Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans

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ABSTRACT

We conducted a gene therapy trial in 10 patients with adenosine deaminase-deficient severe combined immunodeficiency (ADA-deficient SCID) using two slightly different retroviral vectors for the transduction of patients’ bone marrow CD34+ cells. Four subjects were treated without pre-transplant cytoreduction and remained on ADA enzyme replacement therapy (ERT) throughout the procedure. Only transient (months), low level (<0.01%) gene marking was seen in peripheral blood mononuclear cells (PBMC) of two older subjects (15 and 20 years old), whereas some gene marking of PBMC has persisted for the past nine years in two younger subjects (4 and 6 years). Six additional subjects were treated using the same gene transfer protocol, but after withdrawal of ERT and administration of low-dose busulfan (65-90 mg/m²). Three of these remain well, off ERT (5, 4, and 3 years post-procedure), with gene marking in PBMC of 1-10%, and ADA enzyme expression in PBMC near or in the normal range. Two subjects were restarted on ERT due to poor gene marking and immune recovery and one had a subsequent allogeneic hematopoietic stem cell transplant. These studies directly demonstrate the importance of providing non-myeloablative pre-transplant conditioning to achieve therapeutic benefits with gene therapy for ADA-deficient SCID.
INTRODUCTION

Adenosine deaminase (ADA) is an enzyme involved in purine metabolism and is essential for lymphocyte development, survival and function. ADA deficiency is an autosomal recessive inherited disorder that can result in severe combined immunodeficiency (SCID). Affected infants with ADA-deficient SCID are usually diagnosed by six months of age, presenting with failure to thrive and recurrent opportunistic infections, due to profound pan-lymphopenia and the virtual absence of humoral and cellular immunity. ADA-deficient SCID is almost always fatal by two years of age, if immunity is not restored.¹⁻³

The long-standing treatment of choice for ADA-deficient SCID is a hematopoietic stem cell (HSC) transplant from an unaffected, HLA-matched sibling.⁴ However, in most cases, suitable HLA-matched related donors are not available. HSC transplants from a closely HLA-matched, unrelated donor (MUD) or a haploidentical (parental) donor, using T-cell depletion, may be performed. However, outcomes from MUD or haploidentical transplants are significantly worse than those from matched siblings because of graft vs. host disease and engraftment failure.⁵

An alternative, non-transplant option is enzyme replacement therapy (ERT) with polyethylene glycol-modified bovine adenosine deaminase (PEG-ADA), given 1-2 times per week by intramuscular injection.⁶ Since this therapy became available in the late 1980s, more than 150 patients have been treated worldwide.⁵ ERT is life-sustaining and can restore protective immune function. However, its effects may be limited and most patients do not achieve full immune reconstitution.⁷ The high cost of ERT and the required life-long injections limits its access and may create severe financial burdens on patients and their families.

Because of the limitations of these conventional therapies, gene correction of autologous HSC has been explored as a potentially curative treatment for ADA-deficient SCID. The use of a patient’s own HSC by-passes the donor availability and immunological problems associated with allogeneic transplants. In the 1990’s, clinical trials for ADA-deficient SCID were performed
in Europe and in the US using γ-retroviral vector-mediated transfer of the human ADA cDNA into bone marrow or cord blood CD34+ HSC. Unfortunately, these studies achieved only low numbers of ADA-gene corrected blood cells. Based on ethical considerations of risk and benefit, these early trials did not involve pre-transplant cytoreductive conditioning with chemotherapy, which may aid engraftment but carries risks of side-effects. Additionally, subjects continued to receive ERT, which may have blunted the selective advantage of emerging gene-corrected T-lymphocytes. No significant clinical benefits occurred.

By the end of the 1990’s, improved techniques for isolation and gene modification of human HSC fostered the implementation of new clinical trials of gene transfer to autologous HSC for treatment of ADA-deficient SCID. These trials yielded clinically beneficial immune reconstitution with marrow conditioning with busulfan or melphalan at non-myeloablative dosages used to achieve a higher level of engrafted stem cells.

In 2001, we initiated a clinical trial using autologous bone marrow CD34+ cells with improved γ-retroviral vectors and more effective methods for gene transfer to the stem cells. In prior studies in murine models of HSC gene transfer, we demonstrated improved expression and resistance to silencing of retroviral vectors carrying the myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR) featuring specific modifications (deletion of a negative control region and alterations of the adjacent primer binding site). It was unclear, however, that such modifications would confer the same benefits on expression in human cells. Therefore, in our clinical trial we compared two vectors either using the non-modified MPSV LTR or its modified version, MND. The CD34+ cells for each subject were divided into two equal aliquots, separately transduced with one of the two vectors and re-mixed for clinical administration. Four subjects received intravenous infusion of gene-modified autologous bone marrow CD34+ cells without conditioning and remained on ERT. Following the positive results reported by Aiuti et al., six subsequent subjects underwent the procedure under an amended protocol that included
withdrawing PEG-ADA and administrating busulfan prior to infusion of the gene-corrected cells. We demonstrated that only the latter approach led to substantial levels of gene-corrected T-lymphocytes expressing ADA enzyme activity and supporting immune reconstitution.
MATERIALS AND METHODS

Clinical trial protocol: This Phase I/II clinical trial used retroviral-mediated gene transfer to CD34+ cells from bone marrow of ADA-deficient SCID infants and children (n=10) (**Figure 1A**). The major eligibility criteria included lack of a suitable matched sibling donor and adequate organ function. Cell processing followed a common protocol at both sites. The isolated CD34+ cells in each subject were divided into two aliquots, each transduced separately with one of two MMLV-based γ-retroviral vectors carrying the human ADA cDNA (MND-ADA and GCsapM-ADA, **Figure 1B**) (See Supplemental Methods). After transduction, the two cell aliquots were recombined as the “Final Cell Product” for intravenous infusion. Clinical trial objectives included assessments of safety [toxicities from the procedure and exposure to replication-competent retrovirus (RCR)] and efficacy [transduction/engraftment of HSC, ADA gene expression, and assessment of immune function]. Quantitative polymerase chain reaction (qPCR) primers specific for each retroviral vector were used to measure the frequency of cells containing vectors. Subjects were actively followed for the initial two years and then were enrolled into a clinical trial to monitor long-term outcome after gene transfer for FDA-mandated 15 year follow-up. The two clinical trial performance sites were the Children’s Hospital Los Angeles (CHLA) Bone Marrow Transplant Unit and the Clinical Center of the National Institutes of Health (NIH), Bethesda, MD.

