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Long-Term Persistence of a Polyclonal T Cell Repertoire After Gene Therapy for X-Linked Severe Combined Immunodeficiency

H. Bobby Gaspar *et al.*

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Editor's Summary

Out of the Bubble

As part of a normal day, most people will flush a toilet, open a door, or drink from a water fountain without even thinking about it —or about the lurking pathogens poised to infect us. We are afforded this luxury because of our immune system, which responds rapidly and specifically to just about anything thrown at it. Yet, for people with severe combined immunodeficiency (SCID), who carry a mutation that thwarts adaptive immunity, everyday activities can be deadly. Like the famous "bubble boy," some people with SCID choose to live in a germ-free environment. Yet, matched hematopoietic stem cell (HSC) transplantation, which can replace the patient's ailing immune system with functional cells from a related donor, can offer these patients a normal life. Sometimes, however, donor relatives aren't available. Now, two new studies provide clinical support for treatment options that may allow SCID patients without matched donors to live relatively normal lives as well.

One such treatment option is gene therapy. Removing HSCs from SCID patients, repairing the underlying genetic defect in these cells, and returning the repaired cells to the original host can replace the faulty immune system in SCID patients without the graft rejection or graft-versus-host disease that follows transplantation of cells from unrelated donors. Gaspar *et al*. do just that for two types of SCID: X-linked SCID (SCID-X1) and adenosine deaminase –deficient SCID (ADA-SCID). The authors repaired the underlying genetic defect in 10 of 10 patients with SCID-X1 and in 4 of 6 patients with ADA-SCID, resulting in the development of a functional polyclonal T cell repertoire that persisted for at least 9 years after therapy. The procedure produced minimal side effects and permitted all patients to attend typical schools. One patient in the SCID-X1 cohort developed a blood cancer, acute lymphoblastic leukemia (ALL), a complication observed in previous SCID-X1 gene therapy studies, but this patient is currently in remission. No cases of ALL developed in the ADA-SCID cohort.

The promising results of these and similar studies, albeit with an increased risk of ALL in SCID-X1 patients, support the development of new safer and more efficient vectors for this and other kinds of gene therapy. Long-term follow-up of patient participants in early gene-therapy trials such as the ones described here is critical for scientists to decipher the parameters of success and failure for gene therapy in general —and for SCID-specific treatments to bubble over into the clinic.

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Long-Term Persistence of a Polyclonal T Cell Repertoire After Gene Therapy for X-Linked Severe Combined Immunodeficiency

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X-linked severe combined immunodeficiency (SCID-X1) is caused by mutations in the common cytokine receptor γ chain. These mutations classically lead to complete absence of functional T and natural killer cell lineages as well as to intrinsically compromised B cell function. Although human leukocyte antigen (HLA)–matched hematopoietic stem cell transplantation (HSCT) is highly successful in SCID-X1 patients, HLA-mismatched procedures can be associated with prolonged immunodeficiency, graft-versus-host disease, and increased overall mortality. Here, 10 children were treated with autologous $CD34^+$ hematopoietic stem and progenitor cells transduced with a conventional gammaretroviral vector. The patients did not receive myelosuppressive conditioning and were monitored for immunological recovery after cell infusion. All patients were alive after a median follow-up of 80 months (range, 54 to 107 months), and a functional polyclonal T cell repertoire was restored in all patients. Humoral immunity only partially recovered but was sufficient in some patients to allow for withdrawal of immunoglobulin replacement; however, three patients developed antibiotic-responsive acute pulmonary infection after discontinuation of antibiotic prophylaxis and/or immunoglobulin replacement. One patient developed acute T cell acute lymphoblastic leukemia because of up-regulated expression of the proto-oncogene LMO-2 from insertional mutagenesis, but maintained a polyclonal T cell repertoire through chemotherapy and entered remission. Therefore, gene therapy for SCID-X1 without myelosuppressive conditioning effectively restored T cell immunity and was associated with high survival rates for up to 9 years. Further studies using vectors designed to limit mutagenesis and strategies to enhance B cell reconstitution are warranted to define the role of this treatment modality alongside conventional HSCT for SCID-X1.

