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Immunologic reconstitution during PEG-ADA therapy in an unusual mosaic ADA-deficient patient

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Abstract

We report detailed genetic and immunologic studies in a patient diagnosed with adenosine deaminase (ADA) deficiency and combined immune deficiency at age 5 years. At the time of diagnosis, although all other lymphocyte subsets were depleted, circulating CD8⁺ T cells with a terminally differentiated phenotype were abundant and expressed normal ADA activity due to a reversion mutation in a CD8⁺ T cell or precursor. Over the first 9 months of replacement therapy with PEG-ADA, the patient steadily accumulated mature naïve CD4⁺ and CD8⁺ T cells, as well as CD4⁺/FOXP3⁺ regulatory T cells, consistent with restoration of a functional cellular immune system. While CD19⁺ naïve B cells also accumulated in response to PEG-ADA therapy, a high proportion of these B cells exhibited an immature surface marker phenotype even after 9 months, and immunization with neoantigen bacteriophage φX174 demonstrated a markedly subnormal humoral immune response. Our observations in this single patient have important implications for gene therapy of human ADA deficiency, as they indicate that ADA expression within even a large circulating lymphocyte population may not be sufficient to support adequate immune reconstitution. They also suggest that an immature surface marker phenotype of the peripheral B cell compartment may be a useful surrogate marker for incomplete humoral immune reconstitution during enzyme replacement, and possibly other forms of hematopoietic cell therapies.

Introduction

Between 30–40% of patients with autosomally inherited severe combined immunodeficiency (SCID) lack adenosine deaminase (ADA), an enzyme of purine nucleoside metabolism expressed at highest levels in immature lymphoid cells. The marked depletion of thymocytes and circulating T, B, and NK cells in affected patients is thought to result primarily from an

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intracellular accumulation of deoxyadenosine triphosphate (dATP) to levels that inhibit DNA replication and induce apoptosis; abnormal signal transduction mediated by adenosine may also contribute to impaired lymphocyte function (reviewed in [1;2;3]).

About a fifth of ADA deficient patients have a more insidious course before being diagnosed with combined immune deficiency. This milder “delayed onset” phenotype is usually associated with inheritance of at least one *ADA* missense mutation that greatly diminishes but does not completely ablate enzyme function[4;5;6]. In a few cases, reversion of one inherited *ADA* mutation has occurred in lymphocytes, but not in other somatic cells[7;8;9;10]. By “reactivating” one *ADA* allele, this reversion protects lymphocytes from metabolic toxicity and imparts a strong selective survival advantage. Mosaicism for reversion has been considered to be a naturally occurring model for what can potentially be achieved with gene therapy for *ADA* deficiency. However, immune function in mosaic patients has been variable, and their response to enzyme replacement therapy has not been evaluated.

Here we report a patient who presented at age five years with chronic lung disease and evidence suggesting combined immunodeficiency, but with a normal total peripheral blood lymphocyte count. His red cells had undetectable *ADA* activity, but his circulating lymphocytes, remarkably, were nearly all CD8⁺ and had a normal level of *ADA* activity. Due to his age, chronic lung disease, and lack of a human leukocyte antigen (HLA)-matched related or unrelated donor, he was started on enzyme replacement therapy with PEG-*ADA*. We have investigated the basis for his *ADA* expressing CD8⁺ T cells, and have monitored lymphocyte phenotype and response to immunization with bacteriophage ϕ X174 to assess immunologic reconstitution during the first 9 months of enzyme replacement therapy.

Materials and Methods

Subjects

The Immunodeficiency Clinic at Seattle Children's Hospital and Regional Medical Center (CHRM) is a referral center for patients with primary immunodeficiency disorders. The five-year-old male patient with *ADA* deficiency SCID was followed in this clinic from March 2006 until the present. The studies described were performed with the patient's parent's informed consent according to an IRB approved protocol.

Biochemical studies

ADA activity in erythrocytes, lymphocytes, and plasma, and the levels of total adenosine and deoxyadenosine nucleotides (AXP and dAXP, respectively) in erythrocytes, were measured as described previously[11;12]. Amplification of *ADA* exons from genomic DNA was performed using primers and conditions previously reported[4;6]. DNA sequence was determined using an ABI 3730 instrument with BigDyeTMv1.1 terminator sequencing chemistry.

Cell preparation

Heparinized peripheral blood samples from the patient and normal controls were processed for peripheral blood mononuclear cell (PBMC) isolation by Ficoll-Hypaque density gradient centrifugation. The purified PBMCs were frozen for later analysis or freshly used for multiparameter flow cytometric assay as described below, depending on the timing of sample acquisition.

TCRv β spectratyping

TCRv β spectratyping was performed at the Fred Hutchinson Cancer Research Center Immune Monitoring Lab, a core facility supported by the Seattle Cancer Care Alliance, using standard methods.