The protocol (ClinicalTrials.gov:NCT00018018) was approved by the Institutional Review Boards and Institutional Biosafety Committees at CHLA and NIH, by the NIH Office of Biotechnology Activities Recombinant DNA Advisory Committee (#9908-337), and was conducted under BBIND #8556 from the U.S. Food and Drug Administration. The National Heart, Lung, and Blood Institute Cell Therapy/Gene Therapy Data Safety Monitoring Board served as the safety monitoring entity. All human trial participants (or legal guardians for minors) gave written informed consent in accordance with the Declaration of Helsinki.
Retroviral Vectors: Two similar MMLV-based γ-retroviral vectors were used to express the human ADA cDNA in the absence of a selectable marker gene (Figure 1B). In the GCsapM-ADA vector, ADA cDNA expression is driven by the non-modified MPSV LTR and the MMLV primer-binding site (pbs) is retained.\textsuperscript{21} In the MND-ADA vector, ADA cDNA expression is driven by the “MND LTR” which consists of the MPSV LTR with a 66bp “negative-control-region” deletion eliminating a YY-1 binding site, and substitution of the MMLV pbs with the pbs from an endogenous murine retrovirus (dl597rev).\textsuperscript{18-20} (See Supplemental Materials).

Clinical Monitoring for Toxicities: Subjects were monitored for safety and toxicities during and after busulfan administration, when given, (see Supplemental Materials) and after the reinfusion of the transduced cells. Clinical complications and abnormal laboratory values were graded using the Division of AIDS (NIAID, NIH) Table for Grading Severity of Pediatric Adverse Experiences, April 1994. Subjects were tested for exposure to RCR by qPCR assay for GALV sequences in peripheral blood mononuclear cell (PBMC) samples (baseline and after 3, 6, 12 and 24 months) at the National Gene Vector Laboratory. Tested samples from all ten subjects were below the limit of detection (<10 GALV copy number per 0.2 μg DNA).

Immunological Monitoring and ADA Enzyme assays: Immune function tests and ADA assays are described in Supplemental Materials.

qPCR determination of vector copy number: qPCR was performed with primers and probe designed to specifically amplify integrated proviral sequences of either the MND-ADA or the GCsapM-ADA vector. A common sense primer and a common TAMRA probe were used to detect both MND-ADA and GCsapM-ADA; the anti-sense primers were specific to their respective vectors. All reactions utilized Universal Master Mix [Applied Biosystems, Inc. (ABI),
Fullerton, CA], 350ng of template DNA, and were run under default conditions on the 7900 or 7500 Sequence Detector System (ABI). Results were compared to copy number standards (see Supplemental Materials and Methods).

Data and Statistical analyses: Pearson's correlation (r) was used to assess the linear correlation between the administered busulfan dosage (mg/m²) and the resulting busulfan AUC. Continuous outcomes, such as VCN, PBMC ADA activity, erythrocyte %dAXP, absolute lymphocyte counts, and serum IL-7 levels, were collected repeatedly over two years. To account for the correlated/unbalanced structure of the data, we performed generalized multivariate linear regression analysis on these outcomes. This approach allowed us to model the mean response as a smoothly changing function of time and other experimental factors. Within this framework, we performed estimation and hypothesis testing of the mean responses at different time point and various experimental covariate settings. For all statistical investigations, tests for significance were two-tailed, with a statistically significant p-value threshold of 0.05. Statistical analyses were carried out using SAS version 9.2.
RESULTS

Subjects. Ten subjects (age 15 months to 20 years) with ADA-deficient SCID were enrolled between 2001-2009 (Table 1). All were initially diagnosed based on biochemical demonstration of severe ADA deficiency. Mutations in the ADA gene locus were documented in nine subjects and most are known severe null or minimal activity mutations,\textsuperscript{24} with one patient not genotyped. Patients had been treated with ERT since diagnosis until enrollment in the study, from 1-14 years. In the first four subjects enrolled in 2001-2002 (subjects #201-204), ERT was not discontinued and busulfan was not administered. In the last six subjects enrolled between 2005-2009 (subjects #301-306), ERT was withdrawn and busulfan was administered prior to re-infusion of gene-modified CD34+ cells.

CD34+ cell dosages and transduction efficiency. The numbers of cells transduced and reinfused were limited by the amount of BM harvested (10-15 ml/kg) and the resultant numbers of CD34+ cells collected, isolated and present at the end of transduction. The CD34+ cell dosages ranged from 0.7-9.8x10^6 cells/kg (Table 1). There were no toxicities associated with the reinfusion of the cells in any of the subjects.

The vector copy number (VCN) of GCsapM-ADA and MND-ADA detected by qPCR in each subject’s transduced cells prior to re-combining for reinfusion were between 0.1-1.8 VCN/cell (Table 1). The transduced CD34+ cells had ADA enzyme activity in the range present in normal human PBMC (58-128 nM/min/10^8 cells), demonstrating expression by the vector (Table 1). There was not a consistent advantage to transduction by either vector as assessed by these in vitro assays.