INTRODUCTION

X-linked severe combined immunodeficiency (SCID-X1) accounts for about 40 to 50% of all SCIDs and is caused by mutations in the gene encoding the interleukin-2 receptor γ chain (*IL2RG*) (*1*). This molecule is a key subunit of the cytokine receptor complex for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 and is designated the common cytokine receptor γ chain (γc) (*2*, *3*). In the absence of γc signaling, many aspects of immune cell development and function are compromised, leading to the absence of T and natural killer (NK) cells and persistence of dysfunctional B cells ($T^-B^+NK^-$ SCID), although atypical individuals with partial T cell development have also been identified. If a genotypically matched donor is available, allogeneic bone marrow transplantation can successfully treat SCID-X1 patients, with a long-term survival rate of more than 90% (*4*, *5*). These high survival rates are partly because of efficient engraftment in the absence of myelosuppressive conditioning. In contrast, survival in a large cohort of patients treated by mismatched hematopoietic stem cell (HSC) transplantation has been shown to be significantly lower (72% 10-year survival for patients treated after 1995, SCETIDE European registry) and often is associated with poor long-term immunological recovery with minimal HSC engraftment (*6*). Poor response is particularly observed for patients with severe infections at the time of treatment. Furthermore, chemotherapeutic conditioning is often used to facilitate HSC engraftment in the mismatched setting, but may itself be associated with late toxic effects.

SCID-X1 is a particularly attractive target for gene therapy because a profound growth and survival advantage is conferred to corrected cells. Hence, effective gene transfer to a small proportion of bone marrow precursor cells can result in a substantial correction of the immunological deficit, as is observed in patients with rare somatic gene reversion events, who accumulate normally functioning lymphocytes (*7*). Here, we report the clinical outcome in 10 children treated in a phase I/II clinical trial using conventional gammaretroviral vector technology.

RESULTS

Ten children with molecularly defined SCID-X1 underwent gene therapy at a median age of 10 months (range, 4 to 46 months) (Table 1). Patients were discharged from the hospital between 1 and 105 days after infusion of cells and maintained on prophylactic immunoglobulin and antibiotic support until immunological recovery was observed. The lack of any conditioning in the protocol allowed four patients to be discharged the day after receiving gene-transduced cells, and these individuals have only been followed as outpatients.

During T cell recovery, two patients developed a widespread but transient erythematous rash, which resolved with either topical or oral

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Table 1. Characteristics and treatment of the study participants. PCP, *Pneumocystis jirovecii* pneumonitis; mGvHD, maternal graft-versus-host disease; RSV, respiratory syncytial virus; ND, not done.

Patient number and age at treatment (months)	Clinical status before treatment	Maternal engraftment and sequelae	Mutation	γc expression before treatment*	Number of cells infused ($\times 10^6$ per kg)	% Infused cells positive for CD34 and γc	Clinical status after treatment	Latest follow-up (months after treatment)
Patient 1 (10)	PCP, rotavirus infection, failure to thrive	Yes	R289Stop	++	22.5	27.1	Alive	107
Patient 2 (10)	Parainfluenza III, rotavirus infection, failure to thrive, mGvHD	Not analyzed	S238D	–	25.7	18.4	Alive Cutaneous sarcoid	101
Patient 3 (4)	RSV, eczema	—	Y125C	+	13.7	57.7	Alive	99
Patient 4 (46)	PCP at 10 months; well at time of treatment	—	R289Stop	++	6.9	52.1	Alive	92
Patient 5 (10)	PCP	No	R222C	++	34.1	20.2	Alive	81
Patient 6 (7)	RSV, PCP, enterococcus, septicemia	No	PolyA	–	30.8	18.9	Alive	78
Patient 7 (6)	PCP	Not analyzed	Met1I	–	13.0	ND†	Alive	69
Patient 8 (13)	PCP, failure to thrive	No	C182Y	+	23.7	ND	Alive T-ALL (in remission)	54
Patient 9 (7)	Respiratory infections, failure to thrive	No	S108P	+	24.4	ND	Alive	51
Patient 10 (12)	PCP, parainfluenza, norovirus infection, chronic cough, failure to thrive	Not analyzed	Deletion	–	8.4	ND	Alive	54

*Expression γc as shown by surface expression on gate lymphocytes: ++, normal; +, low; –, absent. †γc and CD34 measured independently.

