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The Different Extent of B and T Cell Immune Reconstitution after Hematopoietic Stem Cell Transplantation and Enzyme Replacement Therapies in SCID Patients with Adenosine Deaminase Deficiency

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The lack of adenosine deaminase (ADA) leads to the accumulation of toxic metabolites, resulting in SCID. If the disease is left untreated, it is likely to have a fatal outcome in early infancy. Because hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy with pegylated bovine ADA (PEG-ADA) are both provided in our hospital, we undertook a retrospective longitudinal comparative study of the extent of lymphocyte recovery in two groups of treated ADA-SCID children. Together with classical immunological parameters, we quantified the output of the new B and T cells from the production sites using the κ-deleting recombination excision circle and TCR excision circle assay, and we monitored T cell repertoire diversification. We found that immune reconstitution was different following the two treatments. The stable production of κ-deleting recombination excision circle+ lymphocytes sustained an increase in B cell number in HSCT-treated patients, whereas in PEG-ADA–treated patients, it was accompanied by a significant and progressive decrease in circulating CD19+ lymphocytes, which never reached the levels observed in age-matched children. The mobilization of TCR excision circle+ cells, though lower than in controls, was stable with time after HSCT treatment, leading to a constant peripheral T cell number and to the diversification of the T cell repertoire; however, it was compromised in children receiving prolonged PEG-ADA therapy, whose T cells showed progressively narrowing T cell repertoires. The Journal of Immunology, 2010, 185: 000–000.

A

denosine deaminase (ADA) deficiency is a rare autosomal inherited disorder characterized by abnormalities in immune system development and function, as well as a variety of systemic defects (1, 2). The initial and most devastating presentation of the disease is due to the immune defects and, therefore, affected infants characteristically present with severe opportunistic infections, failure to thrive, and an immunological profile consistent with SCID (3).

ADA is a purine salvage enzyme expressed in all tissues that is capable of catalyzing the deamination of deoxyadenosine (dAdo) and adenosine to deoxyinosine and inosine, respectively (4). Conflicting data exist on the relative contribution of ADA metabolites in the pathogenesis of the immune dysfunctions observed in ADA-deficient patients, but the biochemical hallmarks of this deficiency indicate that dAdo accumulation in intracellular and extracellular compartments is the primary cause of lymphotoxicity. dAdo is converted intracellularly to deoxyadenosine triphosphate, which inhibits ribonucleotide reductase (an enzyme necessary for DNA replication and repair), induces apoptosis in immature thymocytes, and interferes with TdT activity, thereby limiting V(D)J recomposition and Ag receptor diversity (5–7). Furthermore, dAdo accumulation inactivates the enzyme S-adenosylhomocysteine hydrolase and inhibits the transmethylation reactions necessary for effective lymphocyte activation (8). Collectively, dAdo accumulation induces several potentially deleterious effects, including abnormalities in lymphocyte development, viability, and function, which are the major causes of severe lymphocyte depletion. Indeed, in ADA deficiency, there is a profound depletion of T, B, and NK lymphocytes compared with any other form of SCID (1).

Untreated ADA-SCID is fatal in the first years of life and necessitates early interventions. Allogeneic hematopoietic stem cell transplantation (HSCT) has long been considered the mainstay of ADA-SCID treatment. HSCT is highly successful, with survival rates of ~90% if a matched sibling or matched related donors are available. However, survival following matched unrelated donor or haploidentical procedures drops to 63 and 50%, respectively, with a significant rejection/nonengraftment rate in unconditioned procedures (9). Posttransplant immune reconstitution confers competent immunity in terms of lymphocyte number and function and Ab production (9). Unlike other SCID forms, two other treatment options are available for ADA-SCID, namely enzyme replacement therapy with pegylated bovine ADA (pegadimase bovine, Adagen [Sigma-Tau Pharmaceuticals, Gaithersburg, MD]), or, in this study, PEG-ADA and somatic gene therapy. Following PEG-ADA
therapy, approximately two-thirds of patients display a significant clinical improvement with normal growth and are free of infections (10). However, the extent of immune recovery is variable (11), and in a significant number of patients, continuous Ig replacement therapy is required over a longer time period, with some patients remaining lymphopenic due to low T cell numbers (12). Initially, PEG-ADA treatment was also applied in addition to gene therapy performed on lymphocytes. However, the successful transduction of hematopoietic stem cells with ADA-expressing retroviral vectors demonstrated that gene therapy alone is efficacious in correcting ADA deficiency (3, 9, 13–15).

To our knowledge, the immunological parameters of ADA-SCID patients treated with different types of therapies have never been compared, likely because ADA is a rare disease, and the numbers of patients who underwent gene therapy is very small. In the present retrospective longitudinal study, we compared the extent of immune recovery in ADA-deficient patients who received HSCT or were treated with PEG-ADA. The evaluation of the release of new B and T cells from the bone marrow and the thymus was performed using a duplex quantitative real-time PCR protocol that combines the well-established and widely used method of measuring thymic function, the TCR excision circle (TREC) assay (16), and the more recently proposed κ-deleting recombination excision circle (KREC) assay (17–19).