Busulfan Administration. Six subjects were given busulfan (65-90 mg/m\textsuperscript{2}) after bone marrow harvest and prior to infusion of gene-modified cells to enhance engraftment. Busulfan levels
were measured and used to calculate the area-under-the-curve (AUC) for busulfan exposure. We observed highly consistent pharmacokinetics with a strong correlation between the administered dose (mg/m²) and the resulting busulfan AUC achieved (r= 0.95, p=0.003) (Supplemental Table 1). In the first three subjects (301-303), a busulfan dosage of 75 mg/m² was given (equating to 2.8-3.1 mg/kg), with AUC of 3,006-3,034 uM*min. Subject 304C was obese at >20% above the ideal body weight (IBW) for height and had a history of hepato-steatosis with mildly elevated serum transaminases at baseline. Therefore, the dosage of busulfan was adjusted based on IBW to 65 mg/m² (or 1.9 mg/kg). The resulting busulfan AUC (2,377 uM*min) was lower for this subject than for the three recipients of 75 mg/m², suggesting that the IBW dosage adjustment may not have been needed. Nonetheless, this subject had relatively high elevations of her serum transaminases at one month (Figure 2), which may have been more severe had she received a non-adjusted dose. In an effort to increase engraftment of gene-corrected cells in the last two subjects (305C, 306N), the busulfan dosages were increased to 90 mg/m² (~4.0 mg/kg), which produced concordantly higher AUC (4,060-4,873 uM*min).

No clinical toxicities were observed as a result of the busulfan; specifically, there was no associated nausea, vomiting, anorexia, seizures or hair loss. All of the subjects who received busulfan experienced transient neutropenia, thrombocytopenia, and elevation of liver enzymes. The nadir of neutropenia (between 200-700/mm³) occurred between days 22-26 after busulfan dosing (Figure 2A), except for subject 302C, who is discussed below. No adverse consequences of neutropenia were observed. However, brief courses of G-CSF treatment were employed in subjects 301N, 303N, and 306N to help resolve low ANC that persisted 70-100 day after busulfan administration. The nadirs of thrombocytopenia were more variable and occurred between days 18-37 after dosing, with the lowest recorded platelet count of 97,000/mm³ in 305C, who had the highest busulfan AUC (Figure 2B). Subject 304C, who received the lowest
dose of busulfan (65 mg/m²) and had the lowest busulfan AUC, experienced the smallest
decreases in neutrophil and platelet counts. Peak elevation of the liver enzymes alanine
aminotransferase (ALT) and aspartate aminotransferase (AST) occurred between days 20-33
after busulfan administration (Figures 2C and 2D). In subjects 304C and 306N, the increase in
liver enzymes reached grade III on the DAIDS toxicity scale; however liver synthetic function
remained consistently unaffected, and the transaminitis resolved without intervention by two
months after treatment.

Clinical Outcomes: The four subjects who remained on PEG-ADA and did not receive busulfan
(#201-204) experienced no adverse events. They remain clinically stable on continued ERT,
now 9-10 years after the procedure. In contrast, the six subjects who were withdrawn from ERT
and received busulfan (#301-306) experienced transient neutropenia and thrombocytopenia,
and moderate but self-limited increases in serum transaminases, as described above. Other
complications occurred in three subjects, as described below.

Three of the subjects (301N, 303N and 305C) remain clinically well and have been off ERT
since the procedure, currently 3-5 years later. These subjects remain on
trimethoprim/sulfamethoxazole prophylaxis for Pneumocystis jiroveci and intravenous gamma-
globulin (IVIg), except for subject 301N who has been off IVIg since 6 months after treatment.

Three of the subjects experienced adverse events following the procedure. Subject 302C,
who has been reported previously,25 was found to have trisomy 8 mosaicism in bone marrow
cells (including a sample taken prior to treatment with busulfan) and underwent a MUD HSC
transplant 8 months after the gene transfer procedure and is clinically well. Data from this
subject are not included in the subsequent analyses.

Two subjects developed infectious complications 4-5 months after the procedure. 304C was
noted to have fever, otitis media, and left lower lobe pneumonia. She was hospitalized for
intravenous antibiotics, which resulted in resolution of the symptoms without sequelae. 306N developed fever and diarrhea on Day +135 after the procedure and was admitted to the hospital. Adenovirus was detected by PCR in stool and blood samples and became undetectable after eleven weeks of intravenous cidofovir. As per protocol, both 304C and 306N were re-started on ERT at the time of the infectious complications to hasten immune reconstitution, and ERT has continued through the 24 months of follow-up.

Quantification of VCN in PBMC and granulocytes. To assess the transduction and engraftment of ADA gene-modified CD34+ cells, blood samples were obtained at serial time-points and VCN in PBMC and granulocytes were determined using qPCR. In the four subjects who did not receive busulfan conditioning (#201-204), there was only minimal and short-term presence of vector-containing granulocytes, with no vector in the myeloid cells beyond one year (Figure 3A). For the two older subjects treated at 15 and 20 years old (201C and 203N), there was no detectable vector-marking in PBMC beyond the first year. In contrast, the two younger subjects treated at 6 and 4 years old (202N and 204C), did have persistence of PBMC containing both the MND-ADA and GCsapM-ADA vectors, albeit at very low frequencies (10^{-3}–10^{-5} VCN/cell). Vector-marked PBMC have remained intermittently detectable at these levels for 8-9 years of evaluation (data not shown).

The subjects who received busulfan (#301-306) showed significantly higher levels of vector-containing cells in both the granulocyte and PBMC fractions (p<0.05 from month +10 onward, Figure 3B). In this group, the levels of vector marking in PBMC were 100-fold higher (0.01-0.1 VCN/cell – or 1-10% of cells) than in granulocytes (0.001 VCN/cell- or 0.1% of cells) (p=0.0017). Interestingly, the frequencies of vector-marked cells in the two subjects who had ERT resumed (304C and 306N) were similar to those for whom ERT was not re-started (301N, 303N, 305C, p=0.996.
There was a general trend for higher levels of PBMC and granulocytes marked with the MND-ADA vector than with the GCsapM-ADA vector (p=0.0111); only one subject showed persistent gene-marking in granulocytes with GCsapM-ADA beyond one year, whereas all subjects had persistence of granulocytes marked with MND-ADA (Figure 3B). Analysis of the relative gene marking by the two vectors in the individual subjects showed a modestly higher level with MND-ADA than with GCsap-M-ADA (Supplemental Figure 1). T-cell clones grown from peripheral blood of three subjects (301N, 303N, 305C) were found to harbor the MND-ADA vector with greater frequency than the GCsapM-ADA vector (119 vs. 11) (Supplemental Table 2 and Supplemental Figure 2). No significant difference was noted, however, in the ADA enzymatic activity provided by the two vectors in these isolated clones.