corticosteroids. P1, who originally presented with *Pneumocystis jirovecii* pneumonitis, developed acute pneumonitis caused by *P. jirovecii* at 1 year after gene therapy and required ventilator support for 48 hours, after which he made a full recovery. P1 and P7 developed proven pneumococcal pneumonia (at 24 and 52 months after gene therapy, respectively), which responded to antibiotic therapy. P1 displayed intermittent plantar and digital warts, and P4 developed planar warts on his forehead and trunk, which were partially responsive to the Toll-like receptor-7 (TLR-7) agonist imiquimod. P2 developed cutaneous sarcoidosis 7 years after gene therapy, which did not require treatment. At least two children had documented varicella-zoster (chicken pox) infection, which was clinically inconsequential without additional therapy. All children achieved normal centiles for height and weight within 1 year of gene therapy, although P2 has persistent undefined feeding difficulties. P8 developed T cell acute lymphoblastic leukemia (T-ALL) at 24 months after gene therapy and was entered into a 3-year chemotherapy protocol according to UK T-ALL 2003, version 5. After 2.5 years of treatment, P8 remained in clinical and molecular remission. P3, P4, P5, and P9 remained completely free from immunoglobulin replacement; however, P6 and P7 recommenced immunoglobulin replacement after failing to maintain immunoglobulin G (IgG) levels and clinically deteriorating, from which they subsequently recovered. P1 has supplemental therapy during the winter period for persisting rhinitis. All patients continued penicillin V prophylaxis because of the occurrence of pneumococcal pneumonia in two of the cohort. After gene therapy, all patients attended normal nursery or school and had no signs of abnormal development.

Peripheral $CD3^+$ T cell recovery was observed in all patients from 8 weeks after infusion of transduced cells, with 6 of 10 achieving numbers within the normal range by 6 months (Fig. 1A). P2 failed to achieve normality, but reconstitution may have been compromised as a result of serious hemorrhagic complications after gene therapy related to preexisting gut inflammation. P4 was treated at the age of nearly 4 years (having developed *P. jirovecii* pneumonitis within the first year of life), and also achieved subnormal recovery of T cell numbers. At most recent analysis (54 to 107 months after gene therapy), 9 of 10 patients had total $CD3^+$ T cell numbers at or just below the normal range (Table 2). At the time of publication, P8 was on maintenance chemotherapy for T-ALL, and T cell numbers remained low, although were increasing.

Similar patterns were observed for total $CD4^+$ and $CD8^+$ T cells (Fig. 1, B and C), with 6 of 10 having normal $CD4^+$ T cell numbers, and 9 of 10 normal $CD8^+$ T cell numbers at last follow-up (Table 2 and Fig. 1, B and C). The proportion of naïve ($CD45RA^+CD27^+$) T cells was normal or just below normal in 8 of 10 and 7 of 10 patients for $CD4^+$ T cells and $CD8^+$ T cells, respectively (including P8) (Table 2 and Fig. 1, E and F). Most patients maintained a large percentage of naïve $CD3^+CD45RA^+CD27^+$ T cells, and this correlated with higher levels of T cell receptor excision circles (TRECs) as a measure of thymic output. Although there was an initial increase in the numbers of NK cells ($CD16^+CD56^+$ NK cells), this