Materials and Methods

Patients

Among the patients with ADA-SCID followed at the Pediatric Clinic of the Spedali Civili of Brescia (Brescia, Italy) between 2002 and 2010, we selected 13 children, 6 boys and 7 girls (median age 7.1 y; range 2.0–12.5 y), from whom at least three blood samples had been sent to the laboratory over time for the routine analysis of immune status (Table I). At diagnosis, all children showed low total lymphocytes and B and T cell counts, impaired PHA responses, low levels of ADA activity, and low total deoxyadenosine nucleotide in RBCs, compatible with the diagnosis of ADA-SCID. At the time of the last assessment, the median elapsed time from HSCT was 67 mo (range 20–142 mo), whereas PEG-ADA therapy had been performed for a median of 114 mo (range 22–141 mo). The median length of our follow-up was 57 mo (range 11–86 mo) in the five transplanted patients and 41 mo (range 21–73 mo) in the eight patients who received enzyme replacement therapy. Of the five children who received HSCT, three had an HLA-matched family donor, and, therefore, HSCT was performed without conditioning. None of these three patients presented acute or chronic graft-versus-host disease (GVHD). A volunteer donor was found for the two children without a matched family donor. These children underwent a conditioning regimen according to the Working Party on Inborn Errors of the European Blood and Marrow Transplantation Group Guidelines. They received T-depleted bone marrow and cyclosporin A as GVHD prophylaxis, and only patient UPN4 presented an acute GVHD of grade II. In nontransplanted patients, the therapy with PEG-ADA, started at a concentration of 30–60 U/kg twice a week, was then reduced to a maintenance dose of 20–30 U/kg, twice a week. All 13 patients received i.v. Ig (IVIG) for a variable period of time, and this therapy is still ongoing in UPN4 (who was still close to HSCT at the last examination), UPN8 (who did not respond to vaccinations), and UPN6 and UPN9 (due to clinical complications). Furthermore, UPN9 began therapy with anti-CD20 mAb 3 mo before the last examination. Blood samples from 48 asymptomatic children who visited the pediatric outpatient clinic for conditions not related to immunological or infectious diseases were used as controls. Informed consent was obtained from the parents of all participants. Part of the drawn blood was used whole for cytofluorimetric analysis and part to prepare PBMC by Ficoll-Hypaque density gradient centrifugation. Cells were immediately used for immunological studies or separated into aliquots to be stored in liquid nitrogen or kept as dry pellets at −80°C until use.

Ig levels, lymphocyte proliferation assay, cell phenotyping, and chimerism analysis

The assessment of immunological status included the quantification of serum IgG, IgA, and IgM, evaluated using an immunoturbidimetric assay (Abbott Diagnostics, Abbott Park, IL), and the measurement of proliferative responses of PBMCs stimulated with 6.2 μg/ml PHA (Sigma-Aldrich, St. Louis, MO), pulsed with 1 μCi [1H]thymidine, and harvested 18 h later. Specific Ab production was analyzed by ELISA, following patients’ immunization with tetanus toxoid and hepatitis B vaccines, and with measles vaccine only in patients receiving HSCT (20). CD19+ B cell count, as well as CD3+ and naive CD4+CD45RA+ T cell numbers, were determined by flow cytometry (Beckman Coulter, Fullerton, CA). According to Mazzolari et al. (21), IgM <500 mg/dl, IgG <35 mg/dl, IgG <50 mg/dl, PHA response <35,000 cpm, total lymphocytes <1500/μl, CD19 <50/μl, and CD3 <1000/μl were considered abnormally low. In patients receiving HSCT, chimerism was assessed on positively selected CD3+ and CD19+ lymphocytes using an AmpFISTR Identifier PCR amplification kit (Applied Biosystems, Foster City, CA) as described (22).

Real-time PCR for KRECs and TRECs and calculation of the average number of B cell divisions

DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. Primers and probes for KRECs and TRECs were as follows: KRECs signal joint (SJ) forward primer: 5′-TTC ATT AGG TCT CAC-3′; reverse primer: 5′-AGG AGC CAT CTC TTA CCC TAG AGT-3′, and probe 5′-FAM-TGC GCA GGC GCA GGT TGG-TAMRA-3′; TRECs SJ forward primer: 5′-CAC ATT CCT TAC CAT GCT-3′; reverse primer: 5′-TGC AGG TGC CTA TGC ATC A-3′, and probe 5′-FAM-ACA CCT CTG GTT GTA AAG GTG CCC ACT-TAMRA-3′; and TCR α constant gene (TARC) forward primer: 5′-TGG CCT AAC CCT GAT CTT CTT-3′, reverse primer: 5′-GGA TTT AAT CCA AAG GTA GTC TCT CAC-3′, and probe 5′-FAM-TCC CAC ACA TAT CCA AAA GGA TCC GCA CCC-TAMRA-3′. PCR reactions were developed in 96-well optical reaction plates (Applied Biosystems) in a final volume of 25 μl, consisting of 5 μl genomic DNA solution (for which concentration ranged from 50–100 ng/μl), 12.5 μl 2× TaqMan Universal PCR master mix containing AmpErase UNG (Applied Biosystems), and primers and probes for KRECs SJ and TRECs SJ at final concentrations of 900 and 200 nM, respectively. Amplification of the TCRα reference gene was simultaneously done on the same plate using the same concentrations of TCRα-specific primers and probe. Amplification by real-time PCR was performed on a 7500 Fast Real-Time PCR apparatus (Applied Biosystems) and consisted of a first step at 50°C for 2 min, an initial heating at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, and a combined primer/probe annealing and elongation at 60°C for 1 min. KRECs, TRECs, and TCRα copy numbers were obtained by extrapolating the respective sample quantities from the standard curve obtained by serial dilutions (10², 10¹, 10⁰, 10⁻¹, 10⁻²), and of a linearized plasmid DNA containing three inserts corresponding to fragments of KRECs, TRECs, and TCRα.

The number of KRECs or TRECs per PBMC was calculated with the following formula:

\[
\text{KRECs or TRECs/PBMC} = \frac{\text{mean quantity of KRECs or TRECs}}{\text{mean quantity of TCRα/2}}
\]

The mean quantity of TCRα was divided by two because each cell carries two copies of the TCRα gene (i.e., one on each chromosome). As in Chen et al. (23), this value, together with the lymphocyte plus monocyte count (which are the cells contained in PBMC preparation) in 1 ml blood, was used to calculate the number of KRECs or TRECs/ml blood (copies/ml), which was equal to \(\frac{\text{KRECs or TRECs/PBMC} \times (\text{lymphocyte + monocyte count in 1 ml blood})}{\text{mean quantity of TCRα/2}}\).

The average number of B cell divisions was evaluated as reported by van Zelm et al. (17) by calculating the difference (Δ) between the cycle threshold (Ct) number obtained by real-time PCR amplification of SJ, which is the sequence contained in KRECs, and the Ct number obtained post-amplification of the coding joint (CJ), which is sequence generated during amplification of the coding joint (CJ) chain repertoire was analyzed after RNA isolation and cDNA synthesis by PCR following by heteroduplex analysis (24). In this assay, denatured and renatured amplified products of TCRBV chains migrate in polyacrylamide gels as smears in the case of polyclonal TCR repertoire, whereas homoduplex or heteroduplex bands indicate a
Table I. Characterization of the ADA-SCID patients under evaluation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>ADA at Diagnosis (U/g Hb)</th>
<th>Age at HSCT (mo)</th>
<th>Age at Last Examination (mo)</th>
<th>ADA at the Last Examination (U/g Hb)</th>
<th>Donor (Age in y)</th>
<th>Conditioning</th>
<th>Months Since HSCT (mo)</th>
<th>IVIG Therapy Duration (mo)</th>
<th>Clinical Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN1</td>
<td>Male</td>
<td>0.17</td>
<td>1</td>
<td>85</td>
<td>0.87</td>
<td>HLA-id (8 y)</td>
<td>No</td>
<td>84</td>
<td>33</td>
<td>No</td>
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<tr>
<td>UPN2</td>
<td>Female</td>
<td>0.2</td>
<td>4</td>
<td>62</td>
<td>NA</td>
<td>HLA-id (4 y)</td>
<td>No</td>
<td>58</td>
<td>50</td>
<td>No</td>
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<tr>
<td>UPN3</td>
<td>Male</td>
<td>0.19</td>
<td>7</td>
<td>74</td>
<td>0.6</td>
<td>MUD (29 y)</td>
<td>Yes*</td>
<td>67</td>
<td>66</td>
<td>No</td>
</tr>
<tr>
<td>UPN4</td>
<td>Female</td>
<td>0.39</td>
<td>7</td>
<td>40</td>
<td>0.6</td>
<td>MUD (20 y)</td>
<td>Yes*</td>
<td>20</td>
<td>Ongoing</td>
<td>Acute GVHD, grade II</td>
</tr>
<tr>
<td>UPN5</td>
<td>Female</td>
<td>0.34</td>
<td>7</td>
<td>149</td>
<td>0.33</td>
<td>HLA-id (8 y)</td>
<td>No</td>
<td>142</td>
<td>33</td>
<td>No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age at PEG-ADA Start (mo)</th>
<th>PEG-ADA at Present (U/kg/wk)</th>
<th>dAXP at the Last Examination (mmol/ml RBC)</th>
<th>PEG-ADA Therapy Duration (mo)</th>
<th>Clinical Complications</th>
</tr>
</thead>
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<tr>
<td>UPN6</td>
<td>Male</td>
<td>0.26</td>
<td>2</td>
<td>30 × 2</td>
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<tr>
<td>UPN7</td>
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<td>0</td>
<td>4</td>
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<td>30 × 2</td>
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<tr>
<td>UPN8</td>
<td>Male</td>
<td>0</td>
<td>5</td>
<td>124</td>
<td>0.57</td>
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<tr>
<td>UPN9</td>
<td>Female</td>
<td>0.17</td>
<td>2</td>
<td>78</td>
<td>1.04</td>
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<tr>
<td>UPN10</td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>141</td>
<td>NA</td>
</tr>
<tr>
<td>UPN11</td>
<td>Male</td>
<td>0</td>
<td>3</td>
<td>127</td>
<td>NA</td>
</tr>
<tr>
<td>UPN12</td>
<td>Female</td>
<td>1.02*</td>
<td>3</td>
<td>25</td>
<td>1.10</td>
</tr>
<tr>
<td>UPN13</td>
<td>Male</td>
<td>0</td>
<td>1</td>
<td>137</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Busulfan-antithymocyte globulin-cyclophosphamide.
*The patient received blood transfusions.
AIHA, autoimmune hemolytic anemia; BPN, bronchopneumopathy; dAXP, total deoxyadenosine nucleotide; Hb, hemoglobin; id, identical; MUD, matched unrelated donor.
monoclonal or oligoclonal condition. Skewing of the TCR repertoire was scored when an oligoclonal pattern in two or more lanes was found in the heteroduplex gel: skewing in two to five lanes was defined as TCR score 1, whereas skewing in more than five lanes was defined as TCR score 2. In nonskewed cases, polyclonal patterns were seen in all lanes, and these repertoires were defined as having TCR score 0 (25). The analysis of TCR repertoires by spectratyping was performed in selected samples following the method reported by Chiarini et al. (26).