Flow Cytometry

Antibodies and staining—Staining for individual CD4, CD8, and CD19 surface markers was performed on whole blood by the CHRMC Clinical Immunology Laboratory. Staining for multiparameter surface marker analyses was performed on freshly prepared or previously frozen PBMCs. Briefly, PBMCs were stained for 20 min on ice in the dark with the appropriate antibody combination of Alexa Fluor700, APC, cychrome, fluorescein, phycoerythrin, APC-cy7, or biotin-labeled antibodies followed by streptavidin-PE-cy7 (eBiosciences), or streptavidin-PE-cy5 (BD Biosciences). The monoclonal antibodies for the following human cell surface markers: CD19 (Alexa700, H1B19), IgM (PE-cy5, G20-127), IgD (biotin-labeled, IA6-2), BAFFR (PE, 11C1), CD21 (PE-Cy5, B-ly4), CD38 (Percp-cy55, HIT2), CD4 (Alexa700, RPA-T4), CD45RA (FITC, L48), CCR7 (PE-cy7, 3D12), CD31 (biotin-labeled, MBC 78.2), CD8 (Alexa700, RPA-T8), CD28 (PE, CD28.2) and CD45RO (PE-cy5, UCHL1) were obtained from BD Biosciences; CD27 (APC, 0323) and CD62L (APC-cy7, Dreg56) from eBiosciences; IgG (PE) and IgA (FITC) from Southern Biotechnology Inc. For FoxP3 (Biolegend) intracellular staining, the standard protocol from the manufacturer was used following the surface marker staining.

Data acquisition, analysis, and presentation—Multiparameter data acquisition was performed with a FACS-LSRII (Becton Dickinson, Mountain View, CA). FloJo software was used for all aspects of the data analysis. Forward versus side scatter plotting was used to gate viable lymphocytes, and then individual subsets for each major lineage were defined based on the individual gating strategies described below and presented in part in Figures 3-5. In all cases, an attempt was made to acquire at least 10,000 events of the primary surface marker (e.g. CD8, CD4, CD19). If 10000 cells of the appropriate surface marker phenotype were not available, as many as possible cells of that phenotype were acquired. Relative cell numbers were converted to absolute numbers based on the single parameter lymphocyte counts. For presentation, 10,000 CD4⁺, 10,000 CD8⁺, and 7,000 CD19⁺ cells of the respective primary surface marker were plotted for the sample with the greatest number of that cell type per unit volume of blood. For samples from other dates, the number of cells in the same volume of blood (as the sample with greatest number of cells per unit volume) was plotted, thus allowing plotting density to be used for visual assessment of accumulation of peripheral blood cells bearing a given surface marker over time. For CD4⁺ cells, the approximate plotted blood volume was 25 mm³, for CD8⁺ cells, 5 mm³, and for CD19⁺ cells, 140⁺ mm³. For controls for CD4⁺ and CD19⁺ plots, the cells from 50% of these blood volumes (i.e. 13.5 and 70 mm³ respectively) are plotted to illustrate the approximate lower limit of normal; for CD8⁺ cells, the cells from 10 mm³ are plotted to illustrate the approximate upper limit of normal.

Gating strategy

CD8⁺ T cell subset analysis: Donor PBL were stained with antibodies (Abs) to CD8, CD27, CD28, CD45RO and CCR7. CD8⁺ T cells gated from PBL were separated into four quadrants based on CD27 and CD28 staining. From the CD27⁺CD28⁺ quadrant, CD45RO/CCR7 were used to define naive phenotype cells as CD45RO⁻CCR7⁺, and early differentiated cells as CD45RO⁺CCR7⁻. From the CD27⁻CD28⁻ compartment, late differentiated cells were defined as CD45RO⁻CCR7⁻. CD27⁻CD28⁻/CCR7⁻/CD45RO⁺ cells are thought to represent a terminally differentiated form of CD8⁺ T cell.

CD4⁺ T cell subset analysis: Donor PBLs were stained with Abs to CD4, CD27, CD45RA, CD62L, CCR7 and FoxP3 (E). CD4⁺ T cells gated from PBL were separated into four quadrants based on staining of CD27 and CD45RA antibodies as shown. From the CD45RA⁺CD27⁺ quadrant, naïve cells were defined as CD62L⁺CCR7⁺. From the CD45RA⁺CD27⁻ compartment, late differentiated cells were defined as CD62L⁻CCR7⁻. Regulatory T cells were defined as FoxP3⁺ cells from the total CD4⁺ gate.

CD19⁺ B cell subset analysis: Donor PBL were stained with two sets of antibodies:

1. CD19, CD27, IgM, IgD, IgA and IgG. CD19⁺ B cells (10.4%) gated from PBL were separated into CD27⁺ memory B cells (30.8%) and CD27⁻ naïve B cells. IgM and IgD staining in the CD19⁺/CD27⁺ gate were further subdivided as IgM⁺IgD⁺ unswitched memory B cells and IgM⁺IgD⁻ switched memory B cells. The latter subset was also further analyzed by IgG⁺ and IgA⁺ staining to confirm their identification as class switched B cells (data not shown).
2. CD19, CD27, IgM, IgD, CD38 and BAFFR. CD19 and CD27 staining were used to define a CD19⁺/CD27⁻ naïve B cell subset. IgD and CD38 staining was then used to divide naïve B cells into four subsets: naïve mature B cells were defined as IgD⁺/CD38 low cells, with IgD⁺ cells bearing progressively higher levels of CD38 staining classified as intermediate and transitional B cells. Baff-R staining in the CD19⁺ gate is used separately as an index of the overall maturity of the CD19⁺ population. While CD21 was included in the multi-colour staining protocol used, it was omitted from the gating and analysis strategy to reduce the complexity of data presentation.

