Published in final edited form as:

J Allergy Clin Immunol. 2014 March ; 133(3): 799–806.e10. doi:10.1016/j.jaci.2013.12.1043.

B-cell development and functions and therapeutic options in adenosine deaminase–deficient patients

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Abstract

Background—Adenosine deaminase (ADA) deficiency causes severe cellular and humoral immune defects and dysregulation because of metabolic toxicity. Alterations in B-cell development and function have been poorly studied. Enzyme replacement therapy (ERT) and

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Disclosure of potential conflict of interest: J. Puck has received research support from the National Institutes of Health. M. van der Burg has received research support from ZonMW (Vidi grant 91712323). The rest of the authors declare that they have no relevant conflicts of interest.

hematopoietic stem cell (HSC) gene therapy (GT) are therapeutic options for patients lacking a suitable bone marrow (BM) transplant donor.

Objective—We sought to study alterations in B-cell development in ADA-deficient patients and investigate the ability of ERT and HSC-GT to restore normal B-cell differentiation and function.

Methods—Flow cytometry was used to characterize B-cell development in BM and the periphery. The percentage of gene-corrected B cells was measured by using quantitative PCR. B cells were assessed for their capacity to proliferate and release IgM after stimulation.

Results—Despite the severe peripheral B-cell lymphopenia, patients with ADA-deficient severe combined immunodeficiency showed a partial block in central BM development. Treatment with ERT or HSC-GT reverted most BM alterations, but ERT led to immature B-cell expansion. In the periphery transitional B cells accumulated under ERT, and the defect in maturation persisted long-term. HSC-GT led to a progressive improvement in B-cell numbers and development, along with increased levels of gene correction. The strongest selective advantage for ADA-transduced cells occurred at the transition from immature to naive cells. B-cell proliferative responses and differentiation to immunoglobulin secreting IgM after B-cell receptor and Toll-like receptor triggering were severely impaired after ERT and improved significantly after HSC-GT.

Conclusions—ADA-deficient patients show specific defects in B-cell development and functions that are differently corrected after ERT and HSC-GT.

Keywords

Gene therapy; adenosine deaminase–deficient severe combined immunodeficiency; B-cell development; antibodies

Mutations in the adenosine deaminase (ADA) gene are associated with accumulation of its substrates adenosine and deoxyadenosine, leading to lymphopenia (T, B, and natural killer) and a wide spectrum of immune and nonimmune alterations.^{1,2} Bone marrow transplantation (BMT) from an HLA-identical sibling donor is the treatment of choice for ADA-deficient severe combined immunodeficiency (SCID), but transplants from matched unrelated donors are associated with increased morbidity and mortality.³ Enzyme replacement therapy (ERT) with PEG-ADA decreases toxic ADA substrate concentrations and improves the immunologic phenotype. Nonetheless, a variable extent of immune recovery has been reported, and patients undergoing long-term treatment often show a decrease in lymphocyte counts, loss of regulatory T (Treg) cell function, and development of antibodies against bovine ADA.^{2,4–7} Gene therapy (GT) with hematopoietic stem cells (HSCs) engineered with gamma retroviral vectors has been shown to be a successful alternative strategy for patients who do not have access to HLA-identical BMT and for whom ERT was insufficient to maintain adequate immune reconstitution.⁸ Since 2000, more than 40 patients worldwide were enrolled in HSC-GT clinical trials with reduced-intensity conditioning, resulting in long-term multilineage engraftment, sustained systemic detoxification, and improved immune functions.^{8–11}

The lack of ADA induces severe peripheral B-cell lymphopenia, but no information is available about bone marrow (BM) B-cell development in patients with untreated ADA-

SCID. Most patients treated with ERT show an initial B-cell recovery that is not sustained long-term, with reduction in newly produced B-cell counts and oligoclonal B-cell repertoire.^{7,12,13} Additionally, patients with milder forms of ADA deficiency after treatment with PEG-ADA or HSC-GT have shown autoimmune manifestations, which might be related to central or peripheral B-cell tolerance defects.^{1,2,8,14,15}

B-cell development is regulated by the elimination of self-reactive B-cell clones in the BM and during transition from new emigrant to mature naive B-cell stages in the periphery.^{16,17} B cells from patients with ADA-SCID show a high frequency of autoreactive and anti-nuclear antibody (ANA)-expressing clones, loss of central and peripheral B-cell tolerance, and perturbation of checkpoint control during development.¹⁸ Moreover, patients with different primary immunodeficiencies who have autoimmunity show impaired elimination of autoreactive B cells at the transitional B-cell stage^{19,20} and an altered distribution of autoimmune-prone B-cell subsets, including CD21^{low}CD38^{low} B cells.²¹

These observations led us to investigate alterations in B-cell development in ADA-deficient patients and to compare B-cell development and function in patients with ADA-SCID receiving either ERT, HSC-GT, or BM transplantation.