Statistical analysis
To take into account the nonindependence of repeated observations performed in each subject, that blood draws were not performed at the same time during follow-ups in different patients, and that follow-ups often began months post treatment initiation, linear mixed models were chosen for the analysis of the data (27). In the best-fitting model, determined on the basis of a maximum likelihood test, patients were considered the random-effect variable, whereas fixed-effect predictors for each dependent variable (KRECs, TRECs, total lymphocyte count, CD3+, CD19+, CD4+CD45RA+ cell number, and average number of B cell divisions) were the following: the age at therapy initiation, the time interval between the moment of therapy initiation and the evaluation (hereafter referred to as elapsed time), the treatment type, and interaction between treatment and elapsed time. Because the age at therapy initiation and the elapsed time result in the production of new B cells in our cohort of ADA-SCID patients on average, unchanged throughout the entire observation period in patients who received enzyme replacement therapy.

Release of new B cells in ADA-SCID patients undergoing therapy
The production of new B cells in our cohort of ADA-SCID patients was compared with that of age-matched controls, who were divided in two groups because KRECs+ cells were significantly higher (p < 0.001) in children from 2 mo to 3 y of age (median: 226,408 KRECs/ml, 5th–95th percentile: 45,813–675,266 KRECs/ml) in comparison with children from 3–12.5 y (median: 34,697 KRECs/ml, 5th–95th percentile: 7,217–161,197 KRECs/ml). The identification of these two groups, together with the fact that several immune parameters vary with age and that blood draws were not taken at the same times during the follow-ups, led us to plot all data according to patient age. In Fig. 2, the areas corresponding to the fifth percentile, which was used as a cutoff, are indicated as

FIGURE 1. Lymphocytes, B and T cell counts in HSCT- and PEG-ADA–treated patients. All data regarding total lymphocyte (A), CD19+ (B), and CD3+ (C) cell counts recovered since HSCT and PEG-ADA therapy initiation are plotted according to the age of the children. Dotted lines correspond to the cutoff values used by Mazzolari et al. (21): total lymphocytes, 1500/µl; CD19, 50/µl; and CD3, 1000/µl. Horizontal arrows at the top of each graph indicate the time interval during which UPN1, UPN2, UPN3, and UPN5 underwent HSCT and the period when PEG-ADA therapy started in the other patients. * indicates the time when HSCT was performed in UPN4.
Independent of the type of therapy, the number of KRECs in most samples taken before 3 y of age was under the cutoff (Fig. 2A). However, KRECs+ lymphocytes rapidly increased in UPN4 and were also higher than the cutoff in samples taken several years posttransplantation from UPN1, UPN3, and UPN5. Further, two of four patients with KRECs above the cutoff showed a total B cell engraftment (Fig. 2A). In UPN2, the number of lymphocytes containing KRECs remained constantly under the cutoff. In the patients treated with PEG-ADA, the release of B cells from the bone marrow was very heterogeneous. In UPN12, KRECs were low in the first sample (taken 3 d after therapy began), increased 3 mo later, and then decreased in the three following samples. KRECs+ cells also varied substantially among the samples of UPN6 taken at various time points. They were largely stable over the cutoff in UPN11 and UPN13, clearly increased over time only in UPN8, and declined in UPN7, UPN10, and (in particular) UPN9. In this last patient, the decrease in KRECs from a value over the cutoff to undetectable levels at the three last points of the follow-up was not related to anti-CD20 therapy, which was initiated only 3 mo before the last blood sampling. This wide variability likely affected the results of the linear mixed model analysis of the data (elapsed time coefficient for logKRECs: 0.008)

