

# Clinical efficacy of gene-modified stem cells in adenosine deaminase-deficient immunodeficiency

Kit L. Shaw,<sup>1</sup> Elizabeth Garabedian,<sup>2</sup> Suparna Mishra,<sup>1</sup> Provaboti Barman,<sup>1</sup> Alejandra Davila,<sup>1</sup> Denise Carbonaro,<sup>1</sup> Sally Shupien,<sup>3</sup> Christopher Silvin,<sup>2</sup> Sabine Geiger,<sup>1</sup> Barbara Nowicki,<sup>4</sup> E. Monika Smogorzewska,<sup>5</sup> Berkley Brown,<sup>3</sup> Xiaoyan Wang,<sup>6</sup> Satiro de Oliveira,<sup>1,3</sup> Yeong Choi,<sup>1</sup> Alan Ikeda,<sup>7</sup> Dayna Terrazas,<sup>3</sup> Pei-Yu Fu,<sup>1</sup> Allen Yu,<sup>1</sup> Beatriz Campo Fernandez,<sup>1</sup> Aaron R. Cooper,<sup>1</sup> Barbara Engel,<sup>5</sup> Greg Podsakoff,<sup>5</sup> Arumugam Balamurugan,<sup>8</sup> Stacie Anderson,<sup>2</sup> Linda Muul,<sup>2</sup> G. Jayashree Jagadeesh,<sup>2</sup> Neena Kapoor,<sup>5</sup> John Tse,<sup>9</sup> Theodore B. Moore,<sup>3</sup> Ken Purdy,<sup>10</sup> Radha Rishi,<sup>11</sup> Kathey Mohan,<sup>12</sup> Suzanne Skoda-Smith,<sup>12</sup> David Buchbinder,<sup>13</sup> Roshini S. Abraham,<sup>14</sup> Andrew Scharenberg,<sup>15</sup> Otto O. Yang,<sup>1,8</sup> Kenneth Cornetta,<sup>16</sup> David Gjertson,<sup>6</sup> Michael Hershfield,<sup>17</sup> Rob Sokolic,<sup>2</sup> Fabio Candotti,<sup>2</sup> and Donald B. Kohn<sup>1,3</sup>

<sup>1</sup>Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine, UCLA, Los Angeles, California, USA. <sup>2</sup>Genetics and Molecular Biology Branch, National Human Genome Research Institute, NIH, Bethesda, Maryland, USA. <sup>3</sup>Department of Pediatrics, David Geffen School of Medicine, UCLA, Los Angeles, California, USA. <sup>4</sup>Bone Marrow/Stem Cell Transplant, Ronald Reagan Medical Center, UCLA, Los Angeles, California, USA. <sup>5</sup>Division of Hematology, Oncology, and Blood and Marrow Transplantation, Children's Hospital Los Angeles, Los Angeles, California, USA. <sup>6</sup>Department of Biostatistics, David Geffen School of Medicine, UCLA, Los Angeles, California, USA. <sup>7</sup>Children's Specialty Center of Nevada, Las Vegas, Nevada, USA. <sup>8</sup>Division of Infectious Diseases, David Geffen School of Medicine, UCLA, Los Angeles, California, USA. <sup>9</sup>Department of Pharmaceutical Services, Ronald Reagan Medical Center, UCLA, Los Angeles, California, USA. <sup>10</sup>Kaiser Permanente, Santa Clara Homestead Medical Center, Santa Clara, California, USA. <sup>11</sup>Arizona Allergy Associates, Chandler, Arizona, USA. <sup>12</sup>Department of Immunology, Seattle Children's Hospital, Seattle, Washington, USA. <sup>13</sup>Department of Pediatrics, Children's Hospital of Orange County, Orange, California, USA. <sup>14</sup>Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA. <sup>15</sup>Department of Pediatrics, University of Washington, Seattle, Washington, USA. <sup>16</sup>Department of Medical and Molecular Genetics, Indiana University, Indianapolis, Indiana, USA. <sup>17</sup>Departments of Medicine and Biochemistry, Duke University, Durham, North Carolina, USA.

**BACKGROUND.** Autologous hematopoietic stem cell transplantation (HSCT) of gene-modified cells is an alternative to enzyme replacement therapy (ERT) and allogeneic HSCT that has shown clinical benefit for adenosine deaminase-deficient (ADA-deficient) SCID when combined with reduced intensity conditioning (RIC) and ERT cessation. Clinical safety and therapeutic efficacy were evaluated in a phase II study.

**METHODS.** Ten subjects with confirmed ADA-deficient SCID and no available matched sibling or family donor were enrolled between 2009 and 2012 and received transplantation with autologous hematopoietic CD34<sup>+</sup> cells that were modified with the human ADA cDNA (MND-ADA)  $\gamma$ -retroviral vector after conditioning with busulfan (90 mg/m<sup>2</sup>) and ERT cessation. Subjects were followed from 33 to 84 months at the time of data analysis. Safety of the procedure was assessed by recording the number of adverse events. Efficacy was assessed by measuring engraftment of gene-modified hematopoietic stem/progenitor cells, ADA gene expression, and immune reconstitution.

**RESULTS.** With the exception of the oldest subject (15 years old at enrollment), all subjects remained off ERT with normalized peripheral blood mononuclear cell (PBMC) ADA activity, improved lymphocyte numbers, and normal proliferative responses to mitogens. Three of nine subjects were able to discontinue intravenous immunoglobulin replacement therapy. The MND-ADA vector was persistently detected in PBMCs (vector copy number [VCN] = 0.1–2.6) and granulocytes (VCN = 0.01–0.3) through the most recent visits at the time of this writing. No patient has developed a leukoproliferative disorder or other vector-related clinical complication since transplant.

**CONCLUSION.** These results demonstrate clinical therapeutic efficacy from gene therapy for ADA-deficient SCID, with an excellent clinical safety profile.

**TRIAL REGISTRATION.** ClinicalTrials.gov NCT00794508.

**FUNDING.** Food and Drug Administration Office of Orphan Product Development award, R01 FD003005; NHLBI awards, P01 HL73104 and Z01 HG000122; UCLA Clinical and Translational Science Institute awards, UL1RR033176 and UL1TR000124.

**Note regarding evaluation of this manuscript:** Manuscripts authored by scientists associated with Duke University, The University of North Carolina at Chapel Hill, Duke-NUS, and the Sanford-Burnham Medical Research Institute are handled not by members of the editorial board but rather by the science editors, who consult with selected external editors and reviewers.

**Conflict of interest:** K. Cornetta reports personal fees from Cook Regentec that are unrelated to this work and stock ownership in Amgen. M. Hershfield received grants

from Sigma-Tau Pharmaceuticals Inc. (provider of PEG-ADA) while the study was being conducted. D.B. Kohn is a consultant and scientific advisory board member for Orchard Therapeutics, which is developing a different gene therapy approach (lentiviral vector) for ADA-deficient SCID.

**Submitted:** September 8, 2016; **Accepted:** January 24, 2017.

**Reference information:** *J Clin Invest*. <https://doi.org/10.1172/JCI90367>.

## Introduction

Adenosine deaminase (ADA) deficiency is a monogenic disorder with an estimated incidence of 1:200,000 to 1:1,000,000 live births (1) that results in severe immunological deficits as well as variable extraimmune manifestations (2). The ADA enzyme acts on both adenosine and deoxyadenosine, producing inosine and deoxyinosine (3, 4). In the absence of ADA, deoxyadenosine can be phosphorylated, particularly by lymphoid cells, which have high levels of the enzyme deoxycytidine kinase. The resulting deoxyadenosine triphosphate (dATP) accumulation has been shown to inhibit DNA replication and repair and induce apoptosis in immature thymocytes. Thus, infants born with ADA deficiency typically present at an early age with SCID, which is almost always fatal unless treatment is started.