RESULTS

Altered BM B-cell development in patients with ADA-SCID and correction after treatment

To gain information on early B-cell development, we studied the composition of B-cell precursors in the BM of 7 patients with untreated ADA-SCID, 6 ERT-treated patients, and 8 patients undergoing HSC-GT (see Table E1 in this article's Online Repository at www.jacionline.org). We used 13 combinations of 4-color staining to identify pro-B, pre-BI, pre-BII, and immature B cells (Fig 1, A).^{22,23}

Despite the severe peripheral lymphopenia in patients with untreated ADA-SCID, B cells were present during all stages of BM maturation (Fig 1). Pro-B cells were similarly increased in untreated, ERT-treated, and HSC-GT-treated patients (Fig 1, B). Strikingly, a 2-fold increase in pre-BI-cell counts was observed in patients with untreated ADA-SCID compared with control subjects ($48\% \pm 6\%$ vs $18.5\% \pm 7\%$, respectively [mean $\pm$ SD]), with a corresponding decrease in pre-BII and immature B-cell counts. This suggests a progressive loss of mature precursor cells rather than a block in differentiation. Treatment with ERT reduced the percentage of pre-BI cells, with a corresponding recovery in pre-BII-cell counts. Immature B-cell counts were also increased, suggesting an altered progression of B-cell development through maturation. Importantly, the proportion of pre-BI, pre-BII, and immature B-cell subsets was normalized after HSC-GT (Fig 1, C–E, and see Fig E1 in this article's Online Repository at www.jacionline.org).

Selective advantage for gene-corrected B cells occurs at late stages of maturation

In agreement with our previous observations,⁸ on average, the proportion of vector-transduced B cells in patients undergoing HS-GT was 8.9% (range, 0.6% to 32.4%) in BM CD19⁺ cells and 48.3% (range, 8.4% to 76.4%) in peripheral blood (PB). Therefore we assessed whether the observed selective advantage for ADA-transduced B cells occurred

during BM development or at later stages of maturation. Pro-B, pre-BI, pre-BII, and immature B cells from 6 patients (collected 2.6–8.3 years after HSC-GT) were sorted by means of fluorescence-activated cell sorting (FACS) and analyzed by using quantitative PCR (qPCR) for their percentage of gene correction (Fig 2, A). No significant increase in the fraction of gene-corrected progenitor B cells was observed during the early stages of development (Fig 2, A, left panel), whereas the percentage of transduced naive B cells increased, on average, 4.4-fold (Fig 2, A, right panel; median, 24.5%) with respect to immature B cells in the BM (5.6%). In addition to the selective advantage occurring at the transition from immature to naive B cells, we observed a further increase at later stages of maturation because approximately 56% of total CD19⁺ B cells were gene corrected in these patients (Fig 2, B).

Transitional B cells accumulate during ERT treatment

Patients who underwent ERT were divided into short-term (n = 8; 0.4–2.5 years) and long-term (n = 6; 9.1–22.8 years) categories based on their age and duration of treatment. B-cell counts were less than the normal range²⁴ in both the short- and long-term groups (see Table E2 in this article's Online Repository at www.jacionline.org). Nevertheless, all patients undergoing long-term ERT discontinued intravenous immunoglobulin (IVIg) and responded to vaccine antigens. After HSC-GT, B-cell counts were normal in 3 of 14 patients, and 71% of patients discontinued IVIg with good vaccination response. Three patients undergoing ERT and 2 patients undergoing HSC-GT showed signs of autoimmunity (see Table E2). ANA results were positive in 2 asymptomatic patients undergoing HSC-GT, whereas 2 patients undergoing ERT showed ANA or Coombs positivity (data not shown).

