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Normal IgH repertoire diversity in an infant with ADA deficiency after gene therapy

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Author contributions

JS, LY, and MG contributed to the conception and design of the study. MH and DK contributed to the gene therapy protocol and monitoring of ADA. CB, MH, SP, DK, JS contributed to patient care and obtaining clinical data. CB, SB, KC, JY, MG, JS, LY contributed to the analysis and interpretation of sequencing data. CB wrote the first draft of the manuscript. All authors contributed to manuscript editing, read, and approved the final version.

Disclosure of conflicts of interest

Leadiant Biosciences & Orchard Therapeutics (MH).

DK is an inventor on a lentiviral gene therapy for ADA SCID licensed by the UC Regents to Orchard Therapeutics and serves as a member of the Orchard Scientific Advisory Board.

The other authors (CHB, SB, KC, JY, SP, MG, JS, LY) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Approvals

All study participants were enrolled with approvals by the Institutional Review Boards of the University of South Florida and Duke University.

Consent to Participate

Written informed consents were obtained from all participants or parents including the parents of the ADA-deficient child. Additional consent for publication of case report was obtained from the parents of the ADA-deficient child.

Consent for Publication

All authors provided consent for publication.

Code Availability

Not applicable.

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Abstract

Purpose: Adenosine deaminase (ADA) deficiency causes severe combined immunodeficiency (SCID) through an accumulation of toxic metabolites within lymphocytes. Recently, ADA deficiency has been successfully treated using lentiviral-transduced autologous CD34+ cells carrying the ADA gene. T and B cell function appears to be fully restored but in many patients' B cell numbers remain low and assessments of the immunoglobulin heavy (IgHV) repertoire following gene therapy are lacking.

Methods: We performed deep sequencing of IgHV repertoire in peripheral blood lymphocytes from a child following lentivirus-based gene therapy for ADA deficiency and compared to the IgHV repertoire in healthy infants and adults.

Results: After gene therapy, Ig diversity increased over time as evidenced by V, D, and J gene usage, N-additions, CDR3 length, extent of somatic hypermutation, and Ig class-switching. There was emergence of predominant IgHM, IgHG, and IgHA CDR3 lengths after gene therapy indicating successful oligoclonal expansion in response to antigens. This provides proof of concept for the feasibility and utility of molecular monitoring in following B cell reconstitution following gene therapy for ADA deficiency.

Conclusion: Based on deep sequencing, gene therapy resulted in an IgHV repertoire with molecular diversity similar to healthy infants.

Keywords

Severe combined immune deficiency (SCID); Adenosine deaminase deficiency; Gene therapy; B cell repertoire; Immunoglobulin sequencing

Introduction

Adenosine deaminase (ADA), an enzyme in the purine salvage pathway, is found in high levels in lymphoid cells. ADA deficiency is responsible for 10–15% of cases of severe combined immunodeficiency (SCID)[17]. In ADA deficiency, immature lymphoid cells in the thymus and bone marrow accumulate toxic levels of deoxyadenosine triphosphate (dATP), derived from the ADA substrate 2' deoxyadenosine [1, 2]. The dATP inhibits DNA replication and repair, and induces apoptosis resulting in profound depletion of T, B and NK cells. Other effects of 2' deoxyadenosine include aberrant signal transduction and impaired function of surviving lymphocytes [1, 2, 17, 19, 27, 45]. In addition to impaired immunity, these metabolic abnormalities more variably affect the nervous system, skeletal system, lungs, liver, and kidneys [14, 17, 28, 45]. Absent B cells and severe hypogammaglobulinemia are common among infants with ADA deficiency and a major cause of the infectious complications of the disorder.

Without treatment, ADA deficiency is uniformly fatal [17]. However, PEG-ADA enzyme replacement as a bridge to curative gene therapy can be effective in restoring immune function. To date, over 100 patients have undergone gene therapy with 100% survival [28]. First initiated in 1995 [6], modifications to gene therapy protocols, including the use of busulfan to improve engraftment and replacement of γ-retroviral with lentiviral vectors for

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more efficiently transducing CD34+ hematopoietic stem cells, have been implemented in an attempt to improve efficacy and safety [6, 11, 28, 29, 39].

Following gene therapy immune reconstitution can occur quickly with normal numbers of T cells, B cells, and NK cells detected within 6 months post therapy. Over time T cells display a diverse T-cell receptor repertoire with normal functionality [20]. However, the capacity of gene therapy to establish a normal B cell IgHV repertoire in SCID patients with ADA deficiency has not been examined. B cell numbers often remain low after gene therapy even though functional immunity appears to be restored. Up to 90% of patients can discontinue immunoglobulin replacement therapy by 24 months after gene therapy (personal communication with Donald Kohn). Following gene therapy the response to immunizations appears normal [14, 20], but the character of the antibodies with respect to the V-, D-, and J- gene usage and extent of somatic hypermutation is unknown. We applied deep sequencing to examine the IgHV repertoire in a child treated with gene therapy for ADA SCID and compared her repertoire to healthy infants.