Immunization with Bacteriophage ϕ X174

To measure antibody responses to a T-dependent neoantigen, bacteriophage ϕ X174 was administered once before and twice after treatment with PEG-ADA was initiated. Serial blood samples were collected at regular intervals and phage neutralizing antibody activity, expressed as the rate of phage inactivation (K-value, [KV]), determined. Phage-neutralizing antibody resistant to 2-mercaptoethanol is considered to be IgG [13].

Results

Case History

The patient had a history of productive cough and frequent lower tract respiratory infections requiring treatment with antibiotics dating to 3 months of age. In March of 2006, at 5.5 years of age, he was referred to the Seattle Children's Hospital and Regional Medical Center for pulmonary and immunologic evaluation. His presentation was notable for normal growth (c.a. 50% height and weight for age) and development, a lack of similar symptoms in family members, up to date immunizations (including MMR and varicella vaccines), and lack of significant gastrointestinal problems. Diffuse ground glass opacities interpreted as evidence of severe chronic lung disease were evident on high resolution pulmonary CT scan. In addition, sinus CT scans demonstrated evidence of chronic sinus inflammation with mucosal thickening. No abnormalities of bone, liver, or other organ systems were noted on routine chest X-rays, high resolution thoracic CT imaging, or laboratory analysis.

Initial evaluation of the patient's peripheral blood cells and lymphocytes revealed significant thrombocytopenia with a platelet count of 25,000/mm³, an absolute neutrophil count of 774/mm³, a hematocrit of 30%, and a total peripheral blood lymphocyte count of 3382/mm³. Single parameter FACS analysis showed that nearly all peripheral blood lymphocytes were CD8⁺ (absolute count = 3254/mm³), with an absolute CD4 count of only 123/mm³; CD19⁺ B cells and CD16/56⁺ NK cells were also very low in absolute numbers and proportion (<1%).

Lymphocyte proliferation responses at the time of diagnosis were also very diminished relative to controls: PHA-induced ^3H -thymidine incorporation was 13 fold reduced and anti-CD3 induced incorporation was 30 fold reduced; there was no detectable proliferative response to tetanus or candida antigens. Evaluation of humoral immune function revealed a serum IgG of 1500 mg/dl, normal IgA and IgM levels, and elevated IgE of 303 mg/dl. A random titer of anti-diphtheria toxin antibody was 0.03 IU/ml (≥ 0.01 IU/ml being protective), but the patient did not mount an adequate response to any pneumococcal polysaccharide serotype after 23 valent pneumococcal polysaccharide vaccination (0/12 serotypes tested showed an increase in titer). Bone marrow evaluation demonstrated normal cellularity with mild erythroid hyperplasia and mild megakaryocytic hypoplasia. Cytogenetics demonstrated a normal male karyotype with no demonstrable abnormalities; blood lymphocytes were 100% XY male, ruling out maternal chimerism.

Based on the patient's clinical history, unusual T lymphocyte subset distribution, and lack of B cells and NK-cells, a primary combined immunodeficiency disorder was suspected and an etiology was pursued, initially by analysis of purine enzyme levels. His red cells had normal purine nucleotide phosphorylase activity, but absent ADA activity. After the diagnosis of ADA deficiency was established, the patient was started on IVIG, trimethoprim/sulfamethoxazole, and cefuroxime. He also received subcutaneous G-CSF weekly for 3 months until his neutrophil counts normalized. Replacement therapy with intramuscular injections of PEG-ADA was initiated on 5/12/06 at an initial weekly dose of 375 Units, or 20 U/kg. With this treatment regimen, the patient's immunological parameters improved, allowing discontinuation of chronic antibiotic treatment. However, due to continued evidence of poor humoral immune reconstitution, IVIG therapy has been continued to the present time. Overall, the combination of PEG-ADA and IVIG therapies resulted in a markedly decreased frequency of pulmonary and upper respiratory infections, improved appetite and weight gain, and the patient has been able to attend school and resume all normal childhood activities.

Analysis of ADA enzyme levels, metabolites, and genomic sequence

The clinical presentation of this patient, characterized mainly by chronic and recurrent respiratory infections, was similar to that of other ADA deficient patients with a “delayed onset” phenotype. However, the presence of a significant number of peripheral blood lymphocytes was exceptional, as was the finding that they were virtually all CD8⁺ T cells. This prompted a detailed evaluation of ADA and metabolite levels (Table 1) and ADA genotype (Figure 1).