To understand whether different treatments influence B-cell development, we compared the proportion and absolute numbers of PB B-cell subsets of patients undergoing HSC-GT with those of patients undergoing ERT and appropriate age-matched control subjects. The GT cohort was subdivided in 2 categories according to the years of follow-up (1–4 and 4.6–8.9). As shown in Fig 3, the percentage of CD24^{hi}CD38^{hi} transitional B cells was 5.8-fold higher than in control subjects in both the short-term and long-term ERT groups ($P = .0001$; Fig 3, B), whereas absolute counts were increased only after short-term treatment ($P = .001$; Fig 3, C). The percentage of transitional B cells was increased only in the short-term group of patients undergoing HSC-GT but not the absolute numbers ($P = .007$; Fig 3, B). Patients undergoing HSC-GT normalized both transitional B-cell numbers and percentages, as assessed by using a linear mixed effect (LME) model (Fig 3, D), whereas this alteration persisted after long-term ERT ($P = .2232$; Fig 3, D). The percentage of transitional B cells in patients undergoing HSC-GT inversely correlated with transduced B cells ($P = .0401$; Fig 3, E), indicating that the relatively increased transitional B-cell percentages were mainly nontransduced.

We then measured the proportion of naive (CD19⁺CD27⁻), CD27⁺ memory, and switched memory (CD27⁺IgG⁺IgA⁺) B cells (see Fig E2 in this article's Online Repository at www.jacionline.org). Short-term ERT resulted in normal naive B-cell counts, but the long-term generation of naive B cells was not sustained. Decreased memory and switched memory B-cell counts were persistently found in patients receiving short- and long-term

ERT (see Fig E2, *D* and *F*). In contrast, all B-cell subpopulations were reduced at early time points after HSC-GT compared with those seen in control subjects but normal in patients followed long-term ($P = .0003$, $P = .002$, and $P = .003$, respectively; see Fig E2, *B*, *D*, and *F*).

We next analyzed the CD21^{low}CD38^{low} B-cell subset, which was previously shown to be expanded in patients with autoimmunity or immunodeficiency.^{21,25} This population was overrepresented in patients undergoing long-term ERT (8%, $P < .005$) and in patients undergoing HSC-GT ($P = .04$) when compared with control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org). However, no association with autoimmune manifestations was observed in both groups of patients.

Effect of B cell-activating factor on transitional B-cell maturation

B-cell survival, peripheral selection, and maturation largely depend on the activity of B cell-activating factor (BAFF).²⁶ BAFF plasma levels were evaluated in patients undergoing ERT and patients undergoing HSC-GT during follow-up. Shortly after PEG-ADA, BAFF levels were increased ($P < .05$; see Fig E4, *A*, in this article's Online Repository at www.jacionline.org), whereas long-term treatment resulted in levels comparable with those seen in control subjects (median, 0.7 ng/mL; range, 0.4–2 ng/mL). Also, patients undergoing HSC-GT showed increased levels of BAFF (7-fold with respect to control subjects), but its concentration decreased over time ($P < .05$). We then evaluated the level of BAFF receptor (BAFF-R) expression, which has a pivotal role in regulating the size of the mature B-cell pool (see Fig E4, *B*). We observed a 2.8- and a 3.8-fold reduction in BAFF-R mean fluorescence intensity in patients undergoing short-term HSC-GT and ERT (see Fig E4, *C*), which is in accordance with higher BAFF levels and a more immature B-cell phenotype in both groups. BAFF-R instead normalized in the long-term HSC-GT group, which is in accordance with a normalization of BAFF in their plasma.

Reduced *in vitro* proliferation of B cells from ERT-treated patients is corrected after GT

To study the ability of B cells from patients with ADA-SCID to proliferate and differentiate *in vitro*, we purified CD20⁺ B cells and stimulated them through the B-cell receptor (BCR) or Toll-like receptor (TLR). Fig E5 in this article's Online Repository at www.jacionline.org shows carboxyfluorescein succinimidyl ester (CFSE) dilutions in a representative control subject, patients undergoing HSC-GT, and patients undergoing ERT after stimulation with the TLR9 agonist CpG or in combination with immunoglobulin or CD40L ligand (CD40L). TLR stimulation did not induce adequate proliferation in B cells from patients undergoing ERT (Fig 4, *A*), indicating that TLR receptors are unable to signal properly in the absence of functional ADA. The additional BCR stimulation by anti-immunoglobulin antibody was unable to restore appropriate B-cell proliferation in patients undergoing ERT ($P = .003$). In contrast, B cells from patients undergoing HSC-GT responded normally after CpG stimulation, and costimulation of the BCR further increased B-cell proliferation. The addition of CD40L to mimic T-cell/B-cell interaction induced adequate B-cell proliferation in patients undergoing HSC-GT but not patients undergoing ERT. Because the level of gene correction varies between patients, we analyzed patients with different percentages of transduced B cells separately. Their ability to respond to a combination of BCR/TLR stimuli and T-cell mimicking (see Fig E6, *A*, in this article's Online Repository at