DNA from PBMC samples that had vector marking levels >1% was examined by LAM-PCR (National Gene Vector Biorepository) to amplify junctions of vector integrants and flanking chromosomal sequences. No progressive clonal expansions were seen by agarose gel electrophoresis of LAM-PCR products (data not shown). We also used non-restrictive LAM-PCR and high-through-put sequencing to determine the patterns of vector integration sites in PBMC from the conditioned subjects (Supplemental Methods). We found relatively low diversity of integration sites in PBMC from each subject, ranging from 22-145 unique integrants per subject, over 2 years after transplant (Supplemental Figure 3). Stable clones with vectors near known Common Integration Sites (e.g. EVI-1, LMO2) were observed, without clinical consequences during the 2-year observation period.

**PBMC ADA enzyme activity and RBC dAXP levels:** Prior to treatment, PBMC had essentially no detectable ADA activity, consistent with the severe deficiency in these SCID subjects. In the four subjects who did not receive busulfan and remained on ERT (#201-204), there were minimal increases in the PBMC ADA activity in the months after transplant (Figure 4A). In
contrast, PBMC ADA activity of each of the subjects who received busulfan and had ERT withdrawn (#301-306) showed a gradual increase over the months after transplant, to levels from half-normal to normal range (Figure 4B). The levels of ADA activity in PBMC were significantly higher from month+5 onward in recipients of busulfan than in subjects not receiving busulfan (p<0.05). Notably, subject 304C who went back on ERT after 5 months had a similar rise in PBMC ADA activity, suggesting the absence of negative effects of ERT on the persistence and expansion of engrafted gene-corrected cells. In contrast, ADA 306N who also went back on ERT showed substantially less increase in PBMC ADA activity (~12 units at 24 months).

ADA-deficient SCID patients accumulate deoxyadenine nucleotides (dAXP) in erythrocytes, and the percentage of adenine nucleotides that are deoxyadenine nucleotides (%dAXP) are routinely measured to follow the efficacy of ERT. For the subjects remaining on ERT continuously (#201-204), the erythrocyte %dAXP remained at low levels (<1%), indicating adequate detoxification (data not shown). In contrast, the levels of dAXP rose sharply upon ERT withdrawal in subjects #301-306 (Figure 4C). The %dAXP values for the three subjects who remained off ERT for the two years following transplant (301N, 303N, 305C) remained in the range of ~5%, which is similar to levels seen in ADA-deficient SCID patients who have undergone successful allogeneic HSC transplant. The two subjects who resumed ERT (304C and 306N) had significantly lower levels of RBC dAXP (p<0.05 from month+18 onward).

**Immunological Outcomes:** The non-conditioned subjects remaining on ERT had stable ALC in the sub-normal range of ~300-500/mm³, which is typical for patients on long-term ERT (Figure 5A). In contrast, in the conditioned subjects for whom ERT was stopped, the ALC declined sharply in the first months, likely due to combined effects of busulfan administration and toxicity from deoxynucleotide accumulation upon ERT withdrawal. In the three subjects who remained
off ERT (301N, 303N and 305C), the ALC increased over 6-24 months (~300-1,000/mm³). The
two subjects who had their ERT resumed at 5 months (304C and 306N) showed more rapid
increases in ALC, which stabilized at levels similar to those before ERT was interrupted (Figure
5B). The ALC for the group that had ERT stopped were significantly lower than in those
remaining on ERT over the first 10 months (p<0.05).

Lymphocyte subset values at 24 months after the procedure are listed in Table 2 (see also
Supplemental Figure 4 for serial values). The absolute numbers of CD3+ T-cells and T-cell
sub-types [CD4+, CD8+, %CD45RA+/CD4+ (“naïve”)]) were below normal in all subjects,
whether receiving ERT continuously or having had ERT withdrawn prior to gene transfer. T-
lymphocyte subsets were in the ranges of CD3+ 100-500/mm³, CD4+ 75-200/mm³, and CD8+
100-400 cells/mm³. Interestingly, subject 304C who re-started ERT within 5 months of the gene
transfer had the highest numbers of T-cells, B-cells and NK-cells, with normal IgA, suggesting
the dual treatments had additive effects. However, 306N was also re-started on ADA ERT at 5
months, yet had lower lymphocyte numbers.

In vitro lymphocyte proliferation responses to phytohemagglutinin (PHA) remained defective
in the first cohort of subjects (#201-204), who did not have significant numbers of gene-
corrected cells (Table 2). The PHA responses were significantly higher at 24 months in the
second cohort (301-306) (p=0.024), with a general trend to higher PHA responses at times after
one year (Figure 6C). We also assessed the diversity of T-cell receptor rearrangement by
spectratype in some subjects and observed broad, non-restricted repertoires (Supplemental
Figure 5). Analysis of T-cell receptor excision circles (TREC) did not show an advantage for
either treatment on this measure of thymic reconstitution (Table 2). There was a strong
association with achieving higher levels of PBMC expressing ADA and the presence of
CD45RA/CD4+ naïve T cells (Supplemental Figure 4).
The absolute numbers of B-cells were sub-normal in all of the subjects (Table 2). Two of the subjects were not receiving IVIg replacement and had IgG levels in the normal range. One of the six subjects on ERT (203N) was off IVIg. Of the three evaluable conditioned subjects, restoration of serum immunoglobulin production was complete in only one subject (301N) who also responded successfully to immunizations against clinical vaccines to tetanus, diphtheria, *Haemophilus influenzae*, and poliovirus antigens. 303N had low levels of IgA and IgM, whereas 305C had a normal level of IgA, but low IgM. Of the two subjects who had resumed ERT at 5 months, one had normal levels of IgA and IgM (304C), and one continued to have low levels of immunoglobulins (306N). NK-cells numbers were in the normal range in only one of the four non-conditioned subjects (202N) and in one of the two who resumed ERT (304C), and were below normal in the three who remained off ERT (301N, 303N, and 305C).