Allogeneic hematopoietic stem cell transplantation (HSCT) with a matched family donor is the preferred treatment option; however, in the absence of an ideal donor, transplant with mismatched or unrelated donors has significantly adverse survival outcomes (5). Enzyme replacement therapy (ERT) with polyethylene-conjugated ADA, although life sustaining, results in variable immune recovery over time (6, 7) and can be cost prohibitive or not widely available. Gene therapy (GT) for ADA-SCID has been under investigation for more than 30 years as an alternative treatment to HSCT and ERT. The first human GT trial involved transferring a normal ADA cDNA *ex vivo* to peripheral blood T cells using a modified  $\gamma$ -retrovirus ( $\gamma$ -RV) (8–10). Although the presence of cells containing the inserted ADA gene could be documented in the 2 subjects who received this procedure, they remained on ERT, which made it difficult to attribute any clinical improvements to the gene transfer. Subsequent clinical trials began using, as the cell source, hematopoietic stem/progenitor cells (HSCs), which have longer life spans than T cells and the potential of offering a long-lasting cure by producing a broad repertoire of T cells. The first effective gene transfer trial using *ex vivo*-cultured HSCs involved giving the subjects reduced intensity conditioning (RIC) with busulfan to aid engraftment of gene-corrected HSCs and withholding ERT to give the corrected cells a selective survival advantage (11, 12). We and others have adapted this approach in clinical trials to treat children diagnosed with ADA-deficient SCID (13–15). Previously, we demonstrated that RIC pretransplant was essential in these subjects for obtaining levels of engraftment that were efficacious (15).

Here, we report on 10 children who were treated in an open-label, nonrandomized phase II trial, beginning in 2009, using the  $\gamma$ -RV MND-ADA combined with RIC pretransplant and withholding of ERT. We show a high frequency of immune reconstitution, with 9 of 10 subjects having sustained immune reconstitution in the absence of ERT or other interventions.

## Results

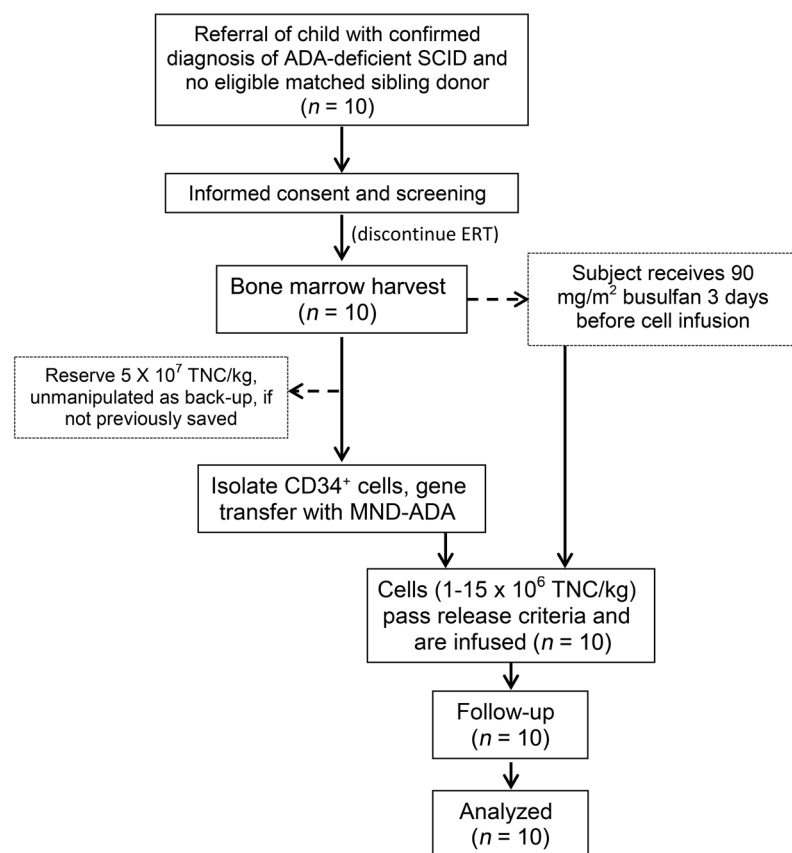
**Subjects.** Ten subjects between the ages of 3 months and 15 years (median, 11.5 months) were enrolled between 2009 and 2012 (Figure 1 and Table 1). Four subjects were diagnosed between 1 and 15 months of age (median, 9 months) when they were hospitalized for failure to thrive or infections; 5 subjects were diagnosed in utero or at birth because a sibling or other family member had ADA deficiency; 1 subject was diagnosed by positive newborn screen-

ing for SCID, which had been recently implemented in the US in California. Conditions for enrollment included confirmed absence of ADA enzyme activity in peripheral blood or fetal cells and evidence of immune deficiency (absolute lymphocyte count [ALC]  $< 200$  cells/mm<sup>3</sup> or T cell lymphoblastic response to phytohemagglutinin [PHA] with  $\Delta$  c.p.m.  $< 5000$ ) prior to initiation of ERT. Genetic mutations in the ADA gene were documented in 9 of 10 subjects (and 1 was not tested). Subjects had been treated with ERT from diagnosis until enrollment in the study (birth to more than 14 years), which included discontinuation of ERT within 1 week of the bone marrow harvest for the gene transfer. All subjects remain alive, at this writing, with normal growth for their age (except for subject 401, who had already neared maximal height at 15 years old, Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/JCI90367DS1>) and are 42 to 84 months (median 57 months) after infusion.

**Busulfan myelosuppression.** RIC was achieved using a standard busulfan dose on day -3 of transplant of 90 mg/m<sup>2</sup> of subject's body surface area, which corresponded to 2.65 to 5.23 mg busulfan/kg body weight (Table 2). Exposure to busulfan was determined by calculating AUC, and levels ranged widely, from 2427 to 6714  $\mu$ mol/l per min (median 5344  $\mu$ mol/l per min, Table 2). The thrombocytopenia followed a consistent pattern among subjects (Supplemental Figure 2A) with nadirs between days 19 and 27 after busulfan and subsequent recovery without need for platelet transfusions. Neutropenia nadir occurred between 15 days and 32 days after busulfan dosing (Supplemental Figure 2B) with increasing absolute neutrophil count (ANC) thereafter. Subject 407 experienced prolonged neutropenia (ANC, 250–350 cells/mm<sup>3</sup>) beyond 42 days after transplant and received 1 course of granulocyte CSF (G-CSF) for treatment at 10-month follow-up. Transient transaminitis was observed in 3 subjects (subjects 405, 406, 407) beginning on day 22 after busulfan dosing (and approximately 1 month after ERT withdrawal) and spontaneously resolving by day 60 after transplant (Supplemental Figure 2, C and D).

**CD34<sup>+</sup> cell dosages and transduction efficiency.** Subjects received between 0.6 and 8.4 million (median, 6.8 million) CD34<sup>+</sup> cells per kg of body weight at the time of infusion (day 0, Table 2), which was dependent upon the initial cell collection from the bone marrow harvest and cell recovery after Ficoll-Hypaque mononuclear enrichment, CD34<sup>+</sup> purification, and culture and transduction processes. Vector copy number (VCN) in the transduced product ranged from 0.18 to 2.68 copies per cell and ADA enzyme activity from 40 to 614 units (nmol/min/10<sup>8</sup> cells). Typically, the younger subjects received larger cell doses (Supplemental Figure 3, as a result of higher starting cell numbers from the bone marrow harvest, data not shown), with the exception of subject 402, where there was poor recovery of the mononuclear cells during the Ficoll gradient centrifugation step. Additionally, there was a manufacturing problem with the cell product for subject 407, which resulted in 2 products being given 5 days apart without additional conditioning. There were no toxicities associated with the cell infusion in any of the 10 subjects.

**Safety.** No subjects experienced serious adverse events (SAEs) attributable to the gene-transfer vector or cell product. Replication-competent retrovirus (RCR) was not detected in any subject. There were no leukoproliferative events, although several subjects do have stable persistent clones with vector integrations adjacent



**Figure 1. Diagram of participant flow and number of subjects entered, followed, and analyzed.** TNC, total nucleated cells.

to *MECOM* or *LMO2* loci (Aaron R. Cooper, unpublished observations), which are common integration loci that have been documented in other  $\gamma$ -retroviral GT trials (16–19). At this writing, all subjects remain alive and well and without restricted activity.