www.jacionline.org) is 2-fold reduced in patients with few corrected B cells (<50%) compared with patients with high correction (>50%) or a patient successfully treated with BMT achieving full donor chimerism (see Fig E6, *B*). This finding highlights the critical importance of ADA for the normalization of B-cell function. After BCR, TLR, and CD40L activation, B cells differentiate into immunoglobulin-secreting cells. Therefore we assessed the percentage of IgM immunoglobulin-secreting cells in patients undergoing ERT or patients undergoing HSC-GT by using the ELISPOT assay (Fig 4, *B*). B cells from patients undergoing ERT and patients undergoing HSC-GT stimulated with CpG showed a reduced percentage of IgM-secreting spots with respect to control subjects. After additional BCR engagement, CD40L, or both, we observed a significant improvement in IgM secretion for the HSC-GT group and, to a lesser extent, the ERT group.

DISCUSSION

The goal of the present work was to assess defects in B-cell differentiation and function in patients with ADA deficiency and to evaluate the effect of different treatments. Here we demonstrate that patients with ADA-SCID display specific alterations in early and late B-cell development, which are differently corrected by ERT or HSC-GT. Moreover, we show that intrinsic ADA expression provided by gene transfer is superior to ERT in restoring B-cell differentiation and function (Table I).

Our findings indicate that, despite the severe peripheral lymphopenia, ADA deficiency only leads to a partial block in BM differentiation. After pre-BCR rearrangement and completion of V(D)J recombination, a 2-fold decrease in late developmental stages can be observed in patients with untreated ADA-SCID (see Fig E1), suggesting that B cells become more sensitive to purine toxic metabolites and apoptosis during maturation.²⁷ These alterations are possibly related to defects in V(D)J recombination and DNA damage repair caused by the accumulation of ADA substrates. In fact, previous reports indicate that increased dATP levels influence the frequency of V(D)J recombination and the composition of N insertions in lymphocytes, leading to alterations in antigen receptor and aberrant lymphoid development.²⁸ Moreover, increased intracellular dATP in the absence of deamination retards DNA repair in human lymphocytes and results in the slow accumulation of DNA strand breaks.²⁹ This is consistent with the pattern observed in patients with SCID with a complete defect in V(D)J recombination (eg, recombination-activating gene 1 or 2 deficiency) or X-linked agammaglobulinemia, showing an earlier and more severe block at pro-B and pre-BI stages and a dramatic reduction in immature B-cell counts.^{22,30,31} The hypothesis that human B cells require different levels of ADA during differentiation is supported by the progressive increase of vector-transduced B cells in patients undergoing HSC-GT from immature to naive B cells and even further in late stages.

We found that both HSC-GT and ERT revert the partial BM differentiation block, leading to rescue of the peripheral B-cell pool. Because gene-corrected cells represent approximately 10% of total BM B cells, the normalization of B-cell development is likely mediated by cross-correction from ADA-expressing B cells and other cell lineages. However, our data indicate that intrinsic ADA expression achieved by GT is important for survival, maturation, and function at later stages of differentiation.

Both patients undergoing ERT and patients undergoing HSC-GT in the first years after treatment showed a relative increase in counts of transitional B cells in the periphery, which normalized at later time points in the HSC-GT group. Also, patients who underwent allogeneic BMT showed an increased proportion of transitional B cells early after transplantation, with normalization within 1 year after treatment.^{20,32} After HSC-GT, a delay in B-cell reconstitution with respect to BMT is expected because of the time needed for highly purified transduced HSCs to repopulate the BM, compete with untransduced B cells, and differentiate into functionally mature B cells. Importantly, increased B-cell counts, including memory and switched memory B cells, alongside the reduction in transitional B-cell counts was associated with an increased percentage of gene-corrected B cells in the PB.

The higher proportion of transitional B cells detected in patients undergoing long-term ERT might be sustained by the increased percentage of immature B cells exiting from their BM or the higher BAFF levels found in these patients. Similar to patients with B cell-mediated autoimmune diseases,^{33–35} BAFF might act as a compensatory mechanism to the lymphopenic condition, thus allowing the expansion of transitional or CD21^{low}CD38^{low} B cells and promoting cell-mediated autoimmunity through survival of low-affinity self-reactive B cells.