In contrast to his erythrocytes, extracts of the patient's peripheral blood lymphocytes (PBL) obtained on two occasions had ADA activity well within the normal range (Table 1). Prior to assay, these PBLs were extensively washed to prevent carry over of PEG-ADA from plasma. PBLs prepared in a similar manner from other ADA deficient patients receiving PEG-ADA have possessed <1% of normal ADA activity[11;12]. By degrading deoxyadenosine, these ADA-expressing T cells would be expected to reduce the level of deoxyadenosine nucleotides (dAXP) in the patient's ADA deficient red blood cells (as occurs with selective engraftment of donor T cells following haploidentical marrow transplantation). In the first sample analyzed, obtained 1 week after his first injection of PEG-ADA, red cell dAXP were 0.060 $\mu\text{mol/mL}$ packed cells, or 2 percent of total adenine nucleotides (Table 1). This was significantly lower than we have typically observed in ADA deficient SCID patients after only one week of PEG-ADA therapy. With continued enzyme replacement, red cell dAXP determinations have ranged from undetectable to 0.01 $\mu\text{mol/mL}$ (Table 1).

Sequencing of amplified genomic DNA from the patient's buccal epithelial cells demonstrated compound heterozygosity for two mutant ADA alleles. Thus, both G (normal) and A (mutant) were present at position 2 of codon 156 (Fig 1A), indicating heterozygosity for the R156H

amino acid substitution in exon 5. This mutation has previously been found in several other ADA deficient patients with a “delayed onset” phenotype, and when expressed in *E. coli* provides about 1% of the ADA activity obtained with wild type human ADA cDNA[4;6]. The patient's second mutation is a deletion of G at position 3 of codon 235 (c.800delG) in exon 8 (resulting in the stretch of double sequence in Fig 1B). This deletion, which has not previously been reported, shifts the reading frame, causing mistranslation of codons 235-309, with premature chain termination at codon 310 instead of codon 364. Sequencing of buccal epithelial DNA from the patient's mother indicated that she carried the R156H mutation, but not the deletion in exon 8 (Figure 1 C, D). DNA from the father, who presumably contributed the exon 8 deletion allele, was unavailable.

Neither of the mutant alleles identified in buccal cell DNA could account for the high level of ADA activity in the patient's CD8⁺ T cells. Genomic DNA was therefore prepared from the patient's nucleated blood cells on several occasions in order to sequence exons 5 and 8. Unfractionated IL-2 stimulated T cells cultured from PBL obtained 6 weeks after the start of PEG-ADA treatment expressed normal ADA activity (Table 1) and showed a significantly higher peak for the normal G compared with mutant A at codon 156 (Fig 1E). These cells showed the expected heterozygosity for the deletion in exon 8 (tracing not shown). The most informative DNA sequence data were obtained from purified preparations of CD4⁺ T cells and terminally differentiated CD8⁺ T-cells isolated by FACS sorting 8.5 months after the start of PEG-ADA therapy (by which time the number of circulating CD4⁺ T cells had increased substantially, as discussed below). While DNA from the CD4⁺ T cells showed roughly equal peaks for the normal G and mutant A nucleotides in codon 156 (Fig 1F), as was found with DNA from buccal epithelial cells, DNA from the terminally differentiated CD8⁺ T cells possessed only the normal G residue (Fig 1G). The terminally differentiated CD8⁺ T-cells showed the expected heterozygosity for the deletion in exon 8 (Fig 1H), as was also found in the CD4⁺ T cells (tracing not shown). Collectively, these data and the assays of cellular ADA activity are most easily explained by invoking a spontaneous reversion of the maternally-inherited R156H point mutation to wild type in a CD8⁺ T-cell or precursor which contributed predominantly or solely to the production of the terminally differentiated CD8⁺ lineage T-cell population present at the time of diagnosis.

Lymphocyte recovery after initiation of PEG-ADA therapy

Flow cytometric analysis of the major lymphocyte lineages (CD4⁺ T cells, CD8⁺ T cells, CD19⁺ B cells, and CD16⁺ NK cells) was performed prior to, and over 9 months of PEG-ADA therapy (Figure 2A, data not shown for NK cells). On presentation a large number of CD8⁺ peripheral lymphocytes represented nearly the entire peripheral lymphocyte compartment, with very small numbers of CD4⁺ T cells, CD19⁺ B cells and CD16⁺ NK cells. With onset of PEG-ADA therapy sufficient to maintain erythrocyte dAXP at normal levels (see Table 1), CD4⁺ T cells accumulated steadily by more than 10-fold, to reach a normal range by 9 months of therapy. In contrast, CD8⁺ T cells began at an above normal level, and more than doubled before reaching a plateau after 2 months of PEG-ADA therapy, while CD19⁺ B cells accumulated approximately 20-fold until they reached a plateau after 6 months of PEG-ADA therapy. Thus, initiation of PEG-ADA therapy permitted the accumulation of the major lineages of peripheral T cells and B cells over a time course similar to that previously described [2; 14; 15; 16; 17]. The patient's NK cell numbers also recovered from an undetectable level prior to PEG-ADA therapy to within the normal range after 3 months of PEG-ADA treatment, where they remained for the duration of the 9 month study period (data not shown).