**Adverse events.** Three subjects (subjects 403, 407, and 410) had prolonged neutropenia (grades 2 to 3) lasting 10 to 18 months after engraftment of gene-modified cells (data not shown). This neutropenia may reflect the abnormal myelopoiesis that may occur with ADA deficiency (20) rather than from the GT procedure. Five subjects developed infectious complications requiring initial or prolonged hospitalization at 2 days to 5 months after transplant that resolved with therapy and did not require restarting ERT (Supplemental Table 1). Subject 410 was hospitalized for 5 days at approximately 20 months after transplant for observation of rash and fever associated with vaccination and antibiotic therapy. No other hospitalizations have been recorded to date.

**ADA enzyme activity and ERT.** ERT was discontinued approximately 7 days before bone marrow harvest. The oldest subject at time of treatment (subject 401) restarted ERT at 6 months after GT because his peripheral blood mononuclear cell (PBMC) ADA enzyme activity did not reach normal levels and his lymphocyte counts did not recover (Figure 2A and Figure 3A). All other subjects have remained off ERT to the time of this writing (3 to 6 years as of May 2016). Subjects 404, 408, and 410, who were all 3 months old at the time of GT, developed the highest levels of PBMC ADA activity, which was sustained throughout follow-up (Figure 2A). Except for subject 401, all subjects have PBMC ADA activity in the normal range of the assay, although the time to normalization varied from 1 to 24 (median, 6) months after transplant. Endogenous ADA activity from the GT was sufficient to deplete toxic adenosine metabolites (measured by the portion of adenine nucleotides that were deoxyadenosine nucleotides [dAXP] in peripheral blood) to below 6% in all subjects, except for subject 401, who shows 0% dAXP from exogenous ERT (Figure 2B).

**Cell counts.** The rate and level of ALC recovery corresponded to ADA activity (Figure 3A). Subjects 408 and 410 had the most rapid rise and recovery of their ALC compared with the remaining subjects. Subjects 401 and 403 (the oldest at time of GT at 15 and 8 years, respectively) had the lowest ALC recovery. Although all subjects who remained off ERT showed increases of their ALC after GT, only subjects 408 and 410 reached low normal (10th percentile) counts in absolute CD3<sup>+</sup> and CD4<sup>+</sup> T cells, B cells (CD19<sup>+</sup>), NK cells (CD56<sup>+</sup>16<sup>+</sup>), and naive T cells (CD4<sup>+</sup>/CD45RA<sup>+</sup>) for their age (Figure 3, B–G, and Supplemental Figure 4) at 12 months of follow-up. Of interest, subject 401, who resumed ERT, did have low normal levels of B and NK cells, although he continued to

**Table 1. Subject characteristics**

Subject	Age at diagnosis (months)	Months on Adagen prior to GT	Age at transplant (months)	Gene mutation	Follow-up time <sup>a</sup> (months)
401	11	173	184	R211H / R211H	84
402	1	3	4	T+2>G in IVS7/unknown-identified	80
403	7	90	97	Not performed	72
404 <sup>b</sup>	0	3	3	T+2>G in IVS7/unknown-identified	60
405 <sup>b</sup>	0	8	8	R211H / R211H	60
406 <sup>b</sup>	0	17	17	G216R / G216R	54
407 <sup>b</sup>	0	14	14	c.955-959delGAAGA/ c.996-997delTA	54
408 <sup>b</sup>	0	3	3	R211H / R211H	54
409	15	5	20	L107P / c.975+5delG	42
410 <sup>c</sup>	0	2	3	L107P / c.790delT	42

<sup>a</sup>As of June 2016. <sup>b</sup>Had affected sibling. <sup>c</sup>By newborn screen.

**Table 2. Infusion of the transduced cell product**

Subject	mg/kg Bu (= 90 mg/m <sup>2</sup> )	Bu AUC $\mu$ mol/l per min	CD34 <sup>+</sup> cell dose ( $\times 10^6$ /kg)	Transduction efficiency (VCN)	ADA activity (nmol/min/10 <sup>8</sup> cells)
401	3	Not done	0.6	0.6	40.3
402	4.9	5437	1.7	0.71	54.4
403	2.65	3871	0.92	0.185	44
404	4.2	3532	7.1	1.96	191.7
405	4.4	5469	7.62	2	124.5
406	3.8	2427	8	2.6	614.4
407 <sup>A</sup>	3.93	3232	4, 1.6	1, 2.27	62.2, 296.6
408	4.14	5344	6.85	2.68	427.54
409	4.18	5608	2.86	1.22	307.8
410	5.23	6714	8.41	2.38	567

<sup>A</sup>Due to a manufacturing error, subject 407 received 2 separate cell products, 5 days apart, without additional RIC. Bu, busulfan.

have very low T cell counts. No subject in the CD8<sup>+</sup> T cell subset had normal counts (Figure 3D and Supplemental Figure 4C).

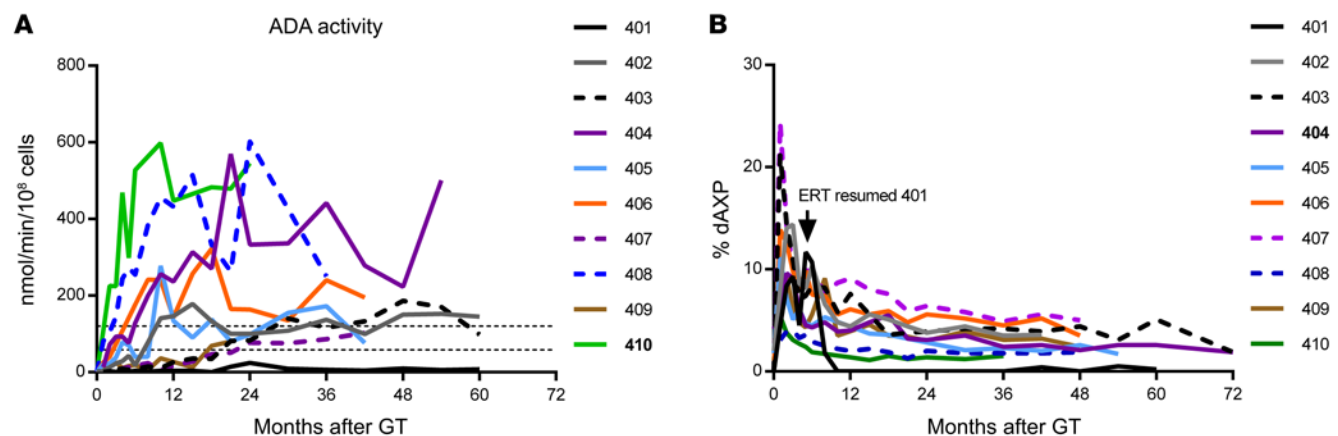
**T cell function.** T cell activation was assessed by measuring the subjects' lymphocytes' ability to respond to the lectin PHA. All but 2 subjects (subjects 401 and 407) had normal responses to PHA (Figure 3H). Despite having low numbers of T cells, subjects 403 and 406 had positive responses, suggesting that even a small number of corrected T cells was sufficient to restore this broad cellular response. Subject 401, despite being on ERT, failed to have a response; this result is consistent with a previous observation that long-term ERT does not fully restore immune function (6).