In patients undergoing HSC-GT, B-cell development proceeded to mature functional B cells and a normal proportion of memory and immunoglobulin-producing B cells. Interestingly, the restored ability of plasmablasts to proliferate and produce IgM *in vitro* correlated with their level of transduction, indicating that endogenous ADA is required for full correction of the B-cell defect. In contrast, the few naive B cells isolated *ex vivo* from patients undergoing ERT did not properly respond to BCR or TLR stimulation, proliferate, and secrete immunoglobulins. These data are in agreement with our previous finding that *in vitro* inhibition of ADA enzymatic activity in normal human B cells blocks responses to TLR and BCR stimulation.¹⁸

Development of autoantibodies and autoimmune manifestations have been reported after long-term ERT^{12,14,15} and have been associated with an incomplete immune recovery, decrease in absolute B-cell numbers, and an oligoclonal B-cell repertoire.^{2,6,12,36} An in-depth analysis of B-cell tolerance and antibody repertoire in 3 ADA-deficient patients treated with ERT showed an increased frequency of both polyreactive and ANA-expressing clones, indicating defects in central and peripheral B-cell tolerance in patients with ADA deficiency.^{2,18} Moreover, we previously demonstrated that an impaired Treg cell function contributes to the loss of peripheral tolerance in patients undergoing ERT, as well as in ADA^{-/-} mice rescued with PEG-ADA therapy. In these animals the abolished Treg cell function leads to the development of immune dysregulation with abnormal serum immunoglobulin levels, antiplatelet antibodies, and autoantibodies directed against multiple organs.⁵

Herein we found a significant increase in the percentage of CD21^{low}CD38^{low} B cells in patients undergoing ERT, a population highly represented among those with systemic lupus erythematosus²⁵ and common variable immunodeficiency,³⁷ resulting in enriched autoreactive clones refractory to BCR triggering and unable to upregulate activation

markers. Similar to mouse T1 B cells,³⁸ CD21^{low}CD38^{low} B cells escape central B-cell tolerance mechanisms and remain unresponsive in the periphery. Inflammatory responses might create a favorable environment to break tolerance and eventually activate CD21^{low}CD38^{low} B cells.²¹ An alternative explanation is that CD21^{low}CD38^{low} B cells represent a developmental stage that precedes that of transitional B cells.²⁰ Also, patients treated with HSC-GT had a higher proportion of CD21^{low}CD38^{low} cells, suggesting a possible predisposition to autoimmune manifestations because of the escape of autoreactive immature B cells that are not eliminated in the periphery.

In the present study we did not observe an increase in autoimmune manifestations or autoantibody production in the ERT group. Because of the low number of patients who had autoimmunity and the limited size of the patient cohort, a direct correlation between clinical signs and biological observations (Treg cell numbers, transitional B cells, and BAFF levels) could not be drawn in both the ERT and HSC-GT groups. A combination of predisposing factors might favor the onset of immune dysregulation. It is also plausible that infections might contribute to immune dysregulation, although we did not observe an increased infection rate in patients with autoimmunity.

In the GT cohort it is possible that systemic detoxification after GT and the metabolic cross-correction of uncorrected B cells by gene-corrected cells in BM allows the survival of potentially autoreactive B cells and their egress in the periphery. The coexistence of noncorrected autoreactive B cells, which produce higher levels of ANA *in vitro*,¹⁸ and gene-corrected functional T-cell help might explain why some patients with ADA-SCID have autoimmune manifestations after HSC-GT. Finally, it cannot be excluded that tolerance defects established under ERT treatment are insufficiently controlled after GT.

In summary, our findings provide important insights into defects in B-cell maturation in ADA-deficient patients. These alterations are partially corrected after ERT because B cells remain mostly at the transitional stage and retain severe defects in proliferation and antibody secretion *in vitro*. GT with HSC provides progressive normalization of B-cell development with recovery of B-cell functions. The majority of treated patients were not taking IVIg, showing specific antibody responses and no autoimmune manifestations. Maintenance of long-term B-cell reconstitution remains to be determined, especially in patients with immunologic defects. Increased HSC-GT efficiency and engraftment of gene-corrected B cells could further improve immune reconstitution at early time points after follow-up and reduce the risk of autoimmunity.

METHODS

Patients with ADA-SCID and clinical trial

BM samples of naive patients with ADA-SCID were collected on the occasion of diagnostic procedures from the remaining cell material, according to the informed consent guidelines of Rotterdam University Hospital. BM of control subjects was obtained according to the informed consent guidelines of the Medical Ethics Committees of the Leiden University Medical Center. For patients undergoing ERT or those after HSC-GT, PB and BM were collected after obtaining parental informed consent on the occasion of diagnostic procedures

or safety follow-up, according to the San Raffaele Hospital–approved research protocol for pathogenetic studies in immunodeficient patients.