Methods

Case Presentation.

A Caucasian female was born at term gestation to non-consanguineous parents. Her newborn screen for T cell excisional circles (TREC) was undetectable and she was evaluated for possible SCID at 22 days of life. Lymphocyte enumeration was consistent with $T^{-}B^{-}NK^{-}$ SCID with an absolute lymphocyte count of 44 cells/mm^3 (Table 1). ADA activity was measured in T cells at that time using methods as previously described [3] with undetectable ADA levels and elevated deoxyadenosine nucleotide levels of 52.6% μmol/ml. Genetic analysis identified compound heterozygosity for two severe mutations in the ADA gene, S291L (c.872C>T) and c.955–959 delGAAGA [35]. Prophylaxis for opportunistic infections, as well as subcutaneous immunoglobulin replacement, was initiated. Prior to gene therapy medical problems included chronic rhinovirus infection and seborrheic dermatitis, but no serious opportunistic infections. PEG-ADA was initiated at 2 months of age because she had no matched siblings to serve as bone marrow donors. At 4.5 months of age the child underwent gene therapy performed at Mattel Children's Hospital at the University of California Los Angeles using lentiviral-transduced autologous CD34+ cells at a dose of 7.16 $\times 10^6$/kg and vector copy number of 3.66 [ClinicalTrials.gov #NCT01852071] [12]. Enzyme replacement therapy (ERT) was discontinued one month after gene therapy. Clinical and laboratory evaluations were done monthly for the first 6 months following gene therapy and then every other month over the next two years, at which time the child transitioned to a separate long-term follow-up gene therapy study [ClinicalTrials.gov #NCT04049084]. Regular assessments of complete blood counts, renal and liver function, immunoglobulin levels, lymphocyte enumeration, and ADA levels were obtained (Table 1). Immunoglobulin levels, diphtheria, and tetanus titers were performed in the Duke University Hospital Clinical Immunology Laboratory. T cell function was monitored before and after gene therapy using phytohemagglutinin (PHA) stimulation of peripheral blood mononuclear cells (PBMC) measure in counts per minute (cpm) also performed in the Duke University Hospital clinical laboratories.

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Assessment of IgHV repertoire.

Peripheral blood samples were obtained at 22 and 28 months of life (18 and 24 months after gene therapy) to evaluate the IgHV repertoire. Routine vaccination per American Academy of Pediatrics schedule was started 18 months post gene therapy with pre-immunization blood draw at that time and post immunization blood obtained at 24 months post gene therapy which was also 2 months following completion of immunizations. A control cohort consisted of seven 12-month-old healthy infants who received American Academy of Pediatrics recommended immunizations of the first year of life and 6 fully vaccinated healthy adults, ages 17 to 23 years. These participants were enrolled in a study of immune development in healthy infants [ClinicalTrials.gov # NCT02568579]. All study participants were enrolled with approvals by the Institutional Review Boards of Duke University; written informed consents were obtained from all participants or parents including the parents of the ADA-deficient child. PBMC were obtained for RNA extraction from cryopreserved cells using established protocols [47].

Generation of amplicon libraries of immunoglobulin heavy chain variable region (IgHV) and NexGen sequencing.

Total cellular RNA was extracted from PBMC using RNAqueous-4PCR kit (Ambion, Austin, TX). Amplicon libraries of IgHM, IgHG and IgHA IgHV were generated from 500 ng of total RNA for each individual by RT-PCR using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA). Immunoglobulin isotype-specific libraries were constructed using reverse primers specific to IgHM (Cμ15), IgHG (Cγ16) or IgHA (AHCR) constant regions respectively [5, 23]. VH_{2-7}-FR1, a cocktail of V heavy (VH) gene family-specific forward primers located in 3’ frame work 1 (FR1) [43] was used for both first and second round PCR amplifications. Ten percent of first round products were used for 2nd round PCR amplification with Q5® High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA). Adaptor P5 was added to the 5’ end of each forward VH family-specific primer, while adaptor P7 with a 5’ 6-mer barcode was incorporated at the 5’ ends of the reverse primers, Cμ2 for IgM, Cɤ1 for IgG and Ca for IgA to generate IgHV amplicons ranging from 321 – 381 bp for IgM, 290 – 350 bp for IgG, or 299 – 359 bp for IgA [18, 23]. First-round RT-PCR included RT at 50°C for 30 min followed by PCR denaturation at 95°C for 2 min, followed by 20 cycles of 95°C for 30 sec, 62°C for 35 sec, and 68°C for 1 min, and a final extension at 68°C for 10 min. Second-round PCR included denaturation at 98°C for 30 sec, followed by 30 cycles of 98°C for 30 sec, 72°C for 35 sec and 72°C for 1 min, with a final extension at 72°C for 2 min.