TCR $\gamma\beta$ spectratyping studies were performed on the patient's bulk peripheral blood lymphocytes at time points 3 and 8 months after initiation of PEG-ADA therapy. These analyses demonstrated a substantial restriction of the patient's TCR $\gamma\beta$ repertoire at the 3 month

time point (Figure 2B, left panels), consistent with a dominant oligoclonal CD8⁺ population at that time. Moreover, the TCR $\nu\beta$ repertoire demonstrated a significant expansion upon reassessment at the 8 month time point (Figure 2B, right panels), suggesting that the observed increased T cell numbers correlated with accumulation of significant populations of naïve T cells in response to PEG-ADA treatment.

Multi-parameter flow cytometry analysis of CD4⁺, CD8⁺, and CD19⁺ lineage subsets were performed at the same points as single parameter analyses. In the data presented, the total number of patient cells plotted corresponds to identical sample volumes - thus changes in the overall dot density in the plots correspond to the accumulation of cells over time.

CD4⁺ T cell subsets (Figure 3)—Current models of human T cell differentiation suggest that the peripheral blood CD4⁺ T cell pool includes a continuum of differentiated cells, with mature naïve CD4⁺ T cells generally found to be CD27⁺/CD45RA⁺, and more differentiated cells CD27⁻/CD45RA⁻. Based on these characteristics, red gate boxes in Figure 3 denote putative naïve and late differentiated cells. In the normal control (row 1), “naïve” phenotype CD4⁺ T cells represent approximately 40-50% of total circulating CD4⁺ T cell pool, with smaller pools of early and later differentiated cells. Regulatory T cells, defined here as the FOXP3⁺, represent 3-5% of CD4⁺ T cells.

Figure 3, rows 2-6 show the multi-parameter analyses of CD4⁺ T cell subsets in the patient. Prior to initiation of PEG-ADA therapy (figure 3, row 2), there were very few CD4⁺ T cells, the vast majority of which exhibited a late differentiation surface marker phenotype. In addition, 12% of the CD4⁺ T cells present initially were FOXP3⁺, suggestive of an acutely activated or regulatory T cell phenotype[18;19;20]. With PEG-ADA therapy, the total number of CD4⁺ cells rapidly increased into the low normal range, including a large population of naïve phenotype CD4⁺ T cells, while regulatory T cells gradually declined in proportion into the 3-4% range, similar to the normal adult control. These data suggest a quantitatively and qualitatively normal reconstitution of the peripheral CD4 T cell compartment over 9 months of PEG-ADA therapy, consistent with the observed expansion of the TCR $\nu\beta$ repertoire between the 3 months and 8 months time points.

CD8⁺ T cell subsets (Figure 4)—Human CD8⁺ T cell differentiation likely also occurs along a continuum, with mature naïve cells generally found to be CD27⁺/CD28⁺, and more differentiated cells CD27⁻/CD28⁻: thus, red gate boxes in figure 4 denote putative naïve, early differentiated, and late differentiated, cells respectively. The normal control (row 1) shows a large population of naïve CD8⁺ T cells, which represent approximately 40-50% of the total circulating CD8⁺ T cell pool, with smaller pools of early and later differentiated cells accounting for the remainder.

Figure 4, rows 2-6 shows the CD8⁺ T cell subsets present in our patient. Prior to initiation of PEG-ADA therapy (Figure 4, row 2), he exhibited an extremely large population of CD8⁺ T cells with a CD27⁻/CD28⁻/CCR7⁻ phenotype, which would be considered as highly or terminally differentiated, and essentially no CD27⁺/CD28⁺/CCR7⁺/CD45R0⁻ naïve CD8⁺ T cells. During PEG-ADA therapy, the patient accumulated CD8 cells with a naïve surface marker phenotype to a level similar to that found in the adult normal control. Similar to what was observed for the CD4 compartment, these data indicate a quantitatively and qualitatively normal reconstitution of the CD8 T cell compartment over 9 months of PEG-ADA therapy.

Over the period of PEG-ADA treatment, the proportion of CD27⁺/CD28⁻/CCR7⁻/CD45R0⁻ late differentiated CD8⁺ T cells decreased, although a large population of terminally differentiated CD27⁺/CD28⁻/CCR7⁻/CD45R0⁺ cells persisted. The existence of the CD27⁻/CD28⁻/CCR7⁻/CD45R0⁺ population at the time of diagnosis and its persistence through PEG-ADA therapy

suggested that these cells might bear a revertant ADA allele. As noted above, sequencing of DNA from CD27⁺/CD28⁻/CCR7⁻/CD45RO⁺ CD8 T cells sorted from a blood sample obtained after 9 months of PEG-ADA therapy confirmed that this was the case (Fig 1G).