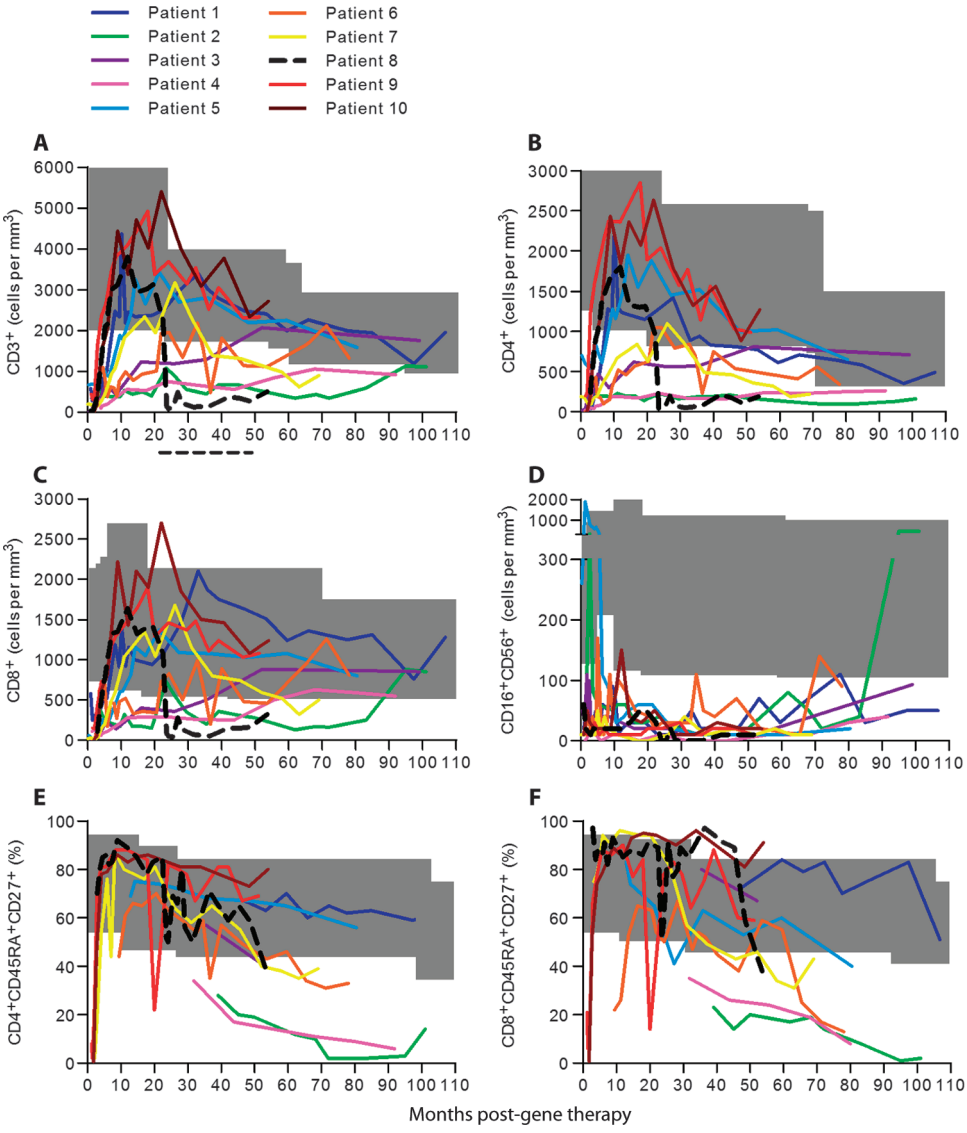

Fig. 1. T cell populations and NK cells after gene therapy. (**A** to **F**) Long-term follow-up of absolute numbers of $CD3^+$ T cells (A), $CD4^+$ (B), and $CD8^+$ (C) T cell subsets; $CD16^+CD56^+$ NK cells (D); and naïve $CD4^+CD45RA^+CD27^+$ (E) and $CD8^+CD45RA^+CD27^+$ (F) T cells for each of the 10 patients. Patient 8, who developed T-ALL, is highlighted in bold. Shaded areas indicate reference values for age-matched controls.

increase was generally not sustained over the longer term (Fig. 1D). Where evaluable, the levels of marking in sorted $CD16^+CD56^+$NK cells also decreased over time, which indicates a diminishing contribution of transduced progenitors to this compartment (copy number ranging from undetectable to 0.06). T cell proliferative responses to phytohemagluttinin (PHA) were normal in all patients except P2 compared to control values (Fig. 2A). In terms of T cell receptor (TCR) repertoire complexity, all patients achieved polyclonality, with all TCRVβ families represented at normal levels (Fig. 2B, table S1, and fig. S1). Furthermore, a mathematical method to score divergence from a normal Gaussian pattern of TCR complementarity-determining region 3 (CDR3) lengths showed that 6 of 10 patients were within the normal range at their most recent follow-up (including P8) (table S1 and fig. S1). P2 and P4 exhibited the most restricted repertoire; both of these patients achieved lower levels of immunological reconstitution in terms of total $CD3^+$ T cell numbers and naïve T cell populations. Transgene copy number determined by quantitative polymerase chain reaction (qPCR) in sorted $CD3^+$ T cell populations was <2 (including in the leukemic clone derived from P10), and the levels of cell surface γc expression were also normal (Table 2).

Total peripheral blood $CD19^+$ B cell numbers were within the normal range compared to age-matched controls in most patients, as expected (Table 2 and Fig. 3A). After gene therapy, the proportion of memory $IgM^+IgD^-CD27^+$ cells and isotype-switched memory $IgM^-IgD^-CD27^+$ B cells remained low in all patients tested, whereas most B cells retained a naïve IgD^+CD27^- immunophenotype (Table 2). Consistent with these findings, specific serological responses to vaccination with MMR (measles, mumps, and rubella), tetanus, and pneumococcal vaccines (polysaccharide and conjugate) were often suboptimal and poorly sustained, indicative of residual B cell dysfunction (table S2). Serological response to pneumococcal infection in P1 was also minimal (table S2). Despite these findings, P3, P4, P5, and P9 remain completely free from immunoglobulin replacement, and P1 received it intermittently (Fig. 3B). Transgene copy number in sorted $CD19^+$ ($CD20^+$) B cells remained low, as expected, in all patients (ranging from undetectable to 0.03 at latest follow-up), and no correlation could be made between the level of marking in this compartment and functional humoral immunity.