Amplicons were gel purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and equal molar ratio-pooled libraries constructed and sequenced by the Interdisciplinary Center for Biotechnology Research at the University of Florida using MiSeq Reagent Kit v3 (600-cycle) in the MiSeq System (Illumina Inc., San Diego, CA) as a 300-bp paired end run and 6 cycles for index reads. Raw reads with a quality score of Q30 (1 in 1,000 probability of incorrect base call) or above were demultiplexed, and pair-end reads were stitched using MiSeq Reporter v2.3.

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Sequence quality control and analysis.

A bioinformatic pipeline was developed to analyze the IgHV deep sequences. Raw reads were filtered initially using Geneious Pro 5.6.5 (Biomatters Ltd., Auckland, New Zealand) to exclude raw reads with more than one mismatches in either primer, and/or reads with a sequence length less than 250 bp or longer than 450 bp, and then by BioEdit (Ibis Biosciences, Carlsbad, CA) to identify and exclude reads with ambiguous nucleotide[s] [48] or out of reading frame. Quality sequences were submitted to IgSEQ, a custom computation program suite that we developed. IgSEQ queries IMGT/V-QUEST and IMGT/JunctionAnalysis and perform analysis for key features including SHM, N-insertion at junctions, CDR3 length diversification and usage of V-, D- and J-gene families [8, 21, 22, 48].

Statistical analysis.

One-way ANOVA or unpaired t-test were used to identify differences among multiple groups or between two groups, respectively. Statistics were considered significant when $p < 0.05$.

Results

Immune reconstitution following gene therapy.

Figure 1 shows that by 6 months post gene therapy, signs of immune reconstitution with increasing numbers of T lymphocytes and NK cells were evident. B cells increased after gene therapy but failed to reach the normal ranges for age (Table 1). The child's chronic rhinovirus infection resolved after 2.5 months of PEG-ADA replacement therapy. Antibiotic and antifungal prophylaxis were weaned between 8- and 10-months post-gene therapy. Subcutaneous immunoglobulin replacement was discontinued, and immunizations were started at 1.5 years post-gene therapy. All vaccines received between 18 and 24 months after gene therapy are shown in Figure 1. She demonstrated an ability to make both tetanus and diphtheria specific antibodies (Table 1). At 24 months post-gene therapy she had a PBMC vector copy number of 1.24. Table 1 shows immunoglobulin levels, vaccine titers to tetanus and diphtheria, proliferation responses to mitogens at 24 and 44 months post gene therapy, which were normal for age. ADA levels were 43.1 nmol/h/mg and dAXP (μmol/ml) was 1.7% at 24 months, remaining in this ranges at 44 months. Live vaccines were administered starting at 30 months post gene therapy and were well tolerated. The child was able to recover from typical childhood viral infections without intervention and has shown normal growth and development at 4 years of age (Figure 1, Table 1).

Distribution and Extent of somatic hypermutation.

As shown in Figure 2A, the overall extent of SHM (CDR1-3, total mutations/100 bp) was significantly lower in healthy infants compared to adults. In healthy infants total SHM/100 bp in IgHM ranged from 2.15 to 3.24 compared to adults 2.84 to 4.96 ($p = 0.0009$). IgHG and IgHA SHM/100 bp were also lower in healthy infants compared to adults; IgHG 2.85 to 4.14 and IgHA 3.17 to 4.37, respectively compared to adults; IgHG 7.64 to 11.60 and IgHA 7.25 to 10.31 ($p < 0.0001$ and $p < 0.0001$), respectively. SHM for the infant following

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gene therapy fell within the range for heathy infants. SHM/100 bp were 2.10, 4.07, 3.97 at 18 months post gene therapy; and at 24 months 4.43, 4.32, and 4.30 for IgM, IgG, and IgA respectively. Among healthy infants or adults, the extent of SHM was greater in IgHG and IgHA compared to IgHM (Figure 2A). In healthy infants, the distribution of SHM across HV regions varied but was greatest in IgHG and IgHA CDR1 and CDR2, similar to adults. However, healthy infants had higher levels of SHM in CDR1 and CDR3 for IgHM. Following gene therapy at 18- and 24-months patterns of SHM were similar to healthy infants as the extent of SHM in the child following gene therapy was greatest in CDR1 and CDR2 for IgHG and IgHA.

CDR3 length variation and V-, D- and J-gene family use.

A major determinant of CDR3 length is TdT activity in bone marrow during early B cell differentiation resulting in nucleotide insertions (N-insertions) at the V-D junction (N1) and D-J junction (N2) (Figure 2B) [37]. N1 and N2 lengths were similar across isotypes in healthy adults. In healthy infants, N1 length was longer than N2 length across isotypes ($p = 0.002$, $p = 0.009$ and $p = 0.035$ for IgHM, IgHG and IgHA respectively). Comparing healthy infants to adults, N1 lengths were longer in adults in IgHG ($p = 0.03$) and IgHA ($p = 0.03$) but shorter in IgHM ($p = 0.04$). Adult N2 sequences were longer in IgHA compared to healthy infants ($p = 0.0001$). Post gene therapy average N1 and N2 lengths fell in the range seen in healthy infants (Figure 2B).