**TCR repertoire.** Quantitative spectratyping to identify T cell receptor (TCR)  $\beta$ -variable (TRBV) CDR3 region rearrangement distribution at the molecular level was performed on subjects 401, 402, 404, and 405 at distinct time points after GT (Supplemental Figure 5, A-D). Regular Gaussian distribution of TRBV peak length across all families was observed in subjects 402, 404, and 405, indicating that those subjects had normal TCR diversity by 12 to 24 months after GT. Subject 401 had abnormal-appearing TRBV peak distribution seen in a small number of families (BV04, BV10, BV30), despite having been restarted on ERT for

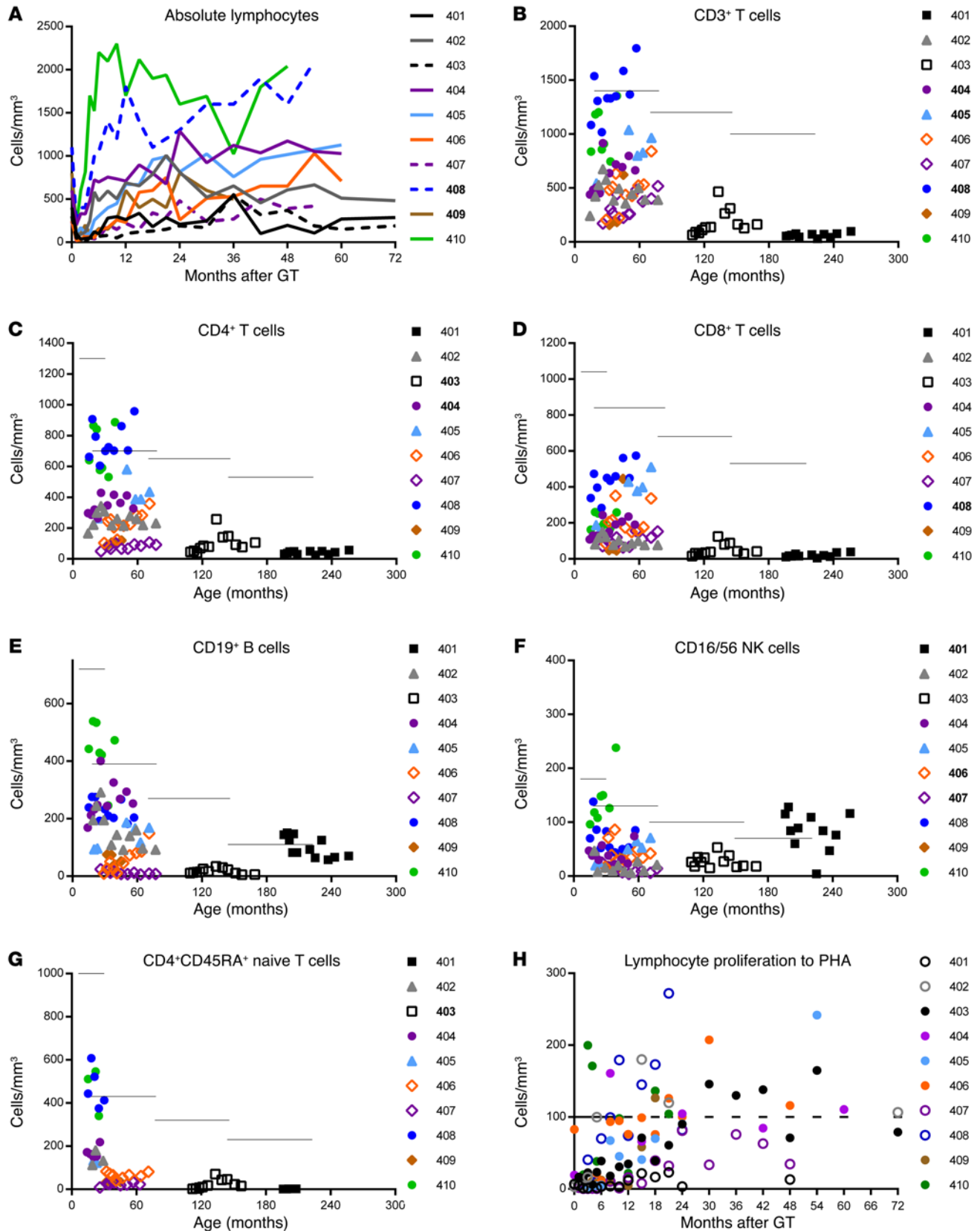
approximately 18 months prior to analysis. The distribution of TRBV families in CD4<sup>+</sup> and CD8<sup>+</sup> T cells was measured in subjects 403, 406, and 407 by flow cytometry at several time points throughout their follow-up (Supplemental Figure 6). Subjects 407 and 408 also had TCR excision circle (TREC) measured, and only subject 408 had normal TREC levels (10,145 TREC copies/million CD3<sup>+</sup> T cells; normal  $\geq 6794$ ) for her age. In subject 407, TREC levels were undetectable.

**B cell function.** The subjects' ability to make IgM and IgA after GT was assessed by measuring serum immunoglobulin levels. At 12 months after GT, all but 3 subjects (subjects 401, 403, and 407) had normal levels of IgM (Figure 4A), whereas only 3 subjects (subjects 404, 408, and 410) had normal levels of IgA (Figure 4B). This outcome corresponded to the subjects' B cell counts (subjects 404, 408, and 410 had B cells  $> 200$  cells/mm<sup>3</sup>), suggesting that correction of humoral function required robust B cell reconstitution.

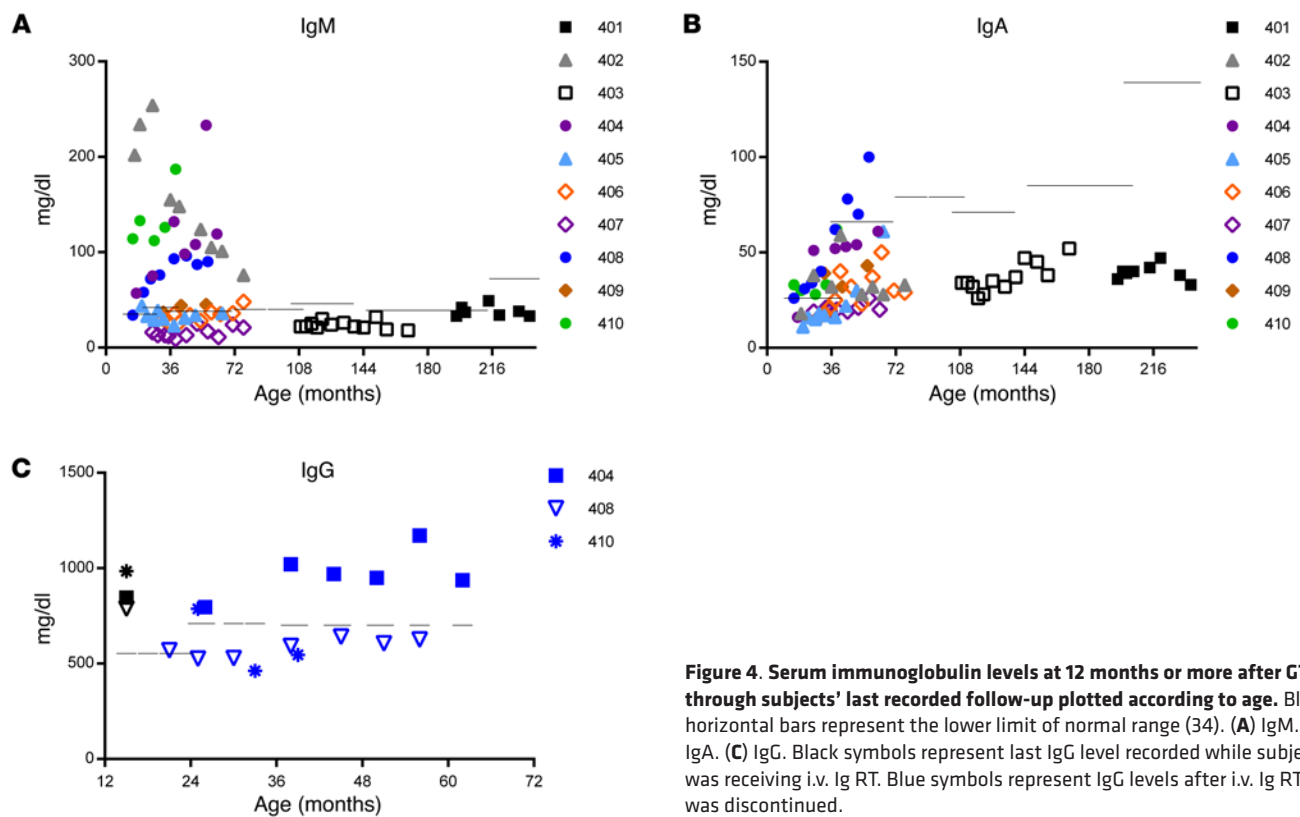
Subjects 402 and 404 had immune responses to the neoantigen bacteriophage  $\phi\chi 174$  tested (21) and B cell studies performed (Table 3 and Supplemental Figures 7 and 8). Subject 402 (who remains on i.v. Ig replacement therapy [RT]) received a lower total



**Figure 2. ADA enzymatic activity in PBMCs and percentages of deoxyadenine nucleotides in erythrocytes.** (A) ADA enzyme activity in PBMCs was measured biochemically. The low and high normal reference range for the ADA enzyme assay in human PBMCs is indicated by the 2 parallel broken lines. (B) Adenine and deoxyadenine metabolites were measured in erythrocytes by high-pressure liquid chromatography and the percentage that were dAXP (dAMP + dADP + dATP) were plotted as %dAXP. The time when ERT was resumed for subject 401 is indicated.