Patients with ADA-SCID undergoing HSC-GT were enrolled in phase I/II clinical protocols approved by the San Raffaele Scientific Institute's Ethical Committee and Italian National Regulatory Authorities. HSC-GT treatment was performed as previously described.^{E1} Data from patients undergoing HSC-GT were collected from 2008 to 2011. Since April 2012, GlaxoSmith-Kline has become a sponsor of ADA-SCID long-term follow-up trial no. 115611 (HSC-GT) conducted at TIGET. A previously described patient^{E1} not studied in the present work showed autoimmune manifestations during PEG-ADA, which persisted after GT.

Patients undergoing short-term ERT received 19 to 80 U/kg/wk ADAGEN (Pegademase bovine; ENZON Pharmaceuticals, Piscataway, NJ) before HSC-GT, where patients undergoing long-term ERT received 10 to 40 U/kg/wk. A subgroup of patients undergoing long-term ERT had previously received (8.5–11.3 years earlier) infusion of transduced PB T cells,^{E2} without evidence of long-term marking in other lineages. The patient with ADA-SCID treated with BMT was previously described.^{E3}

PB from pediatric control subjects was obtained on the occasion of other blood testing after informed consent in the context of a research protocol established at San Raffaele Scientific Institute. Informed consent was approved by the Institutional Ethical Committee of San Raffaele, and forms were signed by all subjects' parents.

Isolation of blood cells, cell purification, and qPCR

PBMCs and BM mononuclear cells were purified by using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient separation. The protocol for the purification of different cell subsets and qPCR for gene-corrected cells was performed as previously described.^{E4} The purity of CD19⁺B cells was greater than 90%.

FACS staining and B-cell sorting

BM progenitor B cells were analyzed on thawed samples from patients with untreated ADA-SCID and patients undergoing ERT or after HSC-GT. Data were compared with reference values obtained from 10 age-matched control subjects (Table E1).

Frozen mononuclear cells from patients with ADA SCID were thawed in RPMI and 10% FBS and directly incubated with 100 μ L of the following mix of antibodies: CD34 fluorescein isothiocyanate (FITC), CD22 allophycocyanin (APC), CD19 phycoerythrin (PE) or APC, CD10 FITC, CD36 FITC, CD20 PE, SmIgM PE, SmIgD FITC, CyIgM FITC, TdT FITC, CyCD179a PE, and CyCD79a PE. Gate exclusion was performed to eliminate T-cell, myeloid cell, natural killer cell, and basophil contaminations. BM B-cell stages were then identified as pro-B (CD22⁺CD19⁺CD36⁺), pre-B1 (CD19⁺CD22⁺CD10⁺TdT⁺CyIgM⁺CD20⁺), pre-B2 (CD19⁺CD22⁺CD10⁺TdT⁺CyIgM⁺CD79a⁺VpreB⁺CD20⁺), and immature B (CD19⁺CD22⁺CD10⁺CD20⁺CD79a⁺SmIgM⁺SmIgD⁺) cells, correcting the percentage of different subpopulations by exclusion of PB B-cell contamination. Calculations were performed as

previously reported.^{E5} Ten thousand events were acquired in the B-cell gate on a BD FACSCanto II (BD Biosciences, San Jose, Calif) and analyzed with DIVA software version 6.0.

Fifty microliters of whole blood was stained for B-cell subsets with the following mix of antibodies: CD19 PeCy7, CD24 FITC, CD38 PerCP5.5, BAFF-R PE, and CD27 APC (all from BD PharMingen). IgG and IgA FITC were purchased from Jackson ImmunoResearch (West Grove, Pa). The blood was lysed for 10 minutes at room temperature, washed in PBS-FACS, and stained directly with antibody mix. In the case of IgG and IgA staining, a previous incubation with medium containing 10% FBS was performed. After staining and washing, the cells were fixed with 150 μ L of PBS-FACS plus 0.2% formaldehyde and read within 24 hours. Thirty thousand events were registered in the lymphogate by using a BD FACSCanto II and analyzed with FlowJo software 2.2 (TreeStar, Ashland, Ore).

Reference values for all stainings were obtained from pediatric and adult donors selected with respect to the age of the patients enrolled in the study.^{E6} Donors with less than 5% B cells were excluded from the analysis. Samples were acquired within 24 hours on a BD FACSCanto II and analyzed with FlowJo software 2.2.

For the isolation of precursor and naive B cells by means of FACS sorting, thawed samples were stained with CD34 PE, CD19 PeCy7, CD10 FITC, and IgM APC (all from BD PharMingen). The subpopulations were sorted with a BD FACSVantage Cell Sorter and tested by using qPCR for their level of gene correction.