IgHM CDR3 lengths displayed a Gaussian distribution in both healthy adults and infants. Infants have a higher percentage of sequences with longer CDR3 length, consistent with longer N1 insertion in healthy infants (Figure 2B). Following gene therapy predominant IgHM CDR3 lengths were evident at 11, 13, and 17 amino acid lengths. The median IgM CDR3 lengths for healthy infants were 14.6 ± 0.4 and for adults were 13.6 ± 0.9 ($p=0.02$). V-, D-, J-gene usage in IgM was similar in healthy infants, adults, and the child post gene therapy with a similar preference for VH3 and VH4, DH2, DH3 and DH6, and JH4 families (Figure 2D).

Assessments of Oligoclonal Expansion.

In order to determine if post gene therapy B cells have the capacity to undergo class switch and oligoclonal expansion the percentage of sequences using the same VDJ segments within a dominant CDR3 peak was evaluated. Unlike IgHM, IgHG and IgHA CDR3 lengths were not evenly distributed in healthy infants and adults with evidence of dominant peaks in both isotypes. As illustrated in Figure 3, within a selected dominant IgHG 15 amino acids length from healthy adult, over 90% of the sequences utilized a single combination of V3D1J3. Similarly, At 18 months post gene therapy in the child, 89% of sequences in the dominant 15 amino acid IgHG peak used V2D3J3. By 24 months post gene therapy, and following immunizations, the dominant 9 amino acid peaks contained highly represented VDJs, V3D2J4 (47.2%) and V3D1J5 (46.7%) of the total sequences. Comparing the total > 45,000 total IgHG nucleotide sequences analyzed at 18 and 24 months post gene therapy failed to detect any shared sequences, although the depth of sequencing underestimates the total sequence diversity which is $>10^{11}$ within an individual (data not shown) [16].

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Discussion

ADA deficiency is one of more common genetic causes of SCID, adversely affecting B and T lymphocytes [15]. SCID variants such as those within the recombinase activating gene (RAG) result in absent B and T cells due to defective VDJ recombination, early in lymphocyte development in the bone marrow and thymus. Lymphocytes in the periphery are abnormal having escaped apoptosis at central checkpoints [15]. The abnormalities of B cells in these disorders are exemplified by their decreased B cell receptor diversity [24, 30]. A study by Lee et al further found that the skewing toward shorter CDR3 length correlated with worsened disease severity in RAG deficient SCID patients [30].

In contrast to RAG deficiency, ADA deficient SCID patients are profoundly T, B, and NK lymphopenic at birth, but effects on lymphocytes continue at later stages of cellular maturation [1, 2, 19]. T cells are adversely impacted in the bone marrow, thymus, and periphery in ADA deficiency [1, 2, 7, 27]. While data in humans are not available, evaluation of ADA−/− mice showed thymocyte apoptosis, abnormal thymocyte distribution, abnormal surface markers on peripheral T cells, inhibition of TCR signaling, and increased TCR-mediated apoptosis [2]. In B cell development there are three points of high levels of cellular replication, when B cells are particularly susceptible to the toxicity of dAXPs: in the bone marrow, in the germinal centers following antigen stimulation, and upon re-activation of memory B cells [27]. In the murine model, ADA deficiency has shown that B cells mature normally in the bone marrow and dATP levels are normal [1]. However, studies of bone marrow from children with ADA deficiency show a progressive loss of mature precursor B cells indicative of a partial block in development [7]. In the germinal centers, dATP levels are elevated along with adenosine and 2’ deoxyadenosine [1]. Levels of dATP and 2’deoxyadenosine within memory B cells have not been reported in untreated ADA deficient SCID patients. The effect of 2’ deoxyadenosine accumulation includes direct activation of pro-apoptotic signaling cascades as well as the inhibition of SAH hydrolase impairing lymphocyte activation after antigen stimulation among B cell subsets [1, 45]. Deoxyadenosine also becomes phosphorylated to form dATP, which impairs DNA synthesis thereby inhibiting lymphocyte proliferation to antigen and directly activating pro-apoptotic pathways [1, 45]. The only available data on the impact of toxic metabolites on the B cell receptor come from an in vitro study where B cell exposure to dATP leads to changes in the B cell receptor including an increase in A-T insertions, a decrease in VDJ recombination, and an overall reduction in diversity [19]. PEG-ADA treatment, now used as a bridge to gene therapy, improves but often does not completely restore immunity, and most individuals on PEG-ADA alone remain lymphopenic with decreased T and B cell function, and about half have had a requirement for immunoglobulin replacement[27]. Long-term PEG-ADA treatment also disturbs B cell subsets with decreased naïve B cells and an accumulation of transitional and CD21lo B cells [7]. Further, some individuals on PEG-ADA display restricted B cell repertoires with oligoclonal expansion [31]. Gene therapy for ADA deficiency normalizes naïve and transitional B cells after long-term follow-up, but CD21lo B cells remain elevated [7].