**Figure 3. Cell counts and lymphocyte proliferation responses after GT.** (A) ALCs plotted from time of GT through subjects' last recorded follow-up. (B–G) Cell counts at  $\geq 12$  months after GT through subjects' last recorded follow-up plotted according to age. Black horizontal bars represent the 10th percentile of normal (33). (B) CD3<sup>+</sup> pan T cells. (C) CD4<sup>+</sup> T cells. (D) CD8<sup>+</sup> T cells. (E) CD19<sup>+</sup> B cells. (F) CD16/56<sup>+</sup> NK cells. (G) CD4/CD45RA<sup>+</sup> naive T cells. (H) Lymphocyte proliferation to PHA expressed as the percentage of lower limit of normal (LLN) for the laboratory that conducted the assay. Black dotted horizontal line is drawn at 100% of normal.



**Figure 4. Serum immunoglobulin levels at 12 months or more after GT through subjects' last recorded follow-up plotted according to age.** Black horizontal bars represent the lower limit of normal range (34). (A) IgM. (B) IgA. (C) IgG. Black symbols represent last IgG level recorded while subject was receiving i.v. Ig RT. Blue symbols represent IgG levels after i.v. Ig RT was discontinued.

CD34<sup>+</sup> cell dose than subject 404 and responded to primary challenge with bacteriophage  $\phi\chi 174$ , but did not respond to secondary or tertiary challenge (Supplemental Figure 7A), whereas subject 404 responded to both primary and secondary challenges (Supplemental Figure 7B). When B cell subsets were measured, subject 402 exhibited a relative deficiency in memory B cells that had class switched to IgA and IgG subtypes compared with subject 404 (8.3% vs. 17.1% IgA and 8.3% vs. 43.7% IgG), despite having normal ratios of memory, mature, and immature B cells (Supplemental Figure 8).

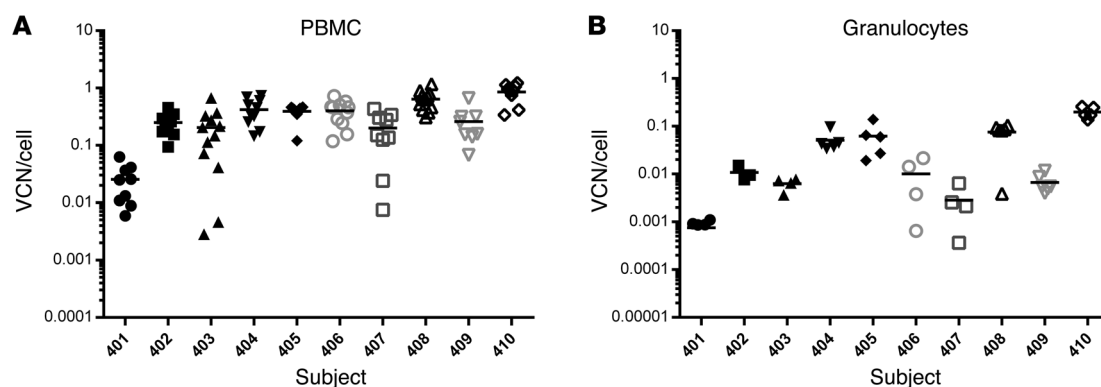
**Discontinuation of supportive medications.** Subjects 402, 404, 405, 408, and 410 were able to discontinue all bacterial and fungal prophylactic medications, and within this group, subjects 404, 408, and 410 were able to also discontinue i.v. Ig RT (Table

3). Additionally, after discontinuing i.v. Ig RT, subjects 404, 408, and 410 were able to maintain total IgG levels at or near the normal range for their ages (Figure 4C). Ability to stop prophylactic medications correlated significantly with higher ADA enzyme activity ( $P = 0.001$ ) and younger age at treatment ( $P = 0.003$ ). However, ability to stop prophylactic medications did not significantly correlate with busulfan AUC during RIC ( $P = 0.110$ ) or with CD34<sup>+</sup> cell dose ( $P = 0.177$ ). Ability to discontinue i.v. Ig RT also correlated significantly with higher ADA enzyme activity ( $P < 0.0001$ ) and younger age at transplant ( $P = 0.008$ ) and did not correlate significantly with busulfan AUC ( $P = 0.425$ ) nor with CD34<sup>+</sup> cell dose ( $P = 0.098$ ). As a result of their good immune reconstitution, subjects 404 and 410 received and responded to

**Table 3. Immunological outcomes**

Subject	ERT	Prophylactic antibiotics	i.v. Ig RT	Immunizations	Special immunological assessments
401	+	+	+	None	
402	-	-	+	None	Bacteriophage $\phi\chi 174^A$ , B cell analysis <sup>B</sup>
403	-	+	+	None	TRBV immunophenotype <sup>C</sup>
404	-	-	-	DTaP, polio, Hib at 22m after GT	Tetanus antibody titer <sup>D</sup> , Bacteriophage $\phi\chi 174^A$ , B cell analysis <sup>B</sup> , TRBV spectratype <sup>E</sup>
405	-	-	+	None	TRBV spectratype <sup>E</sup>
406	-	+	+	None	TRBV immunophenotype <sup>C</sup>
407	-	+	+	None	TREC, TRBV immunophenotype <sup>C</sup>
408	-	-	-	Scheduled	TREC
409	-	+	+	None	
410	-	-	-	DTaP at 19m after GT	Tetanus antibody titer <sup>D</sup>

<sup>A</sup>See Supplemental Figure 7. <sup>B</sup>See Supplemental Figure 8. <sup>C</sup>See Supplemental Figure 6. <sup>D</sup>See Supplemental Figure 9. <sup>E</sup>See Supplemental Figure 5. Hib, Haemophilus influenzae type b; m, months.



**Figure 5. VCN in peripheral blood cells at 6 months or more after GT through subjects' last recorded follow-up. (A) PBMCs. (B) Granulocytes. Mean is shown as horizontal bars.**

immunizations with inactivated vaccines (diphtheria, tetanus, and pertussis [DTaP]; Table 3 and Supplemental Figure 9).

**Quantification of VCN in peripheral blood.** To assess the engraftment and persistence of ADA gene-modified CD34<sup>+</sup> cells, peripheral blood samples were obtained at serial time points, genomic DNA was extracted, and the levels of gene marking in PBMCs and granulocytes (depleted of contaminating lymphocytes) were determined using Droplet Digital PCR (ddPCR, Bio-Rad) to quantify VCN. As expected, the 2 subjects (subjects 408 and 410) with the highest lymphocyte counts also had among the highest gene marking in PBMCs and granulocyte fractions (Figure 5, A and B). Subject 408 had VCN between 0.4 and 0.9 in PBMCs and up to 0.09 VCN in granulocytes (equivalent to 9% of cells, if at single copy/cell). Subject 410 had VCN between 0.5 and 1.1 in PBMCs and up to 0.26 VCN in granulocytes (26% equivalent). Gene marking in granulocytes was significantly higher in subjects who were able to stop prophylactic medications ( $P = 0.024$ ) and i.v. Ig RT ( $P = 0.007$ ) compared with subjects who were not. The remaining subjects had gene marking that was 2-fold or more lower in the granulocyte fractions compared with those seen in subjects 408 and 410.