**Serum IL-7 levels:** Increased serum IL-7 levels have been reported in lymphopenic patients, either untreated SCID patients or following HSC transplant.\textsuperscript{27} We measured serum IL-7 in subject samples. Subjects 201-204 maintained relatively constant IL-7 levels in the months following their procedures (Figure 6). In contrast, subjects who were withdrawn from ERT (#301-306) showed transient 2-fold increases in serum IL-7 levels in the first 2-6 months following gene transfer. After reaching maximum values at +4 months, the IL-7 serum concentration declined to levels similar to those measured in the subjects who had remained on ERT. The changes in serum IL-7 levels in the subjects withdrawn from ADA ERT varied reciprocally with the absolute lymphocyte counts as they declined after PEG-ADA was withdrawn and then rose over the next 6-9 months. These results are consistent with models in which IL-7 levels are modulated by the rate of consumption by lymphocytes and correlate inversely with ALC.\textsuperscript{28}
DISCUSSION

This trial performed a direct comparison between two different approaches to gene therapy for ADA-deficient SCID: the first cohort of four subjects did not receive cytoreductive conditioning prior to reinfusion of transduced bone marrow CD34+ cells and continued to receive ERT, whereas the second cohort of six subjects received non-myeloablative conditioning with busulfan and had ERT withdrawn. Other relevant factors, such as the γ-retroviral vectors used and the method for in vitro gene transduction of the bone marrow cells, remained constant between the two cohorts. Clearly, only the protocol with administration of busulfan and withdrawal of PEG-ADA led to efficacy, as shown by the long-term presence of gene-containing blood cells expressing ADA enzyme activity, which afforded partial immune reconstitution in the absence of exogenous ADA ERT. Absence of conditioning and continuation of ERT did not lead to any adverse effects, but essentially no efficacy was observed. These findings are in accord with those results from prior trials in Italy\textsuperscript{11,12} and the United Kingdom\textsuperscript{14,16} in which non-myeloablative conditioning and ADA ERT withdrawal were performed and subjects realized immune reconstitution. Neither of these prior trials contained a direct comparative group that did not, so the benefits of the approach were based on comparison to earlier studies performed under markedly different conditions.

The relative contributions of the two changed variables (cytoreductive conditioning and ERT withdrawal) are not easily discerned. Administration of busulfan almost certainly was responsible for the higher levels of gene-containing blood cells achieved, by allowing engraftment and persistence of more of the transduced CD34+ cells. PEG-ADA withdrawal may have enhanced the relative frequencies of gene-corrected T-lymphocytes, by leading to lowered numbers of non-corrected lymphocytes, which would dilute the gene-containing cells measured by qPCR. The subjects in the second cohort who had ERT withdrawn, developed
severe lymphopenia in the first months after transplant, associated with elevated serum IL-7 levels and this may have driven expansion of gene-corrected lymphocytes. Subjects remaining on ERT did not become lymphopenic, had stable levels of IL-7 and did not show increases in lymphocyte numbers.

Interestingly, one of two subjects who had ERT withdrawn with gene transfer but then restarted 6 months after the procedure (304C), developed similar levels of gene-marked cells as subjects who remained off PEG-ADA (301N, 303N, 305C). This observation implies that PEG-ADA does not significantly blunt the production of gene-containing lymphocytes or their absolute number, but merely lowers measurements of their relative frequency by diluting them with non-corrected lymphocytes supported in trans by ERT. Based on the absence of a deleterious impact of ERT on production of gene-corrected lymphocytes, it may be advisable to continue ERT for some months after gene transfer to avoid lymphopenia and the associated risks of infection. Of note, two children with ADA-deficient SCID were treated in Japan in 2003-2004 by γ-retroviral-mediated gene transfer to bone marrow CD34+ cells after ERT withdrawal but without pre-transplant chemotherapy.29 These patients slowly developed gene-corrected T-cells, although full immune recovery was not achieved over a 6-year follow-up period, leading the investigators to suggest that both conditioning and the absence of ERT play roles in the kinetics and extent of immune reconstitution.

The partial cytoablative procedure with 3-4 mg/kg busulfan was well tolerated clinically and was minimally toxic. In four out of six subjects treated with busulfan, we observed prolonged neutropenia that promptly responded to G-CSF if given. Persistence of low ANC (200-500/mm³) at 70-100 days after transplant in the three subjects who eventually recovered neutrophil counts in response to G-CSF may be explained as a consequence of the myeloid dysplasia features that have recently been recognized to accompany ADA deficiency.30
We also observed transitory elevation of transaminases that reached values over 5-times normal in two subjects (304C and 306N), who had previous histories of mild hepato-steatosis or hepatomegaly of undetermined nature. While these complications were self-limited and without clinical sequelae, they are significant in that they did not correlate with AUC of busulfan and may reflect the specific hepatic sensitivity of some ADA-deficient patients.\textsuperscript{31} Besides these complications, our observations confirm that the dosage of busulfan first used by investigators in Milan affords an effective enhancement of engraftment, without significant acute toxicity. An interim analysis of data obtained in subjects 301-304, indicated that administration of 75 mg/m\textsuperscript{2} busulfan resulted in 2-3 mg/kg dose and modest engraftment of gene-marked cells. For this reason, we increased the dosage to 90 mg/m\textsuperscript{2} for the following subjects. This resulted in 4-5 mg/kg dosage (Supplemental Table 1), without increased toxicity.