B cell receptor (BCR) diversity in this child with ADA deficiency treated with gene therapy was compared to healthy infants following routine childhood immunizations, as well as

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healthy adults. Normal BCR diversity generated in the bone marrow is initiated by VDJ recombination and TdT mediated N-additions. Diversity is further expanded upon after antigen stimulation and subsequent Ig affinity maturation in the germinal centers and periphery [24]. We hypothesized that if gene therapy response was complete, V, D, and J gene usage, N-additions, and CDR3 length should be similar to healthy infants. If gene therapy did not fully restore the BCR repertoire, differences between the child receiving gene therapy and healthy infants would be expected. In the case of this child, the response to gene therapy was complete and BCR diversity at all stages of B cell development was similar to both healthy infants and adults.

VDJ recombination begins in the fetus and all V, D, and J gene segments are used by the second trimester of gestation; However, N-addition diversity is limited in the fetus and infant with increasing N-additions and longer CDR3 lengths achieved by adulthood [33, 36, 37, 41, 49]. Our data of healthy infants and adults confirm similar use of V, D, and J gene segments in infants and adults as well as similar TdT mediated N-additions. IgHM CDR3 length displays a Gaussian distribution in infants, adults, and in ADA deficiency following gene therapy. Furthermore, V, D, and J segments usage, patterns of N-additions, and SHM rates were similar to healthy infants.

The ability to undergo SHM and affinity maturation is a key factor which differentiates B cells from T cells. SHM can be detected in the fetus by 12 weeks gestation and class switching upon exposure to antigens is evident by late second trimester [32, 33]. Affinity maturation and SHM increases with age and antigenic re-challenge such that adult B cells display a greater degree of SHM than infants' B cells [24, 32, 33, 44, 49]. Similarly, B cells with a T-dependent response will exhibit more SHM than B cells that undergo a T-independent response[44]. In this study, healthy infants have overall lower levels of SHM compared to adults. Following gene therapy for ADA deficiency our patient had the capacity to undergo oligoclonal expansion and Ig class switch as evidenced by expansion of the same VDJ combinations within predominant CDR3 lengths in IgHG before and after immunization. In addition, SHM is similar to healthy infants in all isotypes. Taken together, gene therapy was fully restorative in the B cell compartment of the ADA SCID child.

The child in this report with ADA deficient SCID tolerated lentiviral gene therapy after a brief course of PEG-ADA. While vector copy number was assessed only in PBMC from this child and not specifically for B cells, in a subset of participants in the gene therapy clinical trial for ADA deficiency the vector copy number within isolated r T, B, and NK cells was similar to total PBMC (personal communication with Donald Kohn). As seen in other studies of ERT [4, 10, 13], bone marrow transplantation [4, 9, 38] and/or gene therapy for ADA deficiency [14, 20, 39], B cell numbers improved but remained below the normal range for age while adequate B cell function was demonstrated. While the IgHV repertoire has been analyzed in PIDDs previously [30, 34, 42, 46], this is the first study to examine the IgHV repertoire following gene therapy. The results provide clear evidence of the normalization of the B cell receptor repertoire in this previously ADA deficient child. The results further support the ability to mount an effective antibody response to both vaccines and environmental antigens following gene therapy, providing important baseline data for longitudinal monitoring of individuals who receive gene therapy in the future.

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While this is a single case report, the conclusions of this study provide proof of principle for the application of deep sequencing of the IgHV repertoire in infants who have been treated with gene therapy for ADA deficiency. The strength of the study is the comparison of the results in the child receiving gene therapy to healthy infants and healthy adults who received standard immunizations. The study was not designed to compare the results to other therapies for ADA deficiency such as PEG-ADA or hematopoietic stem cell transplantation. However, our study provides strong rationale to use assessment of the IgHV repertoire to compare immune reconstitution for the various therapeutic approaches for SCID. Recently, the international expert panel on the management of late effects in patients with SCID after allogenic hematopoietic cell transplantation pointed out the need to identify and validate biomarkers of B cell immune reconstitution [24]. Furthermore, analysis of the B cell receptor diversity is one research priority that could be applied to assess B cell function and used as a clinical tool to evaluate the ability to discontinue immunoglobulin replacement post therapy [25, 26]. Our study using deep sequencing of the IgHV repertoire could help to differentiate B cell responses to transplantation versus gene therapy or even help with clinical decision making during immune reconstitution. The approach using deep sequencing to assess the IgHV repertoire and specifically evaluate SHM after gene therapy for ADA deficiency is novel. Sequencing the B cell repertoire over time from a cohort of patients after ADA gene therapy could provide important tool in long term clinical monitoring.