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FIGURE 2. Values of KRECs and CD19+ cells and average number of B cell divisions in HSCT- and PEG-ADA–treated patients at different time points during follow-up. Values of KRECs (A) and CD19+ B cells (B) are plotted according to the age of the patients. Dark and light gray boxed areas include data under the cutoff determined as the fifth percentile of values observed in two groups of age-matched controls (2 mo to 3 y and 3–12.5 y old) who had statistically different numbers of KRECs+ and CD19+ lymphocytes. The average number of B cell divisions (C) was calculated as the ΔCt between the KREC SJ and the genomic CJ; the gray area contains the values lying within the 5th–95th percentile of average proliferations found in controls. Horizontal arrows at the top of each graph indicate the time interval during which UPN1, UPN2, UPN3, and UPN5 underwent HSCT and the period when PEG-ADA therapy started in the other patients. * indicates the time when HSCT was performed in UPN4. D, complete donor B cell engraftment; M, mixed B cell engraftment.

FIGURE 3. Correlation of CD19+ cells with the number of KRECs and with the average number of B cell divisions. The number of peripheral CD19+ cells was correlated with the number of cells containing KRECs (A) and with the average number of B cell divisions (B). The lines were fitted by simple linear regression of non-transformed data and appear curved in A due to the log scale of the horizontal axis.
[95% CI: −0.004–0.021; \( p = \text{NS} \)] in HSCT-treated patients and −0.008 [−0.002–0.108; \( p = \text{NS} \)] in PEG-ADA–treated children, indicating that, collectively, KRECs did not significantly increase or decrease over time in either group of treated children.

To verify whether this heterogeneous pattern of KRECs production affected the peripheral B-lymphocyte number, we assessed the number of CD19+ lymphocytes at the same time points at which KRECs were evaluated. The cutoff level for CD19+ lymphocytes, calculated as the fifth percentile of control samples, was 496/μl in children from 2 mo to 3 y of age and 245/μl in children from 3–12.5 y (shown in Fig. 2B as dark and light gray boxes, respectively). Although samples with CD19+ cells over the cutoff were found only in UPN1, UPN4, and UPN5, there was a small but significant increase \( (p = 0.025) \) over time in CD19+ cells with a coefficient of 1.630 (95% CI: 0.205–3.054) in HSCT-treated children. In PEG-ADA–treated children, CD19+ lymphocytes were always below the cutoff (with the exception of the first UPN6 sample obtained at 4 mo of age), and the elapsed time coefficient was −1.522 (−2.267 to −0.077; \( p = 0.004 \)), indicating that, on average, CD19+ cells significantly decreased post treatment initiation.

Because the pattern of variation in the number of KRECs and CD19+ lymphocytes over time appeared rather similar in most patients of both groups, we calculated the correlation coefficient between the level of CD19+ cells and the number of KRECs in each treatment group, and we found a strong positive association (Fig. 3A). This suggests that the release of new B lymphocytes may be an important determinant of the number of CD19+ cells. Furthermore, to evaluate whether cell proliferation contributes to CD19+ cell number, we calculated the ΔCt for the KRECs SJ and for genomic CJ, which represent an estimation of the average number of cell divisions in the B cell population. In several samples from both groups, the average number of B cell divisions fell within the 5th–95th percentile \((2.9–4.1; \text{gray box of Fig. 2C})\) calculated in the controls. However, there was no statistical difference of average number of divisions over time between the two groups of patients, and there was no relationship between the level of CD19+ lymphocytes and the average number of B cell divisions (Fig. 3B).

With the exceptions of a single time point for UPN1 and UPN9, IgG levels were also within the normal range post discontinuation of IVIG therapy, though at the time of the current study, IVIG therapy was still ongoing in UPN4 (who received HSCT) and UPN6, UPN8, and UPN9 (who were being treated with PEG-ADA) (Fig. 4A, Table I). Abs against tetanus toxoid and hepatitis B Ags in PEG-ADA–treated patients and against measles Ags in transplanted patients were detectable post immunization in all patients who discontinued IVIG therapy. The only exception to this was patient UPN8, who, for this reason, is still undergoing IVIG therapy. IgA and IgM values were more heterogeneous in the two groups, with IgA being constantly low in two patients receiving transplants and in one undergoing PEG-ADA therapy (Fig. 4B).