This trial also involved a direct comparison of two retroviral vectors differing mainly by the presence or absence of the wild-type MMLV primer-binding site (pbs), a sequence associated with profound silencing in murine HSC and embryonic stem cells.\textsuperscript{32-34} While we did not have direct evidence for silencing of either vector, the frequency of PBMC and granulocytes containing the MND-ADA vector was generally higher than that of cells carrying the GCsapM-ADA vector, despite similar initial transfer by the two vectors in the CD34\textsuperscript{+} cells. In addition, the vast majority of T-cell clones isolated from treated subjects contained the MND-ADA vector. These findings suggest that the alternative pbs carried by the MND-ADA vector conveyed favorable expression features that allowed it to out-perform the GCsapM-ADA vector in production or survival of ADA gene-corrected immune cells. Based on these observations, we are now performing a Phase II study for ADA-deficient SCID using only the MND-ADA vector (NCT00794508), but retaining the key features of the Phase I trial with low dose busulfan and PEG-ADA withdrawal prior to treatment.
The outcomes for the six subjects treated with cytoreductive conditioning in the absence of ERT are encouraging: three remain well without ERT, after 3-5 years. From this group, as well as eight additional subjects treated under the ongoing Phase II trial, we have observed that younger subjects (less than 2-4 years old) yield higher numbers of bone marrow CD34+ cells allowing higher numbers of transduced cells to be transplanted and achieve higher numbers of T, B and NK-cells expressing ADA enzyme, compared to the older subjects. This observation may explain also the differences in outcome between the subjects reported here and those reported in the first trial from Milan, where 8 out of 10 subjects achieved significant immune reconstitution. Indeed, for the cohort treated in Milan, the average age of the treated patients was lower and the average CD34+ cell dose was higher than for our subjects.

Importantly, none of the subjects in any of the clinical trials of gene therapy for ADA-deficient SCID using this type of approach (n=38) have encountered the complications from insertional oncogenesis that occurred in trials for other disorders. It is not known whether the better safety profile in the ADA-deficient SCID trials is merely fortuitous or reflects real biological differences. The vector integration site analyses we performed revealed oligoclonal reconstitution, suggesting that a relatively low number of transduced HSC engrafted; this may underlie the incomplete immunological benefits achieved. Further follow-up of these and additional subjects will be needed to understand long-term benefits and risks of autologous transplantation of gene-modified HSC compared to alternatives of allogeneic HSCT or long-term ERT.
ACKNOWLEDGEMENTS

We would like to thank Kathy Wilson MSN, Renna Killen MSN, Mirna Sweeney RN, the CHLA Clinical Immunology Laboratory staff, and the nurses of the BMT Unit at CHLA for their important roles in patient care. At the NIH, invaluable contributions were made by Dr. Cynthia Dunbar, the Cell Processing Section of the Department of Transfusion Medicine, the nurses in 1NW of the Clinical Center and Julia Fekecs for providing the medical artwork. Clinical grade GCsapM-ADA and MND-ADA supernatants used in the trial were produced by W. Jay Ramsey, Laura Tuschong, and Linda Muul in the Clinical Gene Therapy Branch of the National Human Genome Research Institute, NIH. Estuardo Aguilar Cordova and Ken Cornetta provided support with production of clinical grade vector supernatants. Takara Shuzo Inc, Otsu Japan, Amgen Inc, Thousand Oaks CA, and Immunex, Seattle WA all provided essential reagents for CD34+ cell transduction. The National Gene Vector Laboratories and the National Gene Vector Biorepository provided clinical trial support with assays for RCR and LAM-PCR. The UCLA Eli & Edythe Broad Center of Regenerative Medicine & Stem Cell Research Flow Cytometry Core and High Through-Put Sequencing Core were essential for these studies.

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Specific Author Contribution Statement

Fabio Candotti and Donald B. Kohn developed the clinical trial, co-directed the studies, participated in subject enrollment, treatment and evaluations and primarily wrote the paper.

Kit L. Shaw, Elizabeth Garabedian, G. Jayashree Jagadeesh, Barbara C. Engel, and Gregory M. Podsakoff served as clinical and regulatory coordinators for the study.

Robert Sokolic, Shepherd Schurman, and Chimene Kesserwan participated in subject enrollment, treatment and evaluations.

John F. Tisdale, Kenneth I. Weinberg, Gay M. Crooks, Neena Kapoor, Ami Shah, Hisham Abdel-Azim, Alan S. Wayne, Howard M. Rosenblatt, Carla M. Davis, Celine Hanson, Radha G. Rishi provided clinical care to the subjects and participated as clinical co-investigators.

Linda Muul, Denise Carbonaro, Christopher Choi, Xiao-Jin Yu, Monika Smogorzewska, Pei-Yu Fu, Eric Gschweng, Aaron Cooper, Gerhard Bauer, Joanna A. Ireland, Otto O. Yang, and Arumugam Balamurugan performed clinical HSC processing and/or laboratory end-point studies.

Xiaoyan Wang and David Gjertson performed statistical analyses.

Michael Hershfield, R. Michael Blaese, and Robertson Parkman participated in clinical trial development and analysis.

Conflict-of-interest disclosure: M.S.H. receives grant support from Sigma-Tau Pharmaceuticals.
References


Table 1. Subject Characteristics, Treatment and Outcomes.

