natural-killer cells were the first new cells to appear, at

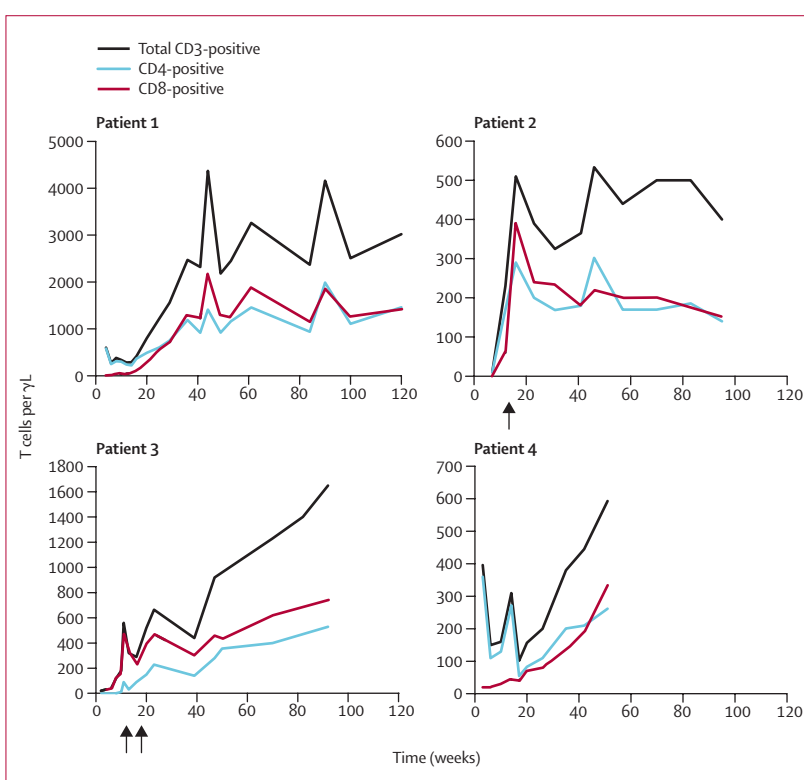


Figure 2: T-cell numbers in the four patients after gene therapy

Physiological normal ranges of total CD3-positive T cells for age-matched controls at the latest analysis for each patient are 900–4500 μ L. Timings of inflammatory events in patients 2 and 3 are indicated by arrows. Note that vertical scales differ substantially for the four patients.

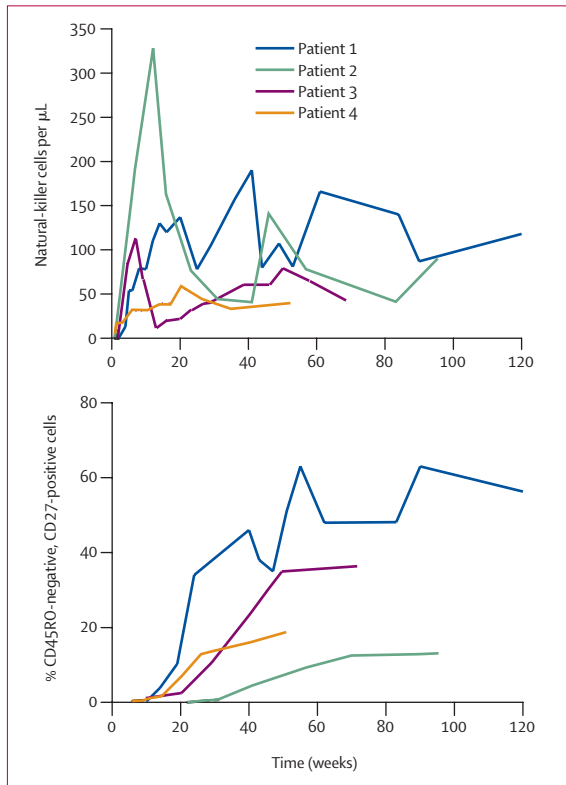


Figure 3: Numbers of natural-killer cells (normal range >100 CD16/56 cells per µL) and naive CD45RO-negative, CD27-positive T cells (proportion of total lymphocyte gate) in patients 1–4

2–4 weeks, and have persisted at the lower end of the normal ranges for age-matched controls (figure 3). The emergence of naive thymic emigrants was indicated by the accumulation of CD45RO-negative, CD27-positive T cells (figure 3) and by the increase and normalisation of numbers of TCR excision circles to within the normal range (data not shown).