**FIGURE 4.** Duration of IVIG treatment, response to vaccination, and values of IgG, IgA, and IgM in HSCT- and PEG-ADA–treated patients at different follow-up time points. The duration of IVIG treatment and the response to the vaccinations are indicated at the top of the upper panels (A). The levels of IgG (A), IgA (B), and IgM (C) are plotted according to the age of the patients. The cutoffs (<500 mg/dl for IgG, <35 mg/dl for IgA, and <50 mg/dl for IgM), depicted as dotted lines, were established according to Mazzolari et al. (21). Horizontal arrows at the top of each graph indicate the time interval during which UPN1, UPN2, UPN3, and UPN5 underwent HSCT and the period when PEG-ADA therapy started in the other patients. *Indicates the time when HSCT was performed in UPN4. nd, not done.
and IgM persistently below the cutoff in four out of five HSCT-treated children and three out of eight patients receiving PEG-ADA therapy (Fig. 4C).

Release of new T cells by ADA-SCID patients under therapy

Thymic output was measured via TREC quantification. Again, patient data were compared with those of the two groups of age-matched controls (i.e., children from 2 mo to 3 y of age [median: 132.154 TREC/ml, 5th–95th percentile: 29,875–431,637 TREC/ml] and children from 3–12.5 y [median: 36,216 TREC/ml, 5th–95th percentile: 11,156–142,143 TREC/ml; p < 0.001]). The fifth percentiles of the two age-matched control groups were used as the cutoff values, and their corresponding areas are indicated as a dark and light gray box in Fig. 5.

Independent of the therapy type, most samples of ADA-SCID patients contained TREC+ cells that were constantly under the cutoff (Fig. 5A), with some exceptions in several samples from patients UPN1, UPN4, and UPN5. UPN4 showed a sharp increase in TREC concentration from 147/ml to 69,939/ml in samples taken from 1–20 mo posttransplantation, which was performed at 20 mo of age. UPN5 had TREC over the cutoff only in the last sample, and UPN1 had rather stable TREC values oscillating around the proposed cutoff. This patient, who was the only one with a mixed engraftment, also showed a higher number of TREC+ cells (93%) at the last examination than UPN2, UPN3, and UPN5, who developed total T cell engraftment (Fig. 5A). In children undergoing PEG-ADA therapy, the number of TRECs displayed a tendency to decrease to very low levels over time, with the levels being variable in the different samples. This phenomenon is particularly evident in UPN10, in whom TRECs over the cutoff in the first available sample fell to almost undetectable levels in the following samples. These opposite trends that were observed in the two treatment groups were verified by statistical analysis, which confirmed the stability of TRECs over time in HSCT-treated children (elapsed time coefficient for logTREC: 0.001 [CI: −0.007 to 0.009]; p = NS) and their significant decrease in patients undergoing PEG-ADA therapy (−0.011 [−0.017 to −0.004]; p < 0.001).

Analogous to what was observed for TRECs, the number of CD4+CD45RA+ (Fig. 5B) and CD3+ (Fig. 5C) lymphocytes was stable in transplanted patients. Again, the only clear exception was UPN4, in whom CD4+CD45RA+ lymphocytes progressively increased from the first to the last sample analyzed. Furthermore, most samples of UPN1 and UPN5 contained CD4+CD45RA+ and CD3+ cells above the cutoff levels (fifth percentile for CD4+CD45RA+ lymphocytes: 530/µl in children from 2 mo to 3 y and 102/µl in children from 3–12.5 y; fifth percentile for CD3+ lymphocytes: 1598/µl in children from 2 mo to 3 y and 750/µl in children from 3–12.5 y). On the contrary, in most of the samples of patients undergoing PEG-ADA therapy, CD4+CD45RA+ and CD3+ cells were below the cutoff levels, with CD4+CD45RA+ lymphocytes that clearly decreased over time in six patients and CD3+ cell counts that were more stable. Indeed, the linear mixed model analysis of the data demonstrated that CD4+CD45RA+ lymphocytes decreased significantly over time in six patients and CD3+ cell counts that were more stable. The persistent behavior of T cells was followed by progressive enlargement of the TCR repertoire (28), with TCR scores decreasing from 2 (clonal pattern) or 1 (oligoclonal pattern) to 0 (polyclonal pattern) in subsequent samples. In UPN2, the progressive enlargement of the TCR repertoire (from TCR score 2