CD19⁺ B cell subsets (Figure 5)—The human peripheral CD19⁺ B cell pool can be roughly divided into memory (CD27⁺) and naïve (CD27⁻) compartments. CD19⁺/CD27⁺ memory B cells can be further subdivided through surface immunoglobulin expression into unswitched (IgM⁺/IgD⁺) and class switched (IgM⁻/IgD⁺) cells. CD19⁺/CD27⁻ naïve B cells can be classified by maturation according to a number of schemata: we chose to utilize IgD and CD38 staining, classifying naïve mature B cells as IgD⁺/CD38⁻, and less differentiated B cells as IgD⁺ with higher levels of CD38 expression. For the normal individual used as a control in Figure 5, the CD19⁺ B cell population contains around 25% CD27⁺ memory B cells and 75% putative naïve B cells. The CD27⁺ memory cells are approximately 2/3 unswitched and 1/3 class switched, while the CD27⁻ naïve cells are primarily CD38 negative-to-low mature B cells, with small populations of presumed immature B cells bearing higher levels of CD38 expression. Of note, CD19⁺ cells in normal individuals express a uniform high level of BAFF-R, the major receptor for the peripheral B cell homeostatic regulatory hormone BAFF.

Figure 5, rows 2-5 illustrate the CD19⁺ B cell subsets in our patient. Prior to initiation of PEG-ADA therapy, he had very few CD19⁺ B cells. However, a surprisingly high percentage of these exhibited a CD27⁺ switched memory phenotype. Following onset of PEG-ADA therapy, naïve CD19⁺/CD27⁻ B cells accumulated rapidly, with the majority exhibiting immature (i.e. intermediate or transitional) phenotypes as assessed by IgD/CD38 staining. CD19⁺/CD27⁺ unswitched memory cells also accumulated, consistent with these cells appearing as a consequence of bone marrow B cell development followed by peripheral maturation within a functional spleen or alternative germinal center-dependent pathways[21;22;23]. Notable observations regarding our patient's peripheral B cell reconstitution after 9 months of PEG-ADA were that: 1) he did not accumulate a quantitatively normal CD19⁺ pool; and 2) his peripheral blood B cells exhibited a persistent immature phenotype, including a consistent reduction in expression of BAFF-R, throughout the 9 month period.

Bacteriophage ϕ X174-induced antibody responses before and after PEG-ADA therapy

In PEG-ADA treated SCID patients immune reconstitution is typically achieved in 6-9 months if consistently normal levels of red cell dAXP metabolites are maintained[2;14;15;16;17]. This metabolic state was achieved in our patient, and he accumulated significant numbers of all major lymphoid cell lineages. However, his peripheral blood CD19⁺ B cell compartment did not reconstitute to the quantitative levels or qualitative cellular distribution observed in normal controls. A previous study has reported that expansion of the transitional B cell compartment occurs in several humoral immunodeficiency states[24]. We were therefore concerned that our patient had not achieved sufficient humoral immune reconstitution to allow discontinuation of IVIG therapy. To determine if the B cell surface marker abnormalities in our patient were associated with humoral immune dysfunction, we investigated his response to immunization with bacteriophage ϕ X174, a neoantigen which has been used to evaluate humoral immune responses in patients on IVIG therapy[25;26]. As can be seen in Figure 6 (filled circles), our patient exhibited a minimal response to immunization with ϕ X174 prior to PEG-ADA therapy. After 9 months of PEG-ADA therapy, reimmunization with ϕ X174 (open circles, see also Figure 6 legend) revealed that he had only gained a very small capacity to amplify and isotype switch, with his overall responses to primary and secondary vaccinations remaining markedly depressed.

Discussion

Here, we report on a patient diagnosed at age 5 years with combined immunodeficiency due to ADA deficiency. He was found to be heteroallelic for two *ADA* gene mutations, R156H in exon 5, and a novel deletion, c.800delG, in exon 8. Remarkably, he had abundant circulating CD8⁺ T lymphocytes that expressed normal ADA activity, findings explained by a survival advantage conferred on this T cell lineage by reversion of the R156H mutation. At what stage of lymphocyte development this reversion occurred is not known, but it must have occurred either in a committed progenitor of CD8⁺, but not CD4⁺, T cells, or in some committed lymphoid progenitor that differentiated predominantly into CD8⁺ lymphocytes. It is noteworthy that a reversion of the same R156H mutation was previously discovered in B cells, but not T cells, in another patient with ADA deficiency who also had a relatively mild phenotype[10].