There have been substantial therapeutic benefits, with clearance of viral or opportunistic infections, and normal growth and development in all four patients (table 1). Patients 1 and 3 have discontinued prophylaxis. Patient 2 (with documented pretreatment maternal graft-versus-host disease) had a recurrence of gastrointestinal bleeding at 16 weeks necessitating limited terminal ileal resection and formation of a temporary ileostomy. The likely cause is exacerbation of an inflammatory process as a result of rejection of

engrafted maternal cells; this idea was supported by the histological appearances of focal ulceration and histiocytic inflammation. Reversal of the ileostomy at 30 weeks led to substantial improvement in clinical condition. Patient 3 developed an erythematous maculopapular rash on the palms and soles, coincident with recovery of CD4-positive T cells; the disorder was clinically and histologically indistinguishable from graft-versus-host disease but there was no documented maternal engraftment. It resolved with administration of topical steroids. In patients 2 and 3, immunological recovery was interrupted when inflammation occurred (figure 2). Lymphocyte numbers remain low in patient 2, whereas they have continued to recover normally in patient 3. At last follow-up, all patients were at home in normal family and social environments, without restriction on activities or exposure.

All patients developed normal T-cell proliferative responses after stimulation with mitogens, candida, or antibodies to CD3/CD28, and in mixed lymphocyte reactions (table 2). All patients showed TCRVβ usage within the normal range at a year or more after treatment (figure 4). Molecular analysis of TCRVβ complementarity-determining region 3 (CDR3) fragment lengths after PCR amplification (TCR spectratypes) revealed progression from oligoclonality to polyclonality within individual Vβ families, reflecting the evolution of a complex and diverse repertoire. Longitudinal analysis also showed physiological expansion and contraction of individual clones, with no evidence of persistent clonal expansion or pathological proliferation (data not shown).

Patients 1 and 3 discontinued immunoglobulin replacement 21 months and 18 months after treatment, when concentrations of IgA and IgM had reached the normal range. Without immunoglobulin treatment, concentrations of IgG rose into the normal range in patient 1 (figure 5). Serological responses to vaccination with tetanus, *Haemophilus influenzae* type B, killed poliovirus (serotypes 1–3), and infection with wild-type varicella zoster virus in patient 1 (specific IgM and IgG) were also normal (data not shown). Total concentrations of IgG were also maintained in patient 3 at latest analysis 3 months after discontinuation of replacement therapy (6.0 g/L). Humoral immunity in other patients has not yet been examined in detail owing to continued administration of immunoglobulin. However, in all four patients, immunological recovery was associated with

Patient	CD3/CD28	Phytohaemagglutinin	Candida	Mixed lymphocyte reaction
1	Normal	Normal	Moderate response	Not done
2	Normal	Normal	Normal	Not done
3	Normal	Normal	Normal	Normal
4	Normal	Normal	Not done	Normal

Normal=normal response compared with control cells.