**IL-7 and hBAFF levels.** Lymphocyte homeostasis and survival are regulated, in part, by cytokine levels. For example, IL-7 is involved in T cell lymphopoiesis and homeostasis (22) and B cell-activating factor of the TNF family (BAFF) promotes B cell maturation and survival (23). We previously reported that serum IL-7 levels were inversely proportional to ALCs in subjects of a GT trial (15). Serum IL-7 levels were particularly increased in the first 4 months after GT after PEG-ADA had been withdrawn and prior to reconstitution of peripheral T lymphocytes. In the current trial, we again measured serum IL-7 levels in subjects and found a similar inverse relationship between IL-7 levels and T cell counts (Supplemental Figure 10A).

BAFF levels have been reported to be inversely correlated with peripheral B cell numbers (23). In the current study, we measured serum BAFF levels from subjects and found that BAFF inversely correlated with absolute B cell counts (Supplemental Figure 10B). T cells began to stabilize at approximately 5 months after GT (with concomitant lowering of IL-7 levels), whereas B cells began to stabilize later, at approximately 8 months, after GT (with concomitant lowering of BAFF levels). These results show that ADA-deficient subjects have the ability to respond to lymphopenia by regulating their cytokine levels.

## Discussion

After more than a decade of ineffective results from early attempts at GT for ADA-deficient SCID, subsequent advances in gene-delivery vectors, stem cell manipulation techniques, and pretransplant conditioning approaches have led to effective therapeutic outcomes (11–15, 24). While some degree of protective immune reconstitution has been achieved in patients, the levels of immune reconstitution have been subnormal in some of the treated patients. Our prior phase I/II trial of GT for ADA-deficient SCID compared 2 different  $\gamma$ -retroviral vectors and examined the benefits of applying RIC, which was shown to be necessary to obtain significant engraftment of gene-modified stem cells and production of lymphocytes expressing ADA (15). The phase II trial presented here builds upon those observations and used the  $\gamma$ -retroviral vector that performed best in the phase I/II trial (MND-ADA) and a slightly increased dosage of busulfan for RIC. The findings extend the prior observations and confirm the clinical efficacy and safety of this approach.

Of the 10 enrolled subjects, 9 showed sustained benefits from the autologous transplant and had sufficient immune reconstitution to remain in good health, free of infections, with normal growth and development, and without need to resume ERT or have an allogeneic HSCT. The one exception was a subject who was 15 years old at the time of treatment who resumed ADA enzyme therapy by 6 months after the procedure due to absence of evidence of immune recovery after GT. This subject received a low cell dose compared with the infants in the trial, and as an older individual, would have decreased thymopoiesis that limited the output of new T cells, even in the presence of gene-corrected cells. For older individuals, efficacy may be achieved by using peripheral blood mobilized stem cells (PBSCs) as the cell source, which would provide a larger cell dose; lentiviruses for more efficient gene transfer and expression; and higher dosages of conditioning chemotherapy to increase engraftment.

The absence of graft-versus-host disease (GVHD) or other immunologic complications of allogeneic HSCT and absence of clinical toxicities from the RIC led to uncomplicated post-transplant courses. This absence of GVHD and the associated morbidity and mortality commonly reported following allogeneic HSCT may provide a clinical advantage to autologous transplantation/GT.

While GVHD is not an issue in autologous GT, a unique adverse event associated with the use of retroviral vectors in this setting is the potential for insertional mutagenesis, where the integrated vector aberrantly activates a host gene in proximity to a vector integration site. Genotoxicity has been seen in other clinical trials for immune deficiencies (25–30) and was caused by vector integrations near cancer-related genes, such as *LMO2*, *CCND2*, and *MECOM*. Although we identified *LMO2* and *MECOM* as common integration sites in this trial, these clones have remained stable throughout the duration of follow-up (Aaron R. Cooper, unpublished observations); no subject has developed any oncogenic event to date. Our experience is similar to that seen in the  $\gamma$ -retroviral trial for ADA-SCID conducted by Aiuti et al. (16), who also observed vector integrations near cancer-associated genes without clonal selection or expansion. Why genotoxicity has been seen in trials for X-linked SCID, X-linked chronic granulomatous disease, and Wiskott-Aldrich syndrome, but not in ADA-SCID, remains unclear. Effects of the specific genetic background of the disease and/or the function of the transgene may be determining factors.

In our study, one-third of the subjects achieved normal levels of different lymphocyte subtypes. In some subjects, the numbers of T cells remained low (e.g., 300–600 cells/mm<sup>3</sup>), whereas other subjects had T cell numbers that were closer to the age-adjusted normal ranges. We also report that 3 of the 9 (33%) subjects who remained off PEG-ADA ERT were also able to discontinue i.v. Ig RT. A similar study conducted by Aiuti et al. (12) reported somewhat higher percentages of subjects reaching normal levels of lymphocyte subtypes (33%–55%) and able to discontinue i.v. Ig RT (5/9, 55%) over the same length of follow-up. With relatively small numbers of subjects in each trial, it is not possible to know whether these outcomes are significantly different. There were differences in the  $\gamma$ -retroviral vectors that express the ADA cDNA from different promoters (GIADA1 vector, which is based on the LXSXN retrovirus containing the Moloney murine leukemia virus [MMLV] long terminal repeat [LTR] in the Aiuti study vs. MND-ADA, which contains a modified myeloproliferative sarcoma virus LTR, in our study). Additionally, on average, larger cell doses were transplanted in the Aiuti study (mean,  $8.2 \times 10^6$  CD34<sup>+</sup>/kg) compared with this present study (mean,  $5 \times 10^6$  CD34<sup>+</sup>/kg); a larger transplanted cell dose could result in better outcomes.

Despite having subnormal levels of lymphocytes, none of the subjects in our study, even those with the lower T cell numbers, have had clinical infections, and thus, the subnormal levels appeared to be sufficient to provide protective immunity, at least during the time observed. Three subjects (subjects 404, 408, and 410), with the most robust levels of immune reconstitution in terms of numbers of T, B, and NK cells, were able to stop receiving i.v. Ig RT. Normal antibody responses to inactivated vaccines have been documented in 2 of these (the third subject has not been tested), and normal TREC levels were documented in 1 subject (and not tested in the other 2). Although thymopoiesis was not documented in 2 subjects of this group, their normal levels of CD4<sup>+</sup>CD45RA<sup>+</sup> naive T cells and good immune reconstitution suggest that thymopoiesis was restored. Whether thymopoiesis was restored in the remaining subjects is unclear. The 3 subjects who performed the best were all diagnosed shortly after birth (2 as second affected siblings and 1 by newborn screening), began ADA

enzyme replacement in the first month of life, and had their GT treatment by 3 months of age. They all received high CD34<sup>+</sup> cell dosages with good gene transfer, based on VCN in the transplant inoculum, and achieved good blood levels of busulfan exposure (AUC); each of these aspects may be an important element in the overall level of engraftment of gene-corrected stem cells and the resulting immune reconstitution.

When evaluating a broader outcome, such as the ability to stop prophylactic antibiotics or i.v. Ig RT, we found that ages at GT ( $P = 0.003$  for stopping antibiotics and  $P = 0.008$  for stopping i.v. Ig RT) and VCN in granulocytes ( $P = 0.024$  for stopping antibiotics and  $P = 0.007$  for stopping i.v. Ig RT) were significant factors, whereas cell dose and AUC were not. These results, based on 10 subjects, suggest that age and transduction efficiency of HSC play important roles, but do not preclude the contributions of cell dose and busulfan exposure to overall success. Interestingly, in a recent report from Cicalese et al. (24) looking at long-term outcomes in  $\gamma$ -RV GT for ADA-deficient SCID, the authors found that intervention-free survival (defined as survival without receiving a post-GT HSCT or continuous ERT for 3 months or more) could not be predicted by age at GT or cell dose. Based on this definition, 9 of 10 subjects in our study have met this end point of intervention-free survival. However, since there has only been 1 event (of restarting ERT), there are not enough data to run any statistical analyses (and thus obtain any statistically meaningful results) on whether intervention-free survival can be predicted by age at GT or cell dose received.