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Availability of Data & Material

Sequencing data is available on dbGAP (Study Accession Number: phs002074.v1.p1).

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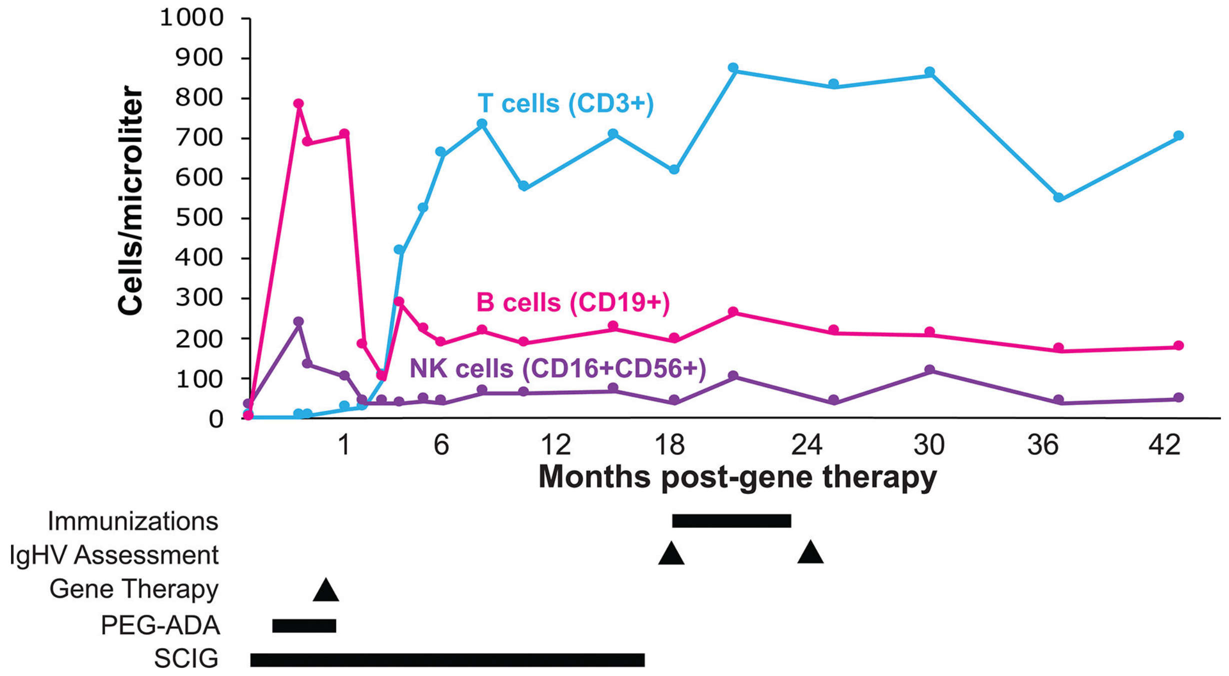

Figure 1.

Immune reconstitution after ADA gene therapy. Absolute counts (cells per microliter) of lymphocyte subsets are shown relative to time (months) after gene therapy. Subsets shown are T cells (CD3+) in turquoise, B cells (CD19+) in magenta, and NK cells (CD16+CD56+) in purple. Clinical care milestones are indicated with black bars/arrows below the graph's x-axis and include immunizations. Vaccinations included DTaP, IPV, HiB, at 18, 19, and 20 months; Hep B at 18, 20 and 22 months, Influenza at 22 months, and PCV-13 at 19 and 22 months post gene therapy. IgHV assessment, gene therapy administration, PEG-ADA administration, and SCIG therapy.