Table 2: Lymphocyte proliferation responses

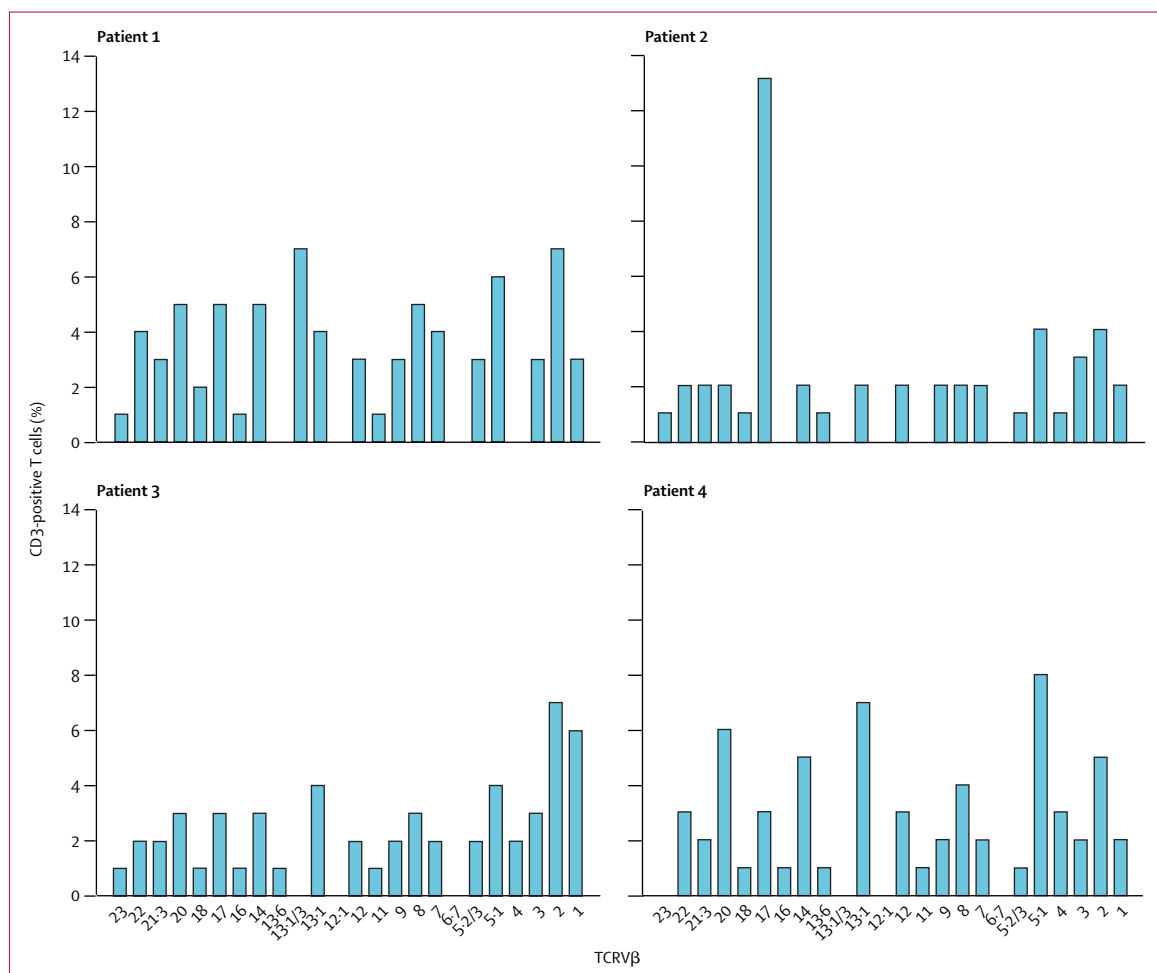


Figure 4: T-cell diversity

the appearance of CD27-positive antigen-experienced B cells in the peripheral blood to 3–8% of CD19-positive cells at latest analysis (figure 5 shows analysis for patient 1). Somatic hypermutation was quantified in κ light-chain transcripts (isotype-independent). In CD27-positive B cells from patient 1, 1.4% of base-pairs from CDR2 were somatically mutated at 10 months after treatment (46 unique mutations of 3363 bp sequenced from 33 clones; 33% of clones mutated). In unselected peripheral-blood B cells, the proportions of mutated $V\kappa A27$ transcripts had increased from less than 2.0% to 5.3% in patient 1, 8.0% in patient 2, 11.8% in patient 3, and 23.3% in patient 4 at latest follow-up (average of two independent assays, normal range for adults, 28–62%). The relation between antibody production, somatic mutation, and B cells containing transgene has not been established.

PCR analysis of cell populations purified by flow-cytometric sorting showed that the proviral copy number in T cells and natural-killer cells was between 1 and 3. Therefore, as expected, all T cells and natural-killer cells probably contained functional transgene. By compar-

ison, on the assumption of a copy number of 1, the proportions of circulating B cells and myeloid cells containing transgene were less than 20% and 1%, respectively (data not shown).