The contribution of this somatic reversion to the patient's phenotype is unclear because the R156H allele is itself associated with a delayed presentation: 9 other patients with this allele whom we have followed were diagnosed at an average age of 5 years (1.1 to 14 years)^[4;6] (and MSH unpublished). The R156H allele encodes a protein with only 1% of wild type ADA enzymatic activity. The large increase in ADA activity resulting from its reversion to wild type in all CD8⁺ lymphocytes apparently diminished the systemic metabolic consequences of ADA deficiency, as indicated by a relatively modest elevation in erythrocyte dAXP at the time PEG-ADA therapy was initiated. However, the magnitude of this detoxifying effect was evidently insufficient to ameliorate the profound depletion of his other lymphocyte populations, which was reversed only by PEG-ADA replacement therapy. In this regard, it can be calculated that the weekly dose of PEG-ADA provides far more circulating ADA activity than would be present in the patient's entire pool of revertant CD8⁺ T lymphocytes. The observation that the detoxifying effect of the revertant population is not able to support immune reconstitution in this patient provides an important contrast between human ADA deficiency and ADA-deficient mice, where hematopoietic stem cell transplantation leading to engraftment with a small percentage of ADA-positive donor lymphocytes has been observed to provide sufficient metabolic rescue to allow host ADA-deficient lymphocyte engraftment. This observation is also of potential relevance to gene therapy of human ADA-deficiency, as it suggests that vector-driven ADA expression in even a large circulating lymphocyte population may be unable to generate sufficient total body detoxification to allow autologous immune reconstitution. Thus, consideration should be given to driving ADA transgene expression throughout hematopoietic development in order to obtain significant immunologic rescue.

During the initial 9 months of PEG-ADA therapy, we observed expansion of the patient's TCRv β repertoire coincident with accumulation of CD4⁺ and CD8⁺ lineage T cells possessing surface marker phenotypes characteristic of normal mature, naïve T cells. While CD19⁺ B cells also steadily accumulated, suggesting that PEG-ADA supported bone marrow B cell development, they exhibited a persistent surface marker phenotype associated with B cells in transitional /immature stages of peripheral B cell development[21;28;29]. Consistent with a corresponding functional immaturity of his CD19⁺ B cells, our patient did not produce a normal humoral immune response to bacteriophage ϕ X174 immunization even after 9 months of PEG-ADA therapy.

The response to immunization with ϕ X174 was previously examined in 10 other patients with ADA deficient SCID[25]. Antibody responses to bacteriophage were uniformly absent prior to treatment in these patients, and remained abnormal in the 6 patients who had received bone marrow transplants, in spite of good T cell reconstitution. In contrast, 3 of 4 patients treated with PEG-ADA developed quantitatively and qualitatively normal antibody responses to ϕ X174. Our patient's limited response to bacteriophage might involve mechanisms similar to

those responsible for delayed or reduced humoral immune responses in some human infants [24]. Alternatively, persisting effects of deoxyadenosine metabolites, abnormal adenosine-related signaling, or other abnormalities in peripheral lymphoid tissue[1;2;3], or dysregulation of B cell regulatory hormones secondary to ADA-deficiency[30], may have compromised recovery of normal B cell maturation during PEG-ADA therapy in this patient. In this regard, BAFF is known to play a crucial role in peripheral B cell homeostasis and peripheral maturation, and thus the reduced BAFF-R expression we observed may have a causal role, as opposed to being a secondary response to peripheral lymphoid abnormalities[30]. Finally, it is intriguing to speculate that revertant CD8⁺ T cells, unique to the present patient compared with those previously studied, may have played some role in impeding recovery of B cell maturation and function during enzyme replacement therapy.

Functional immaturity of the peripheral B cell compartment in association with deficient humoral immune responses has been previously reported in studies of neonates, patients with X-linked lymphoproliferative disease (XLP), and in patients who have undergone hematopoietic stem cell transplantation[24;31]. Our results extend this correlation to this single patient on PEG-ADA therapy, and in addition provide a direct correlation between the surface marker phenotype of the peripheral B cell compartment and a deficient humoral immune response, as demonstrated by bacteriophage ϕ X174 testing. Although caution must be used in extrapolating from this analysis of a single patient, our results further suggest that reduced expression of BAFF-R may be a useful marker for a functionally immature peripheral B cell compartment, as reduced BAFF-R expression was observed in the entire CD19⁺ B cell population of our patient, relative to normal controls.

In summary, the delayed onset phenotype in the present patient is consistent with his maternally inherited R156H mutation. His persisting immune dysfunction at diagnosis in spite of reversion of this mutation in CD8⁺ T cells may have relevance to the development of gene therapy approaches for human ADA-deficiency, as it indicates that achieving normal intracellular ADA activity in only one major branch of the T cell lineage may be inadequate to sustain full immune reconstitution. Consistent with this, maintaining high systemic ADA activity in plasma with PEG-ADA normalized dAXP metabolites and led to a rapid accumulation of naïve phenotype T cells, including non-revertant CD4⁺ and CD8⁺ T cells, and restoration of a quantitatively and qualitatively normal cellular immune system. In contrast to his rapid T cell reconstitution, although B cells steadily accumulated over the 9 months of PEG-ADA treatment, the patient was unable to mount a significant humoral immune response. The lack of humoral immune reconstitution correlated with a persistent immature surface marker phenotype of the patient's peripheral B cells. These latter results support the utility of multi-parameter immunophenotyping of the peripheral T cell and B cell compartments for monitoring immune reconstitution during PEG-ADA therapy, and provide further evidence supporting their use for immune monitoring in related clinical situations, such as after hematopoietic stem cell transplantation or gene therapy.