BAFF ELISA

BAFF was evaluated by means of ELISA (R&D Systems, Minneapolis, Minn), according to the manufacturer's protocol, with 50 μ L of plasma samples from patients and control subjects. BAFF levels were determined within 30 minutes with a microplate reader (Bio-Rad Laboratories, Richmond, Calif) set to 450 nm.

Proliferation assay

CD20⁺ B cells from patients and donors were purified from frozen PBMCs thawed in RPMI and 1% FBS plus 10 μ L/mL DNase (Calbiochem, San Diego, Calif). Purity was greater than 74%. CD20⁺ B cells were labeled with 0.5 μ mol/L CFSE for 8 minutes at room temperature. CFSE-stained B cells (6×10^4) from patients undergoing HSC-GT and donors were plated in 96-well flat-bottom culture plates and stimulated for 72 hours in RPMI 10% Hyclone (Sera; Thermo Scientific, Uppsala, Sweden) with 2.5 μ g/mL CpG2006 (5'-TsCsg sTsGsg sTsTsT sTsTsT sTsTsT sCsTsT sTsTsT sTsTsT sTsTsT-3'; TIB BioMol, Genoa, Italy), 2.5 μ g/mL F(ab')₂ anti-human IgM/IgG/IgA (Jackson ImmunoResearch), and 3 ng/mL CD40L (Alexis Biochemicals, San Diego, Calif). For patients undergoing ERT, 10×10^4 CFSE-stained B cells were plated. Cell culture was performed in a final volume of 200 μ L. The group of control subjects is composed of pediatric and adult donors who were verified to proliferate in the same way after stimulation. Proliferating B cells were stained with anti-CD19 PeCy7 and 7-amino-actinomycin D to exclude dead cells and IgG and IgA APC at the third day of stimulation. A BD FACSCanto II was used to acquire 10,000 events for each condition in the lymphocyte gate. The results were analyzed with FlowJo software 2.2.

ELISpot

Plasmablasts secreting IgM were detected by using a spot ELISA, as previously described.^{E7} Two thousand five hundred stimulated B cells per condition were analyzed, and the spots were counted with an A.EL.VIS Elispot Reader and software.

Statistical analyses

Normality assumption was checked, and parametric or nonparametric tests were applied accordingly to fulfillment of normality assumptions. A 2-tailed Mann-Whitney *U* test was used to assess whether the means of 2 independent groups were statistically different. Data were analyzed with GraphPad Prism, version 4.02 (GraphPad Software, La Jolla, Calif). To analyze longitudinal data, LME models were applied^{E8} to elucidate patients' specific unobservable variability/heterogeneity by including random components. LME models were performed with R statistical software (version 2.15.3, <http://www.R-project.org>). A *P* value of less than .05 was considered significant. One-way ANOVA with the *post hoc* Bonferroni multiple comparison test was performed to assess the significance of differences between means of more than 2 independent groups. When the normality assumption was not met, the Kruskal-Wallis test with Dunn multiple comparison was applied.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Francesca Dionisio for helping with the qPCR, the San Raffaele Hospital Blood Bank staff for their precious contribution to this study, Paola Maria Vittoria Rancoita for helping in statistical analysis, the physicians and nurses of the Pediatric Clinical Research Unit of TIGET for patient's care, and Dr Alessio Palini and staff for FACS sorting.

Supported by the Italian TELETHON foundation (TIGET Core grant A1), the European Commission: Advanced Cell-based Therapies for the treatment of Primary Immuno-Deficiency (HEALTH-F5-2010-261387, CELL-PID), and Italian Ministero della Salute (RF-2009-1485896 conv.055). J.P. acknowledges support from the UCSF CTSI, NIH NCATS 1UL1 RR024131.

Abbreviations used

ADA	Adenosine deaminase
ANA	Anti-nuclear antibody
APC	Allophycocyanin
BAFF	B cell-activating factor
BAFF-R	BAFF receptor
BCR	B-cell receptor
BM	Bone marrow
BMT	Bone marrow transplant
CD40L	CD40 ligand

CFSE	Carboxyfluorescein succinimidyl ester
ERT	Enzyme replacement therapy
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GT	Gene therapy
HSC	Hematopoietic stem cell
IVIg	Intravenous immunoglobulin
LME	Linear mixed effect
PB	Peripheral blood
PE	Phycoerythrin
qPCR	Quantitative PCR
SCID	severe combined immunodeficiency
TLR	Toll-like receptor
Treg	Regulatory T

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Clinical implications

HSC-GT is superior to ERT in restoring B-cell development and *in vitro* B-cell function in patients with ADA-SCID.