Integration of provirus into the chromosome creates a unique sequence defined by the transgene and flanking DNA sequence. Linear-amplification-mediated PCR demonstrated the diversity of clonal integration sites in sorted cell populations (>99% purity). In all four patients there was a polyclonal contribution to the restored T-cell populations; fewer clonal integrants were detectable in B cells, natural-killer cells, monocytes, and granulocytes (figure 6). However, the persistence of multiple gene-modified myeloid cell clones (granulocyte lanes) also suggests gene transfer into multipotent CD34-positive cells. No pathological clonal expansions were detected either by linear-amplification-mediated PCR or by serial TCR repertoire or spectratype analysis, and no insertions in the direct proximity of the LMO-2 gene promoter were found by first-locus-specific PCR screening or by sequencing of more than 200 unique insertion sites (data not shown).

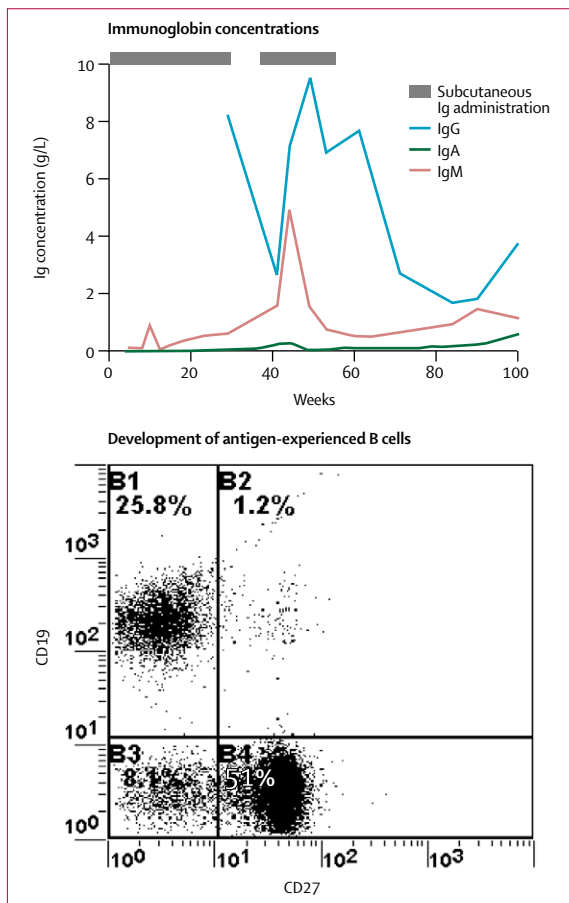


Figure 5: Recovery of humoral immunity in patient 1
 Normal range of immunoglobulin concentrations for age-matched controls: IgG 3.7–15.8 g/L, IgA 0.3–1.3 g/L, IgM 0.5–2.2 g/L. Normal proportion of antigen-experienced B cells (positive for CD19 and CD27) >10%.

Discussion

We found restoration of both cellular and humoral immunity in four patients with SCID-X1 after somatic gene therapy. For two of the patients, withdrawal of prophylactic medication was possible within 1–2 years of treatment. Continued follow-up of the other two recipients will show whether they also achieve complete functional recovery, but all patients have returned to normal social environments and lifestyles. The restoration of immunity is substantial and was achieved without the administration of preconditioning chemotherapy, which is generally used to increase the chances of engraftment and full reconstitution.⁶ The substantial long-term adverse effects of chemotherapy on growing tissues have therefore been avoided.

No major side-effects have been observed. Two patients developed inflammatory reactions at the time of new T-cell emergence, which could be a direct manifestation of local rejection of previously engrafted maternal T cells or indirect CD4-mediated (or CD8-mediated) cytokine-driven reactions, sustained by antigen-presenting cells independently of alloantigen expression in target

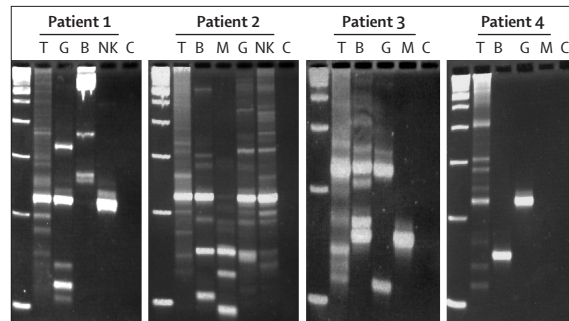


Figure 6: Clonal integration site analysis
 DNA samples of 1–50 ng isolated directly from sorted peripheral-blood leucocytes were analysed at various times over a year after treatment. T=T cells; G=granulocytes; B=B cells, NK=natural-killer cells; C=negative control analysis on 10 µg non-transduced human DNA and water control; M=monocytes. Left lane on each panel, 100 bp DNA marker.