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A. Patient ex 5: buccal

G C A T G C G C C A C
A

B. Patient ex 8: buccal

A G C G G C T G G G
C T G G G A

C. Mother ex 5: buccal

G C A T G C G C C A C
A

D. Mother ex 8: buccal

A G C G G C T G G G

Figure 1. Analysis of DNA sequence in amplified genomic DNA samples

Panels A-D: Buccal epithelial DNA, showing segments from exons 5 (A, C) and 8 (B, D) from the patient (A, B) and his mother (C, D). Panels E and F: segment from exon 5 in DNA isolated from unfractionated IL-2 stimulated T cells (E) and purified, uncultured CD4+ T cells of the patient. Panels G and H: segments from exon 5 (G) and exon 8 (H) from purified, uncultured CD8+ T cells of the patient.

Figure 2. Analysis of peripheral blood lymphocytes after initiation of ADA therapy

A: PBL's were purified from blood collected at each time point, and stained and analyzed using flow cytometry to determine the relative numbers of cells bearing major lymphocyte lineage markers CD4, CD8, and CD19. Absolute cell numbers/mm³ of whole blood were then calculated based on the total PBLs obtained and the initial volume of blood in each sample.

B: PBL's were purified from blood collected at approximately 3 months (left panels) or 8 months (right panels) after initiation of PEG-ADA therapy, and TCRv β repertoires were analyzed by spectratyping.

Figure 3. Time course of peripheral CD4⁺ T cell subset accumulation after initiation of PEG-ADA therapy

PBLs were purified from blood collected at each time point, and then stained and analyzed for CD4 differentiation markers as described in the materials and methods. The density plots illustrate the accumulation of CD4⁺ T cells, CD45RA⁺CD27⁺CD62L⁺CCR7⁺ naïve-recent thymic emigrant cells, CD45RA⁻CD27⁻CD62L⁻CCR7⁻ late differentiation CD4⁺ cells and FoxP3⁺CD4⁺ regulatory T cells of this patient over the 9 month period of PEG-ADA therapy.

Figure 4. Time course of peripheral CD8⁺ T cell subset accumulation after initiation of PEG-ADA therapy

PBLs were purified from blood collected at each time point, and then stained and analyzed for CD8 differentiation markers as described in the materials and methods. The density plots show the accumulation of total CD8⁺ T cells, CD27⁺CD28⁺/CCR7⁺/CD45RO⁻ naïve phenotype CD8⁺ cells, and CD27⁻CD28⁻/CCR7⁻/CD45RO⁺ late differentiation cells of this patient over the 9 month period of PEG-ADA therapy. CD27⁻CD28⁻/CCR7⁻/CD45RO⁺ cells are thought to represent a terminally differentiated form of CD8⁺ T cell, and in this patient represented a population of cells bearing a revertant mutation in the R156H allele.

Figure 5. Time course of peripheral CD19+ B cell subset accumulation after initiation of PEG-ADA therapy

PBLs were purified, and then stained and analyzed for CD19 differentiation markers as described in the materials and methods. The density plots show the accumulation of CD19⁺/CD27⁺ memory B cells, CD19⁺/CD27⁻ naive B cells, CD19⁺/CD27⁺/IgM⁻/IgD⁻ and CD19⁺/CD27⁺/IgM⁺/IgD⁺ switched and unswitched memory B cells respectively, CD19⁺/CD27⁻/IgD⁺/CD38⁺ intermediate/transitional B cells, and CD19⁺/CD27⁻/IgD⁺/CD38⁻ mature B cells over the 9 month period of PEG-ADA therapy. BAFFR expression on the total CD19⁺ population of each time point is also presented in the far right panels.

Figure 6. Bacteriophage ϕ X174 antibody responses before and after 9 months of PEG-ADA therapy
 When immunized with a single dose of bacteriophage Φ X174 prior to PEG-ADA therapy (filled squares), the patient cleared the antigen normally in less than one week and produced a minute amount of phage neutralizing antibody with a peak Kv of 0.19 at one week. This is less than 1% of a normal response. Nine months after he was started on PEG-ADA, no detectable neutralizing antibody was present and he received two additional immunizations with phage Φ X174, six weeks apart (open squares). The peak Kv after the first of those immunizations was 0.83, all IgM, and after the second was 9.0, 1% being of the IgG isotype. Shaded area represents the 95% confidence interval of responses observed in healthy young adult controls.

Table 1

Biochemical Parameters

Date	Weeks on PEG-ADA	Plasma		Erythrocyte		PBL		Erythrocyte Nucleotides		
		ADA	$\mu\text{mol/h/mL}$	ADA	nmol/h/mg	PNP	nmol/h/mg	AXP	$\mu\text{mol/mL}$	dAXP
5/23/06	1	11.7		0.0		1411		2.94		0.06
6/27/06	6	14.9						2.00		0.00
9/19/06	18	16.8						1.97		0.01
2/6/07	38	29.2						2.05		0.01
4/17/07	48	43.4						1.84		0.00
8/21/07	66	39.8						1.03		0.00
Normal values		<0.5		63 $\pm$ 41		1336 $\pm$ 441		1.465 $\pm$ 0.38		<0.002
										<0.2