Because of many variables, more consistent efficacy may be attained by (a) using better vectors for gene transfer to human HSCs (e.g., lentiviral vectors), (b) achieving consistent busulfan exposure levels (by using pharmacokinetic adjustment of dosages), (c) diagnosing the condition earlier by neonatal screening, and (d) using G-CSF-mobilized peripheral blood stem cells, instead of bone marrow, as the source of HSCs for older subjects, which may provide more CD34<sup>+</sup> cells. Nonetheless, the overall success of this approach demonstrates the capacity of autologous HSCT/GT to treat this otherwise fatal congenital immune disorder.

## Methods

**Clinical trial protocol.** In 2009, we began a phase II clinical trial of  $\gamma$ -RV-mediated gene transfer to CD34<sup>+</sup> cells isolated from bone marrow of ADA-deficient SCID infants and children ( $n = 10$ ), who were referred based on their diagnosis of ADA-deficient SCID (Figure 1). The major eligibility criteria included age of 1 month or older; no medically eligible matched sibling donor available; adequate organ function (determined by physical exam, complete blood count with differential, blood chemistry panel, coagulation studies, chest x-ray, and electrocardiogram); no infection with human immunodeficiency virus, hepatitis B virus, cytomegalovirus, or parvovirus B19; and normal cytogenetics. Subjects discontinued ERT within 2 weeks prior to bone marrow harvest. Isolated CD34<sup>+</sup> cells in each subject were transduced ex vivo with an MMLV-based  $\gamma$ -RV vector carrying the human ADA cDNA (MND-ADA) (15). After 5 days of culture, the cells (no more than  $15 \times 10^6$  total nucleated cells per kg body weight) were i.v. infused after the subjects had received 90 mg/m<sup>2</sup> busulfan 2 to 3 days earlier. The clinical trial objectives included assessments of safety (toxicities from the procedure and exposure to RCR) as well as



assessments of efficacy in terms of transduction/engraftment of HSC (by ddPCR, Bio-Rad), ADA gene expression (by enzymatic activity), and assessment of immune function. The protocol involved 2 years of active follow-up, followed by offer of enrollment into a separate study to monitor long-term outcome after gene transfer to meet the FDA-mandated 15 years of follow-up. The 2 clinical trial performance sites were the Mattel Children's Hospital, UCLA Pediatric Hematopoietic Stem Cell Transplant Unit and the Genetics and Molecular Biology Branch of the National Human Genome Research Institute, Clinical Center NIH.

**Vector.** The MMLV-based  $\gamma$ -RV vector MND-ADA uses the "MND" LTR to drive human cDNA ADA expression and has been described elsewhere (15, 31). This vector was chosen for the phase II trial based on its greater persistence in peripheral blood cells compared with a second  $\gamma$ -RV vector used previously in the phase I trial (15). The MND-ADA vector was packaged using the PG13 packaging cell line, which confers the gibbon ape leukemia virus (GALV) pseudotype. Clinical lots of vector supernatant were produced at the Indiana University Vector Production Facility (IUVPF) in X-VIVO 10 medium (Lonza) with 1% HSA and qualified for clinical trial use. Aliquots of the unprocessed supernatant (having approximate titer of  $5.0 \times 10^5$  transducing units [TU]/ml on HT29 cells) were cryopreserved at  $-80^\circ\text{C}$ . Vector potency was determined annually by measuring titer and conferred ADA enzyme activity in transduced HT29 cells.

**CD34<sup>+</sup> ex vivo transduction and characterization.** Bone marrow (up to 20 ml/kg) was harvested in a hospital operating room from subjects under general anesthesia from the posterior iliac crest and collected in Plasma-Lyte (Baxter) and heparin (10 IU/ml final concentration). A fraction of the marrow containing  $5 \times 10^7$  total nucleated cells/kg of subject body weight was cryopreserved as back-up graft without additional manipulation. If a subject had umbilical cord blood stem cells cryopreserved by a clinical lab at the time of birth, these cells were considered sufficient for the back-up graft, provided they consisted of  $5 \times 10^7$  or more total nucleated cells/kg at the time of the GT procedure. Mononuclear cells were enriched from bone marrow by standard Ficoll-Hypaque gradient centrifugation, and CD34<sup>+</sup> cells were then selected using the Isolex 300i (Baxter; subjects 401, 402, and 403) or CliniMACS CD34 Reagent System (Miltenyi Biotec; subjects 403 to 410). The CD34<sup>+</sup> cell enrichment procedures yielded preparations of cells that were 81.4% to 90.6% CD34<sup>+</sup> from starting bone marrow harvests that contained between 1.2% and 7.7% CD34<sup>+</sup> cells.

For ex vivo transduction, CD34<sup>+</sup> cells were seeded at a density of 0.1 to  $0.8 \times 10^6$ /ml into cell culture bags or flasks (T175) that had been coated with recombinant fibronectin fragment CH-296 (20  $\mu\text{g}/\text{ml}$ , Takara Shuzo Co.) and precultured for 40–48 hours at  $37^\circ\text{C}$  in X-VIVO 15 serum-free medium (Lonza) containing stem cell factor (SCF) (50 ng/ml, Amgen Inc.), Flt3 ligand (300 ng/ml, Immunex Corp.), and megakaryocyte growth and differentiation factor (MGDF) (50 ng/ml, Amgen, Inc.) or thrombopoietin (TPO) (50 ng/ml, R&D Systems) and 1% HSA. Precultured CD34<sup>+</sup> cells were then transduced with the MND-ADA  $\gamma$ -RV vector by removing the cells from the preculture flasks and reculturing them into new cell culture bags or flasks that had been preloaded with MND-ADA vector supernatant. Every 20 to 24 hours for 3 cycles, the nonadherent cells were pelleted by centrifugation, resuspended in an equal mixture of freshly thawed  $\gamma$ -RV supernatant and 2 $\times$  cytokine-containing culture medium, and returned to the original culture bags or flasks containing the adherent cells. At the end

of the 5-day culture period, cells were harvested and washed 3 $\times$  with HBSS containing 1% HSA and suspended in 10 to 25 ml Plasma-Lyte 1% HSA as the final cell product for immediate infusion.

Final cell product release criteria for clinical infusion included the following: viability greater than 70%, negative in-process bacterial and fungal stains and cultures, and endotoxin of less than 5 EU/kg by Limulus Amebocyte Lysate Kit (Associates of Cape Cod Inc.) or by the Endosafe-PTS Kit (Charles River Laboratories). Additional characterization of the final cell product to complete the certificate of analyses (COA) after infusion included mycoplasma test (agar cultivable and nonagar cultivable [BioReliance] or MicroSEQ [Life Technologies] PCR Detection Kit), final sterility, measurement of ADA enzyme activity, and quantification of VCN using quantitative PCR (qPCR). For all 10 enrolled subjects, the final cell products met release criteria and were administered by trained medical personnel via i.v. injection over 5 to 10 minutes at hospital bedside without incident. Except for in subject 407, only 1 cell product was administered. Because of a manufacturing error, subject 407 received 2 cell products (from 1 bone marrow harvest) that were infused 5 hours apart. Both products were characterized separately.

**Clinical monitoring and follow-up.** Subjects were monitored for safety and toxicities during and after busulfan administration and infusion of the transduced cells. Typically (unless otherwise described below), subjects recovered from nadir of busulfan-related neutropenia by 30 days after infusion, when they were discharged home. Follow-up evaluations occurred at the subject's primary care physician's office or the performance site and included a physical exam and phlebotomy for clinical and research blood samples at a frequency of monthly for the first 6 months, bimonthly until 1 year, and quarterly until 2 years. Clinical complications and abnormal laboratory values were graded using the *Division of AIDS Table for Grading Severity of Pediatric Adverse Experiences (>3 months of age)* (National Institute of Allergy and Infectious Diseases, NIH, April 1994). Subjects were tested for exposure to RCR by qPCR assay for GALV sequences in PBMC samples from baseline (pretreatment) and 3, 6, 12, and 24 months after cell reinfusion at the National Gene Vector Biorepository (Indianapolis, Indiana, USA). Tested samples from all 10 subjects had fewer than 10 GALV copy numbers per 0.2  $\mu\text{g}$  DNA (i.e., below the limit of detection).