DISCUSSION

This study reports a longer-term follow-up (median months; range, 54 to 107 months) of 10 children with classical SCID-X1 treated by gene therapy using a conventional gammaretroviral vector. Substantial reconstitution of T cell immunity occurred in all patients, although some variability was observed, which may relate to cell dose, clinical condition at the time of treatment, and age. There were no deaths, although one patient developed T-ALL as a result of vector-mediated insertional mutagenesis (*8*). The one child treated at the age of nearly 4 years achieved modest T cell immunological reconstitution compared with those treated in the first year of life. This may reflect age-related restrictions to initiation of normal thymopoiesis as observed previously in an older child with atypical SCID-X1 and a young adult with a failed allogeneic graft (*9*). The level of reconstitution was not compared with a parallel cohort treated by unconditioned human leukocyte antigen (HLA)–mismatched allogeneic HSC transplantation (HSCT). However, reconstitution is

Table 2. Immunological characteristics at last follow-up. ND, not done.

	Patient number										Range*
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
Follow-up month	107	101	99	92	81	78	69	54	51	54	
$CD3^+$ (cells per mm^3)	1,950	1110	1754	920	1,580	1,330	900	**500**	2,330	2,720	700–4500
$CD4^+$ (cells per mm^3)	490	**160**	708	**260**	650	340	**220**	**190**	980	1,270	300–2400
$CD8^+$ (cells per mm^3)	1,280	850	854	550	800	810	500	320	1,080	1,240	300–1600
$CD16^+CD56^+$ (cells per mm^3)	**50**	450	93	**40**	**20**	90	**10**	**10**	**10**	**10**	90–1000
$CD4^+CD45RA^+CD27^+$ (%)	64	**14**	**41**	**6**	56	**33**	**39**	**39**	69	80	42–85
$CD8^+CD45RA^+CD27^+$ (%)	51	**2**	67	**8**	40	**13**	43	60	59	**91**	42–81
$CD4^+CD45RA^-CD27^+$ (%)	21	**60**	ND	**85**	**41**	**64**	**60**	**58**	30	20	9–30
$CD8^+CD45RA^-CD27^+$ (%)	**50**	**87**	ND	**64**	**33**	**68**	**23**	**35**	**30**	7	4–21
$CD4^+$ TRECs (per 10^6 T cells)	79,527	**185**	ND	**6318**	76,803	**26,123**	**26,573**	**4052**	52,391	**23,909**	>30,000 (1–16 years)
$CD8^+$ TRECs (per 10^6 T cells)	53,864	**100**	ND	**1576**	46,978	**3,129**	**16,686**	**2649**	**29,485**	**10,022**	>30,000 (1–16 years)
$CD19^+$ (cells per mm^3)	400	330	250	210	480	290	440	**190**	600	620	200–2100
$IgD^+IgM^+CD27^-$ (%) naïve	**97.14**	**96.06**	ND	**94.00**	**93.00**	**95.62**	**95.78**	**95.72**	**96.10**	**94.96**	58–62†
$IgD^+IgM^+CD27^+$ (%) memory	**2.26**	**3.27**	ND	**5.00**	**5.00**	**3.05**	**2.60**	**2.82**	**2.46**	**3.35**	19–42†
$IgD^-IgM^-CD27^+$ (%) switched	**0.25**	**0.21**	ND	**0.90**	**1.00**	**0.32**	**0.26**	**0.23**	**0.07**	**0.25**	19–35†
IgG (g/liter)	9.0	9.0	7.0	6.5	5.9	5.1	7.7	7.9	**4.4**	7.0	4.9–16.1
IgA (g/liter)	**0.25**	**<0.07**	0.49	4.30	1.19	**0.35**	**0.24**	**<0.07**	0.74	**<0.06**	0.4–2.0
IgM (g/liter)	**0.27**	**0.48**	1.04	0.57	1.39	**0.11**	**0.17**	**<0.05**	**0.48**	**0.16**	0.5–2.0
γc expression (mean fluorescence intensity)	586‡	348	ND	ND‡	324‡	332	513	320	550	480	250–600

*Numbers in bold are out of the normal range of age-matched controls. †Adult control sample. ‡Normal γc expression before gene therapy.

often incomplete in this setting (unless patients are treated early in the first 3 months of life), associated with graft-versus-host disease, and also, where used, from side effects of myelosuppressive chemotherapy (*4*, *5*, *10*). Furthermore, the 10-year survival in a large cohort of patients treated since 1995 was 72% (*6*).

The quality of T cell reconstitution, as determined by the continuing presence of naïve cells and repertoire complexity, in most patients suggests that immune function can be maintained long term after gene therapy. Only in patients with a low level of initial reconstitution does there appear to be an accelerated decline, probably because of low reserve. One patient treated with chemotherapy for T-ALL after first recovering normal T cell immunity maintained a normal proportion of naïve cells, even though numbers were low (on maintenance chemotherapy), and he has retained a highly polyclonal repertoire (fig. S1 and table S1). It is therefore possible that chemotherapy-resistant normal thymopoiesis persists for a substantial period of time in these patients even in the absence of observable transduction of bone marrow HSC.