*Patient Identification Number

<table>
<thead>
<tr>
<th>PIN</th>
<th>Age at Entry</th>
<th>Sex</th>
<th>Mutation(s)</th>
<th>PEG-ADA ERT[^1]</th>
<th>Busulfan Conditioning (mg/m²)</th>
<th>CD34+ Cell Dose (x10³/kg)</th>
<th>VCN[^2] (copy/cell)</th>
<th>ADA enzyme activity[^3] (nmol/10⁶ cells/min)</th>
<th>Years of Follow-up</th>
<th>PEG-ADA ERT Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>201C</td>
<td>15 yr</td>
<td>F</td>
<td>c.955-959del c.529C&gt;A</td>
<td>Continued</td>
<td>No</td>
<td>0.7</td>
<td>n.a[^4]</td>
<td>n.a[^4]</td>
<td>36</td>
<td>10</td>
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<tr>
<td>202N</td>
<td>6 yr</td>
<td>F</td>
<td>Homozygous c.632G&gt;A</td>
<td></td>
<td></td>
<td>9.8</td>
<td>1.46</td>
<td>0.78</td>
<td>70.3</td>
<td>148.5</td>
</tr>
<tr>
<td>203N</td>
<td>20 yr</td>
<td>F</td>
<td>c.302G&gt;T c.872C&gt;T</td>
<td></td>
<td></td>
<td>1.1</td>
<td>not done</td>
<td>not done</td>
<td>8.3</td>
<td>7.6</td>
</tr>
<tr>
<td>204C</td>
<td>4 yr</td>
<td>M</td>
<td>Not available</td>
<td></td>
<td></td>
<td>1.9</td>
<td>0.48</td>
<td>0.25</td>
<td>143</td>
<td>50.3</td>
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<tr>
<td>301N</td>
<td>2 yr</td>
<td>M</td>
<td>c.424C&gt;T c.978A&gt;G</td>
<td></td>
<td></td>
<td>75</td>
<td>4.8</td>
<td>not done</td>
<td>43.4</td>
<td>204.5</td>
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<tr>
<td>302C</td>
<td>3 yr</td>
<td>F</td>
<td>c.302G&gt;A c.632G&gt;A</td>
<td></td>
<td></td>
<td>75</td>
<td>1.5</td>
<td>0.106</td>
<td>34.25</td>
<td>43.55</td>
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<tr>
<td>303N</td>
<td>15 mo</td>
<td>F</td>
<td>c.478+1G&gt;A c.646G&gt;A</td>
<td>Withdrawn</td>
<td></td>
<td>75</td>
<td>1.9</td>
<td>1.2</td>
<td>7.23</td>
<td>88</td>
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<tr>
<td>304C</td>
<td>9 yr</td>
<td>F</td>
<td>c.221G&gt;T c.986C&gt;T</td>
<td></td>
<td></td>
<td>65</td>
<td>1.6</td>
<td>0.3</td>
<td>5.9</td>
<td>10.5</td>
</tr>
<tr>
<td>305C</td>
<td>20 mo</td>
<td>M</td>
<td>Homozygous c.632G&gt;A</td>
<td></td>
<td></td>
<td>90</td>
<td>9.8</td>
<td>0.0565</td>
<td>14.28</td>
<td>85.53</td>
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<tr>
<td>306N</td>
<td>22 mo</td>
<td>M</td>
<td>Homozygous c.955-959del</td>
<td></td>
<td></td>
<td>90</td>
<td>9.5</td>
<td>0.215</td>
<td>54.9</td>
<td>170</td>
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</table>

[^1]: Enzyme replacement therapy (ERT) with polyethylene glycol-conjugated adenosine deaminase (PEG-ADA).
[^2]: VCN: vector copy number in transduced CD34+ cells by qPCR
[^3]: ADA enzyme activity in transduced CD34+ cells
[^4]: For subject 201C, only the GCaspM-ADA vector was used.
[^5]: Status-post matched unrelated donor (MUD) hematopoietic stem cell transplant (HSCT) 8 months after gene transfer for marrow aplasia.
[^6]: Due to development of infection.
Table 2. Immunological Status at Two Years after Autologous Gene Transfer.

<table>
<thead>
<tr>
<th>PIN*</th>
<th>On ERT†</th>
<th>Lymphocyte Subsets b</th>
<th>Serum Immunoglobulins c</th>
<th>PHA (%LLN) d</th>
<th>TREC / ug PBMC DNA e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD3 (ref. range)</td>
<td>CD4 (ref. range)</td>
<td>CD8 (ref. range)</td>
<td>CD19 (ref. range)</td>
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<tr>
<td>201C</td>
<td>Yes</td>
<td>0.371 (1.09-2.20)</td>
<td>0.181 (0.52-1.30)</td>
<td>0.192 (0.55-0.92)</td>
<td>0.002 (0.11-0.57)</td>
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<tr>
<td>202N</td>
<td>Yes</td>
<td>0.068 (1.02-2.60)</td>
<td>0.061 (0.65-1.50)</td>
<td>0.004 (0.37-1.10)</td>
<td>0.062 (0.27-0.98)</td>
</tr>
<tr>
<td>203N</td>
<td>Yes</td>
<td>0.151 (1.09-2.20)</td>
<td>0.095 (0.33-1.30)</td>
<td>0.045 (0.33-0.92)</td>
<td>0.013 (0.11-0.57)</td>
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<tr>
<td>204C</td>
<td>Yes</td>
<td>0.174 (1.09-3.70)</td>
<td>0.067 (0.70-2.20)</td>
<td>0.095 (0.40-1.30)</td>
<td>0.063 (0.39-1.40)</td>
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<tr>
<td>301N</td>
<td>No</td>
<td>0.512 (1.40-3.70)</td>
<td>0.074 (0.70-2.20)</td>
<td>0.415 (0.49-1.30)</td>
<td>0.025 (0.38-1.40)</td>
</tr>
<tr>
<td>303N</td>
<td>No</td>
<td>0.113 (1.40-3.70)</td>
<td>0.070 (0.70-2.20)</td>
<td>0.042 (0.49-1.30)</td>
<td>0.024 (0.39-1.40)</td>
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<tr>
<td>304C</td>
<td>Yes</td>
<td>0.737 (1.20-2.60)</td>
<td>0.254 (0.65-1.50)</td>
<td>0.473 (0.37-1.10)</td>
<td>0.209 (0.27-0.98)</td>
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<tr>
<td>305C</td>
<td>No</td>
<td>0.317 (1.40-3.70)</td>
<td>0.194 (0.70-2.20)</td>
<td>0.090 (0.49-1.50)</td>
<td>0.022 (0.39-1.40)</td>
</tr>
<tr>
<td>306N</td>
<td>Yes</td>
<td>0.174 (1.40-3.70)</td>
<td>0.115 (0.70-2.20)</td>
<td>0.043 (0.40-1.50)</td>
<td>0.073 (0.38-1.40)</td>
</tr>
</tbody>
</table>