tissues.¹⁰ These are reminiscent of reactions resembling graft-versus-host disease that occur after autologous bone-marrow transplantation, which might be potentiated by imbalance of regulatory cells in the graft or inhibition of their function.¹¹ Immunological reconstitution was interrupted in both these patients between 12 weeks and 20 weeks, which suggests that thymopoiesis is fragile at this time and susceptible to disturbance by external factors.

Sustained correction of SCID-X1 after gene therapy with an amphotropic gammaretroviral vector has been reported previously in a study of ten patients.¹² The kinetics of recovery of T cells and natural-killer cells and overall outcomes were similar to those in our study, although follow-up for our patients is shorter. Recovery was slower in patient 4 than in our other three patients, which may have resulted from administration of a lower cell dose. However, another possibility is that the recovery of thymopoiesis is intrinsically compromised in older patients. In mice, developmental arrest at early T-cell-precursor stages (CD44-positive, CD25-negative) results in severe thymic hypoplasia and thymic epithelial-cell disorganisation;^{13–15} although thymic stroma shows much plasticity, there could be a time-dependent restriction on the development of a normal cortical microenvironment and therefore for successful reinitiation of thymopoiesis.¹³ This possibility has important implications for treatment of older patients and those in whom allogeneic stem-cell transplantation has been unsuccessful.

Insertional mutagenesis resulting in retroviral enhancer-mediated activation of the T-cell proto-oncogene LMO-2 has occurred in two children with SCID-X1 previously given gene therapy.^{16,17} The virtually identical configuration of the vector genome used in our study predicts that the risk of mutagenesis, and inadvertent activation of LMO-2 (or other proto-oncogenes) will be similar. This risk cannot yet be clearly defined. At latest analysis, we have not observed any evidence of clinically

manifest insertional mutagenesis, although our follow-up is short in comparison. Slight differences between protocols (culture of cells in serum-free medium, lower concentration of interleukin 3, and a pseudotyped vector) might affect the risk, but we do not believe this possibility to be likely.

The time over which reconstitution will be sustained is unknown and can be determined only by longitudinal observation, as has been the case after bone-marrow transplantation.¹⁸ However, the mature T-cell pool includes long-lasting cells, and clinical benefit can be expected for many years and even for the lifetime of the individual. Sustained thymopoiesis will depend on the successful transduction and engraftment of very early bone-marrow precursors and, most likely, true haemopoietic stem cells. Analysis of clonal integration patterns in myeloid cells suggests that transduced stem cells have been engrafted at low frequency. Future protocols that improve this process, such as the use of shorter culture periods (to prevent stem-cell wastage) and vectors that transduce stem cells with high efficiency, could be beneficial. In our study, increased polyclonality in transduced myeloid and B cells compared with that observed with an equivalent amphotropic vector, suggests that some advantage may be obtained in the long term by use of the gibbon-ape-leukaemia-virus envelope.

Gene therapy is an effective treatment for SCID-X1 and for adenosine-deaminase-deficient SCID.¹⁹ Our findings suggest superior reconstitution and lower morbidity and mortality than with mismatched bone-marrow transplantation. Recruitment of more patients into these studies and analysis of effectiveness and toxicity over longer periods will be necessary to assess the extent of benefit for patients with SCID-X1. With improved protocols and more sophisticated vector configurations, there is potential to establish gene therapy as a standard therapeutic procedure for this type of disease.

Contributors

K L Parsley and K C Gilmour were primarily responsible for establishing and carrying out the clinical transduction protocol. S Howe, D King, J Sinclair, M Schmidt, C Von Kalle, T Barington, M A Jakobsen, H O Christensen, A Al Ghonaium, H N White, and J L Smith were responsible for analysis of patients' samples. G Brouns constructed the retroviral vector. R J Levinsky, R R Ali, and C Kinnon contributed to the design of the study. H B Gaspar and A J Thrasher designed the study, supervised its conduct, and prepared the report.

Conflict of interest statement

We declare that we have no conflict of interest.

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