The occurrence of human papillomavirus (HPV) cutaneous warts in two patients is unlikely to reflect defective T cell reconstitution and is a common finding after allogeneic HSCT for $T^-B^+NK^-$ SCID (*11*, *12*). The mechanisms for the propensity to develop warts are unclear but may reflect persisting intrinsic defects in host-derived Langerhans cells, abnormalities of NK function, or local γc-dependent keratinocyte immunodeficiency. The number of NK cells remained low in most patients despite an initial increase after engraftment. Furthermore, the levels of gene marking in purified NK cells diminished over time, suggesting that there was limited long-term persistence of transduced NK progenitor cells, common lymphocyte precursor cells, and HSCs.

In contrast to T cells, B cell reconstitution resulted in only partial humoral function, even in patients with normal peripheral T cell numbers and function. However, some patients have been able to discontinue immunoglobulin replacement (including P4, who was treated at the age of nearly 4 years), and we have previously shown that somatic mutations of the B cell receptor after gene therapy can be detected (*11*). Both vaccine responses and serological response to pneumococcal infection (in two patients) were suboptimal. In addition, one patient developed acute *P. jirovecii* pneumonitis at a time when T cell numbers and function had apparently normalized. Whether these observations also reflect coincident antibody deficiency (as anecdotally reported for patients with inherited antibody deficiency syndromes) or insufficient T cell help because of incomplete T cell reconstitution is unclear. Although total B cell numbers were normal, the numbers of memory cells in all patients were low. This indicates that restoration of polyclonal T cells alone is insufficient to entirely rescue antibody responses, and highlights the fact that SCID-X1 B cells are intrinsically dysfunctional as a result of deficiency of γc-dependent cytokine receptors (such as IL-21R and IL-4R). A long-term survival advantage for transduced B cell progenitors also does not appear to persist in vivo. Thus, some form of myelosuppression in

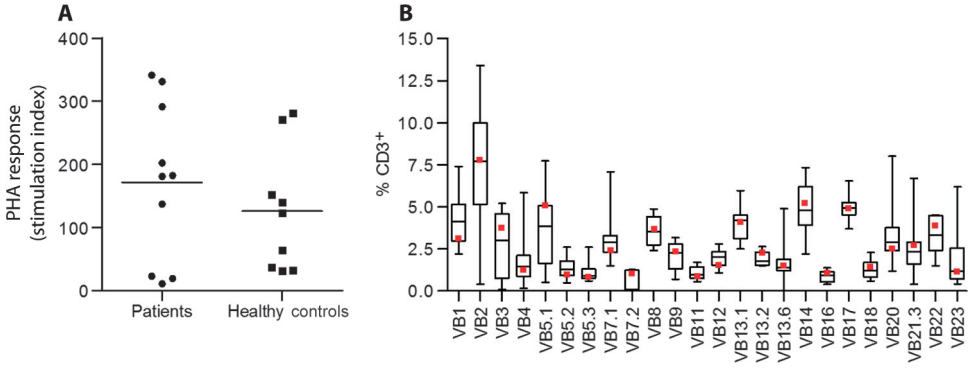

Fig. 2. T cell functionality and diversity after gene therapy. (**A** and **B**) In vitro proliferative responses to PHA compared to background (stimulation index) in patients compared to healthy controls (A) and TCRVβ family diversity in patients at latest follow-up. The mean values for the normal ranges are indicated by red dots (B).