**FIGURE 5.** Values of TRECs, CD4+CD45RA+, and CD3+ cells in HSCT- and PEG-ADA–treated patients at different follow-up time points. Values of TRECs (A), naïve CD4+CD45RA+ (B), and CD3+ (C) cells are plotted according to the age of the patients. Dark and light gray boxed areas include data under the fifth percentile of values observed in two groups of age-matched healthy controls (2 mo to 3 y old and 3–12.5 y old) with statistically different numbers of TRECs, CD4+CD45RA+, and CD3+ lymphocytes. Horizontal arrows at the top of each graph indicate the time interval during which UPN1, UPN2, UPN3, and UPN5 underwent HSCT and the period when PEG-ADA therapy started in the other patients. * indicates the time when HSCT was performed in UPN4. D, complete donor T cell engraftment; M, mixed T cell engraftment.
to 0) was confirmed in three samples by spectratyping analysis, an alternative way to measure TCR repertoire diversity that detects the polymorphisms in the CDR3 region lengths; this technique preferentially found clonal peaks in the samples taken closer to HSCT (upper TCRBV profiles of Supplemental Fig. 1A). In the transplanted UPN4, the increased number of TRECcs observed in the last two samples analyzed (Fig. 5A) was not yet enough to induce a detectable diversification of the TCR repertoire, because all five samples analyzed showed TCR score 2 by heteroduplex analysis (Fig. 6A), and two samples (one obtained 16 mo and the other 20 mo posttransplantation) both showed mono/oligoclonal peaks in the spectratyping analysis (Supplemental Fig. 1B). A different picture emerged from the analysis of the TCR repertoire of PEG-ADA–treated patients. In UPN8 and UPN11, there was a shift from a polyclonal (TCR score 0) or oligoclonal (TCR score 1) TCR repertoire to a restricted one (TCR score 2), whereas constantly restricted heterogeneity of TCRBV chains (TCR score 2) was found in all samples of UPN6, UPN7, UPN9, and UPN13. In UPN12, the restricted repertoire detected in the first sample was substituted by polyclonal cells in subsequent samples. Notably, an increase in TRECcs was also observed in the second sample obtained from this patient (Fig. 5A), and this release of new T cells may have sustained the enlargement of the TCR repertoire observed in the following samples. This interpretation may also explain the highly diverse TCR repertoire detected in both the heteroduplex and spectratyping analyses (Fig. 6A, Supplemental Fig. 1C) of TCRBV segments of UPN10, showing a high number of TRECcs in the first available sample in the absence of peripheral CD3⁺ cell expansion at the same and subsequent time points.

Although the patient response to PHA stimulation was variable in the two groups and even within the same patient (Fig. 6B), a reduced capability of lymphocytes to respond to mitogenic stimulation was observed in most of the ADA-SCID patients who underwent both treatments. This indicated that some functional deficit was still present even in transplanted patients with full donor engraftment and with a number of CD3⁺ and CD19⁺ lymphocytes that rose above the cutoff (UPN4 and UPN5).

**Discussion**

It is known that the immune recovery of patients with ADA-SCID receiving HSCT or PEG-ADA therapy is highly variable, depending on underlying clinical conditions, the age at which treatment is started, treatment duration, the level of residual thymic activity at the time of therapy initiation, and (in the case of HSCT) the donor type (10). Until now, the extent of B and T cell compartment recovery following HSCT or PEG-ADA treatment has never been compared, though this information would be of particular relevance because ADA metabolites are known to affect B and T cell development and function differently (29). Indeed, although the T cells of ADA-SCID patients undergo maturation in the thymus early in ontogenesis, B cells are spared deoxyadenosine triphosphate toxicity by the scavenger activity of macrophages in the bone marrow, and thus, their maturative impairment occurs at later times in the spleen (29). Therefore, we undertook a comparative retrospective longitudinal analysis of B and T cell compartment recovery in two groups of differentially treated ADA-SCID patients, because HSCT and PEG-ADA therapy are both provided in our hospital.