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FIG 1.

BM B-cell development in patients with ADA-SCID. **A**, Representative FACS staining and gating strategy for BM B-cell development. Dotted arrows indicate the stage of development of pro-B (CD22⁺CD19⁻), pre-BI (CD19⁺CyIgM⁻SmIgM⁻), pre-BII (CD19⁺CyIgM⁺SmIgM⁻), immature (CD19⁺SmIgM⁺SmIgD⁻), and mature (CD19⁺SmIgM⁺SmIgD⁺) B cells. **B–E**, Percentage of pro-B (Fig 1, *B*), pre-BI (Fig 1, *C*), pre-BII (Fig 1, *D*), and immature (Fig 1, *E*) B cells in 10 age-matched healthy donors, 7 patients with ADA-SCID, 6 patients undergoing ERT, and 8 patients undergoing HSC-GT corrected for blood contamination. **P* < .05, ***P* < .005, and ****P* < .001, Kruskal-Wallis test with Dunn correction.

FIG 2. Selective advantage of gene-corrected B cells occurs at late stages of maturation. **A**, Percentage of transduced BM and PB B-cell subpopulations determined by means of qPCR (BM, n = 6; PB, n = 5). *Gray line*, Median values. **B**, Percentage of gene-corrected B cells in PB. *Bar* indicates median value.

FIG 3.

Transitional B cells accumulate in patients undergoing ERT and decrease after HSC-GT. **A**, Representative dot plots for age-matched control subjects, patients undergoing HSC-GT, and patients undergoing ERT for CD24^{hi}CD38^{hi} transitional B cells. **B**, Percentage of transitional B cells in patients undergoing short-term HSC-GT (n = 8), patients undergoing short-term ERT (n = 8), patients undergoing long-term HSC-GT (n = 6), and patients undergoing long-term ERT (n = 6). Age-matched control subjects are shown: Controls A (n = 24, 0.5–4 years), Controls B (n = 36, 4.1–13 years), and Controls C (n = 30, 13–25 years).

Median values with 5th and 95th percentiles. $*P < .05$ and $**P < .005$, Mann-Whitney test. **C**, Absolute numbers of transitional B cells in the same groups as in Fig 3, *B*, are shown. Data are presented as median values with 5th and 95th percentiles. $*P < .05$, $**P < .005$, and $***P < .001$, Mann-Whitney test. **D**, Longitudinal analysis for the percentage of transitional B cells and years of F.U. for 14 patients undergoing HSC-GT (*black dots*) and 5 ERT-treated patients (*open squares*). Analysis of longitudinal data was done with LME models and time effect significance. **E**, Longitudinal analysis between the percentage of transitional B cells and the percentage of vector-transduced B cells in the 14 HSC-GT-treated patients studied using LME models, with significance.

FIG 4.

Impaired B-cell proliferation after BCR/TLR triggering in patients undergoing ERT is corrected after HSC-GT. **A**, Percentage of CFSE-diluting B cells in patients undergoing HSC-GT ($n = 4$ for CpG stimulation and $n = 6$ when either immunoglobulin or CD40L were added) and 4 ERT-treated patients compared with 15 control subjects. Data are shown as means $\pm$ SEMs. $*P < .05$ and $**P < .005$, Mann-Whitney test. **B**, Number of IgM-producing B cells in patients undergoing ERT ($n = 5$) and patients undergoing HSC-GT ($n = 5$ for CpG stimulation and $n = 6$ when either immunoglobulin or CD40L were added) compared with 17 control subjects. Data are shown as means $\pm$ SEMs. $*P < .05$ and $**P < .005$, Mann-Whitney test.

TABLE I

Summary of the main B-cell features in the BM and the periphery of patients with untreated ADA-SCID or patients who received long-term treatment with ERT or underwent HSC-GT

Group	BM development	PB	
		B-cell maturation	B-cell function
Untreated	↑ Pre-BI B cells	ND	ND
ADA-SCID	Block of BM development		
ERT	Normal BM development ↑ Immature B cells	↑ Transitional B cells ↓ Naive, memory, and switched memory B cells ↑ BAFF levels	↓ B-cell proliferation ↓ IgM production to TLR7/9 and immunoglobulin
HSC-GT	Normal BM development Selective advantage for gene-corrected cells (later stages)	Progressive normalization of B-cell maturation ↑ BAFF levels	Normal B-cell proliferation ↓ IgM production to TLR7/9

ND, Not done because of severe lymphopenia.