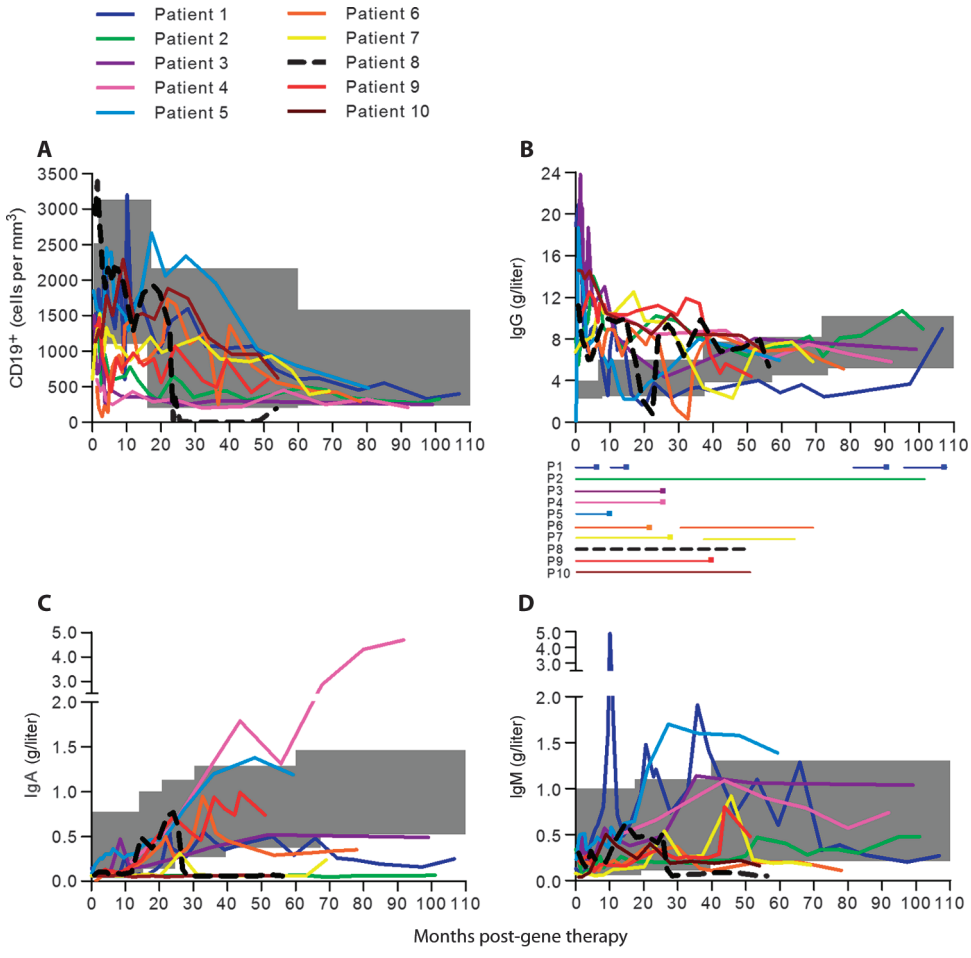

Fig. 3. B cells and serum immunoglobulin levels after gene therapy. (**A** to **D**) Long-term follow-up of absolute numbers of $CD19^+$ B cells (A), and levels of circulating IgG (B), IgA (C), and IgM (D) for each of 10 patients. Patient 8 is highlighted in bold, and colored lines correlating to each of the patients indicate periods of immunoglobulin replacement therapy (B). Boxes indicate where immunoglobulin replacement therapy has ceased.

addition to gene therapy may be necessary to ensure robust humoral reconstitution through enhanced engraftment of transduced HSC or at least long-lived B cell precursors. Either low-intensity conditioning using conventional agents or antibody-targeted immunodepletion is likely to be safe and may be sufficient for this purpose. Alternatively, use of more efficient gene delivery systems may allow effective engraftment of higher numbers of transduced HSC. A similar argument can be proposed for achievement of complete NK cell recovery.

In terms of immunological reconstitution, our findings are similar to those reported recently from another study and therefore provide important independent evidence that gene therapy is clinically effective (*13*, *14*). Hacein-Bey-Abina *et al.* reported long-term follow-up (median, 108 months; range, 96 to 144 months) of nine patients with a similar pattern of T cell, B cell, and NK cell recovery as our study. Four patients developed T-ALL as a result of insertional mutagenesis, and one died (*15*). Even so, the combined survival to date for the 19 patients treated in both studies is 95%. Three of the four patients surviving leukemia recovered T cell numbers and diversity after completion of chemotherapy. Lessons from these studies have now led to the design of improved vectors that are less likely to transactivate dangerous proto-oncogenes, and clinical trials are beginning in both Europe and the United States (*16*, *17*).

New vectors with deleted retroviral enhancer elements promise enhanced safety and efficacy of gene therapy. The additional use of myelosuppression should be considered to ensure long-term functional B cell reconstitution as observed in murine models and also in gene therapy trials for other conditions such as adenosine deaminase–deficient SCID (*16*, *18*, *19*). Overall, gene therapy offers a highly effective option for treatment of SCID-X1 when HLA-matched donors are unavailable and provides long-term reconstitution of a polyclonal T cell repertoire that effectively restores clinical immunity.

MATERIALS AND METHODS

Study entry criteria

Ten patients were recruited on the basis of a confirmed molecular diagnosis of

SCID-X1 and absence of an HLA-identical family or unrelated donor. Regulatory and ethical approval was received from the Gene Therapy Advisory Committee and Medicines and Healthcare Regulatory Agency. Fully informed written consent was obtained from the parents.