The data regarding B and T cell counts indicated that immune reconstitution of the peripheral lymphocytes was only partial after both treatments. However, the recovery of CD19⁺ cells was different between the two groups, with a significant time-related increase in this lymphocyte population in transplanted patients and a decrease in patients undergoing PEG-ADA therapy. In particular, we found that in patients receiving enzyme replacement therapy, the recovery of the B-lymphocyte number, although occurring fairly early [as previously observed by Chan et al. (30)], faltered after several years of treatment. Similarly, CD3⁺ lymphocytes significantly increased (but to a lesser extent) in HSCT-treated patients, likely because the number of this cell subtype declines physiologically with age (31). On the contrary, most of the CD3⁺ cell counts from patients receiving enzyme replacement therapy were consistently under the cutoff level.
Thus, we investigated the B and T cell reconstitution kinetics in these patients by measuring KRECs and TRECs, which are considered a reasonably good estimation of the amount of newly produced B and T lymphocytes (18, 19). Indeed, KRECs and TRECs, which are stable DNA episomes generated during BCR and TCR rearrangements (32), are respectively present in at least 50% of B cells released from the bone marrow (33) and 70% of T cells emerging from the thymus (34), and do not duplicate during subsequent cell divisions (thus being diluted in daughter cells). We found that the number of KRECs in samples obtained from four of the five transplanted children long after HSCT treatment reached the values observed in age-matched controls. However, there was no relationship between the extent of new B cell release and the quantity of donor engrafted B cells, because two of these patients showed a mixed chimerism and two a complete engraftment. Conversely, in about half of the PEG-ADA–treated patients, newly produced B cells decreased to levels under the cutoff in samples taken long after therapy initiation. Therefore, if KREC production results in a CD19+ cell increase, and a scape from the bone marrow, were similar and not correlated with the CD19+ cell number. However, in the PEG-ADA–treated UPN9, when the KREC number fell to nearly undetectable levels, B cell divisions reached their peak. An opposite situation was observed in UPN8, because as KRECs progressively increased, the average number of B cell divisions gradually decreased. These features were found in all patients undergoing PEG-ADA therapy (and in the UPN4 transplant patient), with the exception of UPN11, in whom both KRECs and B cell divisions were stable. These data suggest that B cell proliferation tends to maintain the peripheral homeostasis.

We found low IgA and IgM serum levels in our patients, but this is a common feature both post HSCT and PEG-ADA treatment (10). Indeed, IgA recovery, in particular, has been reported to take several years to achieve (35). It is of note, however, that despite the presence of different numbers of circulating KRECs+ and CD19+ cells, most of ADA-SCID children (independent of the treatment) had a normal ability to produce IgG, even after discontinuing IVIG treatment. This suggests that the capability of B cells to produce Ig is conserved in these patients. However, the maintenance of immune surveillance requires the continuous supply of newly produced lymphocytes, and therefore, the evaluation of KRECs offers a better marker for monitoring the efficacy of B cell compartment regeneration. Thus, our data, indicating that KRECs tended to reach a level comparable to that of age-matched controls only posttransplantation, suggest that humoral immune activity could be long lasting in these patients.

The results of the T cell immune reconstitution analysis indicate that thymic output was compromised in both groups of ADA-SCID patients, because few samples showed a number of newly produced T lymphocytes comparable to that of age-matched controls. In patients treated for a longer duration with PEG-ADA, thymic output decreased progressively to levels that cannot only be ascribed to the physiological thymic activity impairment observed with decreasing age (36, 37). On the contrary, in patients receiving HSCT, the production of TRECs was stable over time (though lower than in controls) and was not different in patients who received the transplant from siblings or matched unrelated donors. Therefore, different from that previously reported in mice and humans (38, 39), the extent of immune reconstitution in our patients was not correlated with the age of the donor. In addition, there was not an apparent relationship between the extent of new T cell release and the quantity of donor-engrafted T cells, because the only patient with a mixed engraftment showed a higher number of TREC+ cells than that found in the other patients with total T cell engraftment.

The diminished thymic output was accompanied by a narrowing of the TCR repertoire in most of our patients treated with PEG-ADA, whereas in HSCT-treated patients, the number of TRECs (even if low) appeared to be sufficient to sustain diversification of the TCR repertoire. An exception was patient UPN4, whose TCR repertoire was restricted, despite the posttransplant phenomenon of thymic rebound that was ongoing. In this patient, the normal number of circulating CD3+ cells in the presence of low levels of TREC+ and CD4+CD45RA+ T cells soon posttransplantation suggests that repertoire narrowing may be due to homeostatic proliferation of T lymphocytes.

As reported (11, 40), independent of the type of therapy, we observed that mitogen responses often fluctuated over time (even within the same patient), with normal responses evident in few patients. Finally, it should be noted that although all children were clinically well and free of opportunistic or severe viral infections, one PEG-ADA–treated patient had chronic bronchopneumopathy and ultimately died of measles complications, and another had autoimmune hemolytic anemia and seizures.

In summary, our data suggest that during prolonged PEG-ADA therapy, thymic function is severely impaired, and bone marrow output is compromised. In contrast, the release of new B lymphocytes is restored post HSCT, and though thymic function is not completely recovered in these patients, the number of released TRECs is enough to diversify their T cell repertoires.

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Disclosures
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References
KRECs and TRECs in ADA-SCID–treated patients


27. KRECs AND TRECs IN ADA-SCID–TREATED PATIENTS


Supplementary FIGURE 1. TCRBV repertoire analysis in selected patients.

The TCRBV repertoire analysis was performed by spectratyping samples obtained from UPN2 at 1, 24 and 58 months after HSCT (A), from UPN4 at 16 and 20 months after HSCT (B), and from UPN10 at 132 and 141 months after PEG-ADA therapy initiation (C).