*Patient Identification Number
†Enzyme replacement therapy (ERT) with polyethylene glycol-conjugated adenosine deaminase (PEG-ADA) at two years.
‡Lymphocyte subsets are presented as number of cells per microliter x 10⁵. Age-based normal reference ranges for lymphocytes subsets are from Shearer et al. ²⁹
§Serum immunoglobulin levels are presented as mg/dl. Age-based normal reference ranges for serum immunoglobulin levels are from Joliff et al. ³⁹
Anne of lymphocyte proliferative response to phytohemagglutinin (PHA), presented as percentage of ³H-thymidine incorporation (c.p.m.) by subject to lower limit of normal for testing lab.
⁴TREC= Thymic recombination excision circles, measured by qPCR as described in Supplemental Materials and Methods. n.d. = not done; Neg = negative for detectable TREC qPCR product. Healthy adult reference range >2STREC/ug. ⁴⁰
Receiving monthly intravenous immunoglobulin (IVIg) replacement therapy.
Figure 1

**A**

- +/- Withdraw PEG-ADA (1-2 wks before GT)
- Harvest autologous bone marrow and isolate CD34+ cells
- Divide cells into two aliquots
- Pre-stimulate x 2 days SCF, MGDF, Flt-3L on rFBN
- Assess safety and efficacy end-points
- Combine transduced cells and infuse I.V.
- +/- Busulfan administration (75-90 mg/m²)
- Separately transduce x 3 days: GCsapM-ADA and MND-ADA

**B**

- MND-ADA
  - MND
  - dl587
  - 5' LTR
  - pbs
  - ψ
  - SD
  - SA
  - HuADA cDNA
  - MND
  - 3' LTR

- GCsapM-ADA
  - MPSV
  - MMLV
  - 5' LTR
  - pbs
  - ψ
  - SD
  - SA
  - HuADA cDNA
  - MPSV
  - 3' LTR

**Figure 1.** Experimental schema and maps of retroviral vectors carrying the normal human ADA cDNA. (A) The experimental schema for the clinical trial is shown. SCF: stem cell factor; MGDF: megakaryocyte growth and differentiation factor; Flt-3L: Flt-3 ligand; rFBN: recombinant fibronectin. (B) The elements of the proviral forms of the two retroviral vectors used to transfer normal human ADA cDNA are depicted. LTR: long terminal repeat; pbs: primer-binding site; ψ: packaging signal; SD: splice donor site; SA: splice acceptor site; MPSV: Myeloproliferative Sarcoma Virus; MND: MPSV LTR, ncr-deleted, coupled to dl587rev pbs; MMLV: Moloney Murine Leukemia Virus; dl587: endogenous murine retrovirus dl587rev; A: common qPCR primer; B: vector-specific qPCR primer; common qPCR probe.
Figure 2. Hematologic values and serum transaminase levels after busulfan administration.

(A) absolute neutrophil counts (ANC), (B) platelet counts, (C) serum alanine aminotransferase (ALT), and (D) serum aspartate aminotransferase (AST) levels over two months.
Figure 3. Quantitative polymerase chain reaction (qPCR) measurements of the average vector copy/cell in blood cell samples obtained after transplant. Separate qPCR analyses were done on granulocytes (Grans) and peripheral blood mononuclear cell (PBMC) fractions with primer/probe sets specific for the MND-ADA vector or the GCsapM-ADA vector provirus.

(A) Results from subjects not receiving busulfan conditioning and remaining on ERT (#201-204) and (B) results from subjects having ERT withdrawn and receiving busulfan prior to transplant (#301-306).
Figure 4

 ADA enzymatic activity in PBMC and % deoxyadenine nucleotides in erythrocytes. ADA enzyme activity in PBMC was measured biochemically. The graphs show (A) the values from subjects not receiving busulfan conditioning and remaining on ERT (#201-204) and (B) the values from subjects having ERT withdrawn and receiving busulfan prior to transplant (#301-306). The normal reference range for the ADA enzyme assay in human PBMC is indicated (gray shaded horizontal bar). (C) Adenine and deoxyadenine metabolites were measured in erythrocytes by high-pressure liquid chromatography and the percentage that were deoxyadenosine nucleotides (dAMP + dADP + dATP) were plotted as %dAXP for the subjects in the group receiving busulfan (#301-306). The times when ERT was resumed for subjects 304 and 306 are indicated.
Figure 5

**A**

Figure 5. *Absolute lymphocyte counts (ALC) and proliferative responses after gene transfer.* (A) Results from subjects not receiving busulfan conditioning and remaining on ERT (#201-204) and (B) results from subjects having PEG-ADAERT withdrawn and receiving busulfan prior to transplant (#301-306). The time when ERT was resumed for subjects 304 and 306 are indicated. (C) Proliferative responses to phytohemagglutinin (PHA) of PBMC from subjects over time. Open symbols represent subjects remaining on ERT and not receiving busulfan; filled symbols represent subjects receiving busulfan with ERT stopped.
**Figure 6.** *Serum IL-7 levels.* Stored serum samples were used to measure levels of interleukin-7 (IL-7) by ELISA. The average IL-7 levels for the subjects remaining on continuous ERT (#201-204) and for the subjects withdrawn for ERT and receiving busulfan (300 series) are shown.
Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans


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