Gene transfer and administration of cells

Details of the gibbon ape leukemia virus–pseudotyped gammaretroviral vector and methodology of gene transfer have been described previously (*11*). Between 7 million and 30 million transduced progenitor cells per kilogram were infused into patients in the absence of myelosuppressive or immunosuppressive conditioning.

Immunological and molecular monitoring

Enumeration of lymphocyte populations was performed by flow cytometric analysis. Whole blood was labeled with combinations of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), or fluorochrome combinations with cyanines (PerCP-Cy5.5, APC-Cy7, and PE-Cy7) (BD Biosciences). Lymphocyte subsets were detected with a six-color multitest reagent containing CD3 FITC, CD16$^+$56 PE, CD45 PerCP-Cy5.5, CD19 APC, CD4 PE-Cy7, and CD8-APC-Cy7. Naïve, effector, and memory T cell populations were detected with CD45RA FITC, CD27 PE, CD45 PerCP, and CD4 or CD8 APC. Naïve, memory, and switched B cell populations were detected with CD19 PE-Cy7, CD27 PE, IgD FITC, and IgM Cy5. Staining was analyzed on a FACSCantoII or a FACSCalibur flow cytometer (BD Biosciences). For cell sorting of T, B, and NK cells, peripheral blood mononuclear cells (PBMCs) were labeled with CD45 APC-Cy7, CD19 APC, CD20 PE-Cy7, and CD3 FITC/CD16$^+$56 PE simultest reagent (BD Biosciences) and sorted on a MoFlo XDP (Beckman Coulter) fitted with two air-cooled solid-state lasers (100-mW Xcyte UV and 200-mW Icyt blue 488 nm) and a Coherent 647-nm red diode (75 mW). Dead cells were excluded with 4′,6-diamidino-2-phenylindole (DAPI) stain (Sigma). The analysis for cell sorting was done on Summit software (BD Biosciences).

Analysis of TCRVβ expression as a percentage of the total CD3$^+$ T population and assessment of T cell functionality after PHA stimulation were performed as described previously (*11*).

Immunoglobulin levels were measured with a Dade Behring nephelometer (BN II) according to the manufacturer's instructions. Immunoglobulin concentrations were compared with normal age-matched ranges. Patients discontinued immunoglobulin replacement usually when IgA levels reached normal values for age-matched controls on two consecutive occasions.

CDR3 TCR spectratyping was performed as described previously (*20*). Briefly, RNA was extracted and complementary DNA (cDNA) was prepared from CD3$^+$, CD4$^+$, and/or CD8$^+$ T cell subsets. Twenty-four Vβ-specific primers were used with a fluorescent-labeled constant region (Cβ)–specific primer to RT-PCR (reverse transcription–PCR)–amplify the CDR3 region of the TCRβ chain. Products were run on an AB3130 Genetic Analyzer (Applied Biosystems) and analyzed with SpA Web-based software (*21*).

Real-time qPCR targeting a specific marker of functional T cells, the TREC, was performed as described previously (*22*). Briefly, DNA was extracted from CD3$^+$, CD4$^+$, and/or CD8$^+$ T cell subsets and subjected to a multiplex qPCR to amplify TRECs and the RNaseP housekeeping gene. By reference to standard curves, generated with a TREC-containing plasmid (*23*) and dilutions of genomic DNA for the RNaseP gene, TREC numbers were calculated for each sample.

Transgene copy numbers in PBMCs, CD3$^+$, CD19$^+$, and CD16$^+$CD56$^+$ lymphocyte populations were determined by qPCR as described previously (*24*). Briefly, genomic DNA was extracted and used as a template for amplification of the transduced γc gene and β-actin housekeeping gene by qPCR. The forward and reverse primers and probe used for amplification were modified versions of those used previously (*24*) and were designed by E. Grassman (Cincinnati Children's Hospital): forward primer, 5′-TGCTAAAACTGCAGAATCTGGT-3′; reverse primer, 5′-AGCTGGGATTCACTCAGTTTG-3′; and probe, 5′-FAM-CCTGGGCTCCAGAGAACCTAACA-TAMRA-3′. By reference to standard curves with plasmids encoding γc and β-actin gene sequences, mean copy numbers of γc per cell were generated for each sample.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/3/97/97ra79/DC1
Table S1. Spectratype DkI values at last follow-up.
Table S2. Antibody responses following immunization in patients post–gene therapy.
Fig. S1. CD4$^+$ T cell spectratype histograms after gene therapy.

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