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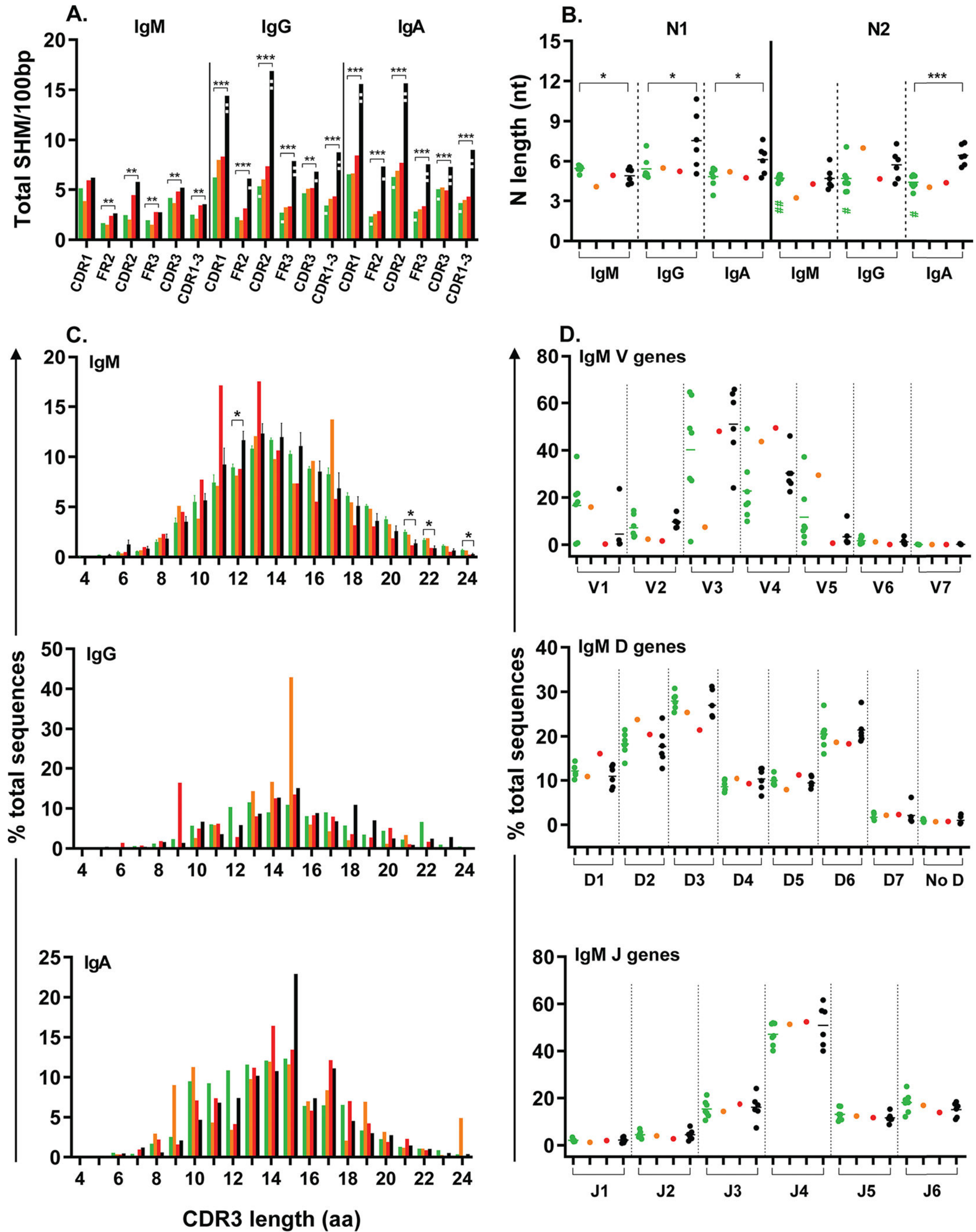

Figure 2.

B cell receptor repertoire usage and diversity in a child after ADA gene therapy compared to healthy infants and adults. Color codes: **green,** 7 healthy 12 month old infants; **orange,** ADA deficient child at 18 months and r**ed**, 24 months post gene therapy (8 weeks after immunizations); **black,** 6 healthy immunized adults. **Panel A** shows the average rate of somatic hypermutation as SHM/100 bp [40] in IgHM, IgHG, and IgHA sequences across complementarity determining regions (CDRs) and framework regions (FRs) [48]. **Panel B** shows non-templated nucleotide insertions (N-insertions) as nucleotide length in N1

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(between V and D genes) and N2 (between D and J genes). **Panel C** shows CDR3 amino acid length (aa) for IgHM, IgHG, and IgHA in amino acids length with the y axis showing the CDR3 length as a percentage of total sequences for each isotype in heathy infants (green), adults (black), and the ADA deficient child at 22 month (orange) and 28 months (red). **Panel D** shows relative percentage of total sequences for V, D, and J gene usage in IgHM. Statistical comparisons between healthy infants and adults was performed with an unpaired t test with *p<0.05 **p 0.005, and ***p 0.0001.