**RIC with busulfan and pharmacokinetic monitoring.** Subjects received phosphenytoin (15 mg/kg i.v. loading dose the night before busulfan was administered and 5 mg/kg i.v. every 12 hours  $\times$  3 doses) as antiseizure prophylaxis and ondansetron (0.15 mg/kg i.v. or PO  $\times$  2) as antinausea prophylaxis. Busulfan was given as an i.v. infusion of 45 mg/m<sup>2</sup> over 2 hours on 2 successive days for subject 401 or 90 mg/m<sup>2</sup> over 3 hours as a single dose for the remaining 9 subjects on day  $-3$  of transplant. Blood samples were taken immediately at the end of the busulfan infusion and 1, 2, 4, 8, and 13 hours later for measurement of serum busulfan levels by HPLC (Children's Hospital Los Angeles Clinical Special Chemistry Laboratory or the Mayo Clinic). The busulfan levels were used to calculate the AUC for busulfan exposure by trapezoidal estimation using the computer program PKSolver (32).

**ADA enzyme activity.** Cells (2 to  $5 \times 10^5$  PBMCs or CD34<sup>+</sup> cells) were washed with HBSS, pelleted, and frozen at  $-80^\circ\text{C}$  until use. Pellets were thawed at  $37^\circ\text{C}$  and resuspended at  $5 \times 10^6$ /ml in M-Per Mammalian Protein Extraction Reagent (Pierce), vortexed, and left on a cell shaker for 30 to 40 minutes. Lysates were then cleared of cellular

debris by centrifugation, and 10  $\mu$ l aliquots were transferred to 0.5-ml tubes and incubated with 10  $\mu$ l of 1 mM  $^{14}$ C-adenosine (50 mCi/ml) at 37°C for 5 to 40 minutes. The products of the enzymatic reactions were then separated by thin-layer chromatography and the conversion of adenosine into inosine determined by using a phosphoimager (Fuji Medical Systems) and expressed as units of ADA (1 unit = 1 nmol of adenosine deaminated per  $10^8$  cells per min).

**VCN determination and ddPCR.** Heparinized peripheral blood samples were separated into PBMC and granulocyte fractions by centrifugation on Ficoll-Hypaque. The granulocyte fraction was depleted of contaminating lymphocytes by depletion with immunomagnetic beads to CD3, CD19, and CD56 (Stem Cell Technologies or Miltenyi). Genomic DNA was isolated from cells using phenol/chloroform method or the PureLink Genomic DNA Mini Kit (Invitrogen). ddPCR was performed using the Bio-Rad QX100 Droplet Digital PCR System. Template DNA not exceeding 100,000 copies of vector or genomic normalization targets was used to set up a 20  $\mu$ l ddPCR reaction with MND and uc378 primers and probes, with 2 $\times$  ddPCR Supermix for Probes (Bio-Rad), 400 nM final concentration of each primer, 200 nM final concentration of each probe, and 1 unit of DraI enzyme (New England Biolabs).

**Immunological monitoring.** Immunological assays for lymphocyte subset numbers by immunophenotype, lymphocyte proliferative responses to mitogens/antigens, and serum antibody/immunoglobulin levels were performed in laboratories certified by Clinical Laboratory Improvement Amendments (CLIA) at the Ronald Reagan Medical Center; the Department of Laboratory Medicine of the Clinical Center, NIH, Bethesda, Maryland, USA; the Laboratory of Cell-Mediated Immunity of the National Cancer Institute, SAIC-Frederick (currently Leidos Biomedical Research Inc.), Frederick, Maryland, USA; or the clinical laboratories of the subject's primary care physicians. TREC analysis was performed at the Mayo Clinic Laboratories. Adenosine and deoxyadenosine nucleotide measurements were performed in the laboratory of Michael S. Hershfield, Duke University.

For additional information, see Supplemental Methods.

**Statistics.** Descriptive statistics of patient characteristics and transduced cell products are summarized and presented in tables. Unpaired *t* tests were used to evaluate differences in age at treatment, busulfan AUC during RIC, and CD34<sup>+</sup> cell dose difference between subjects who stopped prophylactic medications (*n* = 5) and those who did not (*n* = 5). Similar analyses were performed to compare patients who discontinued i.v. Ig RT (*n* = 3) with those who did not (*n* = 7). To correlate these immunological outcomes with longitudinal measurements such as ADA enzyme activity and vector marking in PBMCs and granulocytes, repeated measure ANOVAs were performed. For all statistical investigations, tests for significance were 2-tailed, with a *P* value of less than the 0.05 significance level considered significant. All statistical analyses were carried out using statistical software SAS version 9.4 (SAS Institute Inc., 2013).

**Study approval.** The protocol (ClinicalTrials.gov NCT00794508) was reviewed and approved by the Medical Institutional Review Board 2 at UCLA; the National Human Genome Research Institute Institutional Review Board at the NIH; the institutional biosafety committees at UCLA and the NIH; the NIH Office of Biotechnology Activities Recombinant DNA Advisory Committee (protocol 9908-337); the UCLA Institutional Scientific Peer Review Committee; and the NHLBI's Cell Therapy/Gene

Therapy Data Safety Monitoring Board. The study was conducted under US FDA BB IND 8556. Written informed consent was obtained from the patients or their parent(s) prior to trial participation.

## Author contributions

KLS managed the study and wrote the manuscript. SM, PB, AD, DC, SG, YC, PYF, and AY performed the cell processing and gene transfer. CS, BCF, ARC, AB, SA, LM, and GJJ designed and performed experiments and analyzed data for end-point analysis. SS provided critical regulatory guidance and support. EG, BB, SDO, AI, DT, TBM, KP, RR, KM, SSS, DB, and RS were responsible for coordination of patient care and clinical follow-up. BE, GP, and FC designed the study and secured funding; FC additionally directed the study at the NIH performance site. BN, EMS, NK, JT, RSA, AS, OOOY, KC, and MH provided critical reagents and tests. XW and DG performed the statistical analysis and interpretation of the data. SM, ARC, SDO, FC, and DB critically reviewed the manuscript. DBK designed the study, secured funding, directed the research, analyzed data, and had overall oversight of the trial and manuscript.

## Acknowledgments

The study was supported by grants from the FDA Office of The study was supported by grants from the FDA Office of Orphan Product Development (RO1 FD003005), the NHLBI (PO1 HL73104 and Z01 HG000122), the Clinical and Translation Science Institute (UL1RRO33176 and UL1TR000124), and the California Institute for Regenerative Medicine (CL1-00505-1.2 and FA1-00613-1). Additional support was provided by the UCLA Eli & Edythe Broad Center for Regenerative Medicine and Stem Cell Research, the UCLA Jonsson Comprehensive Cancer Center, the UCLA David Geffen School of Medicine Human Gene and Cell Therapy Program, the UCLA Department of Pediatrics, and the Children's Institute for Innovation and Discovery. The National Gene Vector Biorepository performed assays for RCR and clonal expansion by linear amplification-mediated PCR. We also thank the patients and their parents for participation in this study. This article reflects the views of the authors and should not be construed to represent the FDA's views or policies.

Address correspondence to: Donald B. Kohn, 3163 Terasaki Life Science Building, 610 Charles E. Young Drive South, Los Angeles, California 90095, USA. Phone: 310.794.1964; E-mail: dkohn1@mednet.ucla.edu.

EG's present address is: Office of the Clinical Director, National Human Genome Research Institute, NIH, Bethesda, Maryland, USA.

SM's present address is: CalImmune Inc., Tucson, Arizona, USA.

DC's present address is: Orchard Therapeutics Ltd., London, United Kingdom.

CS's present address is: National Institute on Deafness and Other Communication Disorders, NIH, Bethesda, Maryland, USA.

SG's present address is: Research and Development, Apceth, Munich, Germany.

YC's present address is: Center for Immunotherapy, Roswell Park Cancer Institute, Buffalo, New York, USA.

RS's present address is: Center for Biologics Evaluation, Federal Drug Administration, Silver Spring, Maryland, USA.

BE and GP's present address is: The Children's Hospital of Philadelphia Research Institute, Philadelphia, Pennsylvania, USA.

FC's present address is: Division of Immunology and Allergy, University Hospital of Lausanne, Lausanne, Switzerland.

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