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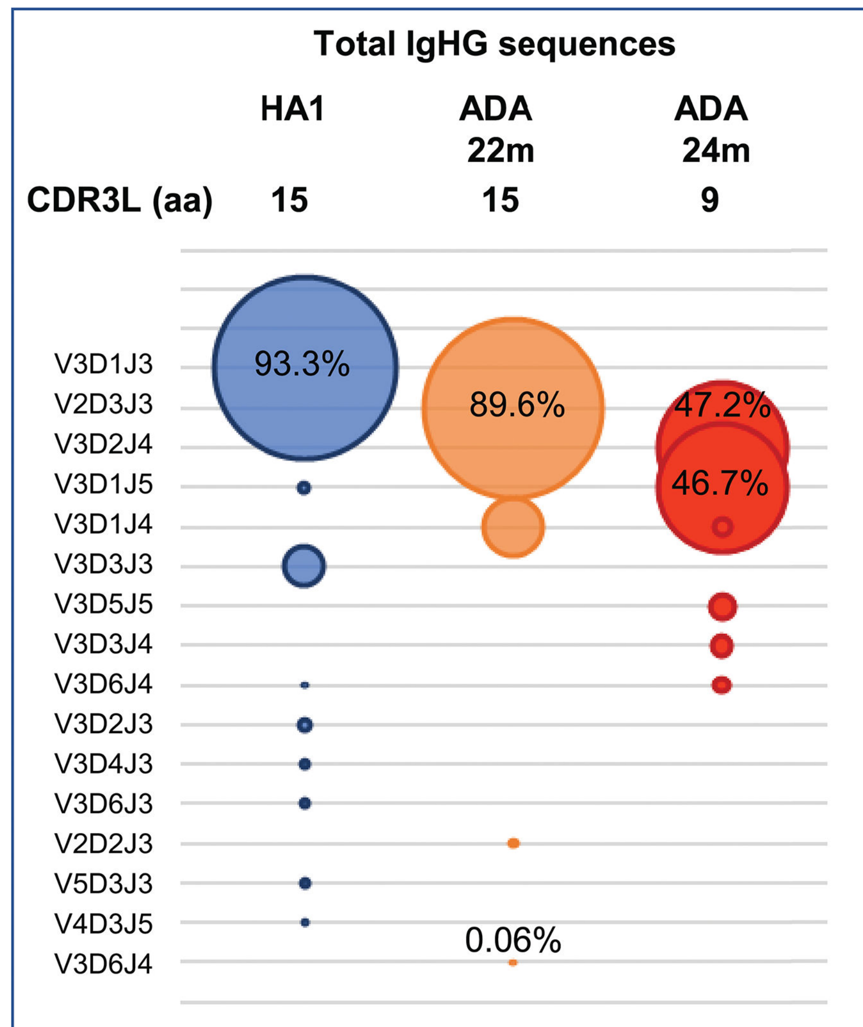

Figure 3. VDJ usage within dominant IgHG CDR3 lengths demonstrates oligoclonality.

IgHG clonality was assessed by fractions of unique VDJ sequences (bubble size) within a dominant CDR3 length for a selected healthy adult (15 amino acids for HA1). Post gene therapy in the ADA deficient child the dominant the dominant CDR3 length at 18 months (pre vaccination) was 9 amino acids containing single highly represented VDJ and 15 amino acids at 24 months (post vaccination) containing two highly represented VDJs. Fractions of VDJ sequences < 0.05% are not shown.

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Table 1.

Child laboratory data from initial presentation and most recent presentation.

	22 days of age	Normal range*	Child at 24 months post gene therapy	Normal range*	Child at 44 months post gene therapy	Normal range*
		Flow cytometry (Absolute count /mm^3 (%))				
Absolute lymphocyte count	44	3,400–7,600	1,107	2,300–5,400	946	2,300–5,400
CD3	4 (9.2)	2,500–5,500 (53–84)	828 (75)	1,400–3,700 (56–75)	700 (74)	1,400–3,700 (56–75)
CD3CD4	4 (9.1)	1,600–4,000 (35–64)	664 (60)	700–2,200 (28–47)	491 (51.9)	700–2,200 (28–47)
CD3CD8	0 (0.7)	560–1,700 (12–28)	108 (10)	490–1,300 (16–30)	153 (16.2)	490–1300 (16–30)
CD16CD56	33 (75.7)	170–1,100 (4–18)	39 (4)	130–720 (4–17)	48 (5.1)	130–720 (4–17)
CD19	2 (5.4)	300–2,000 (6–32)	215 (19)	390–1,400 (14–33)	176 (18.6)	390–1,400 (14–33)
CD4CD45RACD62L	0 (0)	1,200–3,600 (61–94)	410 (62)	420–1,500 (50–85)	261 (53.2)	260–850 (42–81)
		Immunoglobulin levels (mg/dL)				
IgG	555	591–1,583	386	391–1047	456	498–1,332
IgA	<5	0	41	15–95	27	21–129
IgM	8	4–20	53	49–202	57	31–126
		T cell function				
PHA CPM	318		173,095		181,536	
		Specific antibody response (IU/mL)				
Diphtheria	NA	NA	1.04	0.100	0.148	0.100
Tetanus	NA	NA	3.23	> 0.16	1.11	> 0.16
		ADA activity in RBCs				
ADA (nmol/h/mg)	0.0	63.0 +/− 41.4	43.1	63.0 +/− 41.4	56.8	63.0 +/− 41.4
AXP (μmol/ml)	1.0	1.465 +/− 0.38	2.246	1.465 +/− 0.38	1.826	1.465 +/− 0.38
dAXP (μmol/ml)	1.108	<0.002	0.038	<0.002	0.030	<0.002
% dAXP (μmol/ml)	52.6	<0.2	1.7	<0.2	1.6	<0.2

* Normal ranges adjusted for age.