ARTICLE

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IL-3 or IL-7 Increases *ex Vivo* Gene Transfer Efficiency in ADA-SCID BM CD34$^+$ Cells while Maintaining *in Vivo* Lymphoid Potential

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To improve maintenance and gene transfer of human lymphoid progenitors for clinical use in gene therapy of adenosine deaminase (ADA)-deficient SCID we investigated several gene transfer protocols using various stem cell-enriched sources. The lymphoid differentiation potential was measured by an *in vitro* clonal assay for B/NK cells and in the *in vivo* SCID-hu mouse model. *Ex vivo* culture with the cytokines TPO, FLT3-ligand, and SCF (T/F/S) plus IL-3 or IL-7 substantially increased the yield of transduced bone marrow (BM) CD34$^+$ cells purified from ADA-SCID patients or healthy donors, compared to T/F/S alone. Moreover, the use of IL-3 or IL-7 significantly improved the maintenance of *in vitro* B cell progenitors from ADA-SCID BM cells and allowed the efficient transduction of B and NK cell progenitors. Under these optimized conditions transduced CD34$^+$ cells were efficiently engrafted into SCID-hu mice and gave rise to B and T cell progeny, demonstrating the maintenance of *in vivo* lymphoid reconstitution capacity. The protocol based on the T/F/S + IL-3 combination was included in a gene therapy clinical trial for ADA-SCID, resulting in long-term engraftment of stem/progenitor cells. Remarkably, gene-corrected BM CD34$^+$ cells obtained from one patient 4 and 11 months after gene therapy were capable of repopulating the lymphoid compartment of SCID-hu hosts.

Key Words: immunodeficiency, ADA-SCID, lymphoid differentiation, human stem/progenitor cells

INTRODUCTION

The first attempts to cure genetic diseases by gene transfer were started in the early 1990s [1–3]. However, it is only recently that gene therapy has proven to be a curative approach for patients affected by primary immunodeficiencies [4–7]. Hematopoietic stem/progenitor cells with lymphoid potential represent an attractive target for a gene therapy approach aimed at the correction of genetic defects involving the immune system, including severe combined immunodeficiency (SCID) due to the lack of adenosine deaminase (ADA) or X-linked SCID due to the lack of common γ-chain receptor (SCID-X1). Normal lymphoid progenitor cells are endowed with a strong *in vivo* selective advantage over defective cells, as shown by the occurrence of spontaneous somatic revertants *in vivo* in ADA-SCID [8,9], SCID-X1 [10], and Wiskott–Aldrich syndrome patients [11]. This is also highlighted by results in HLA-identical bone marrow (BM) transplants performed in SCID in the absence of preconditioning, which resulted in split chimeras, with the only circulating cells of donor origin being lymphocytes [12,13]. However, while T cells are always of donor origin, B cells remain usually host-derived, resulting often in insufficient B cell functions.

Common lymphoid progenitors are very well characterized in the mouse system [14,15], and their existence in humans is strongly suggested by a number of indica-

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tions [16–18]. Human lymphoid progenitors represent a small subgroup of the broader $CD34^+$ cell population, coexpressing other markers such as CD10, IL-7Rα, CD45RA, and CD7 [19,20]. However, since most of these cell surface molecules are not specific, such progenitors can be defined only by functional tests assessing their capacity to differentiate both *in vitro* and *in vivo* in T, B, and NK cells.

BM stromal microenvironment and growth factors have a crucial role in the maintenance and/or differentiation of hematopoietic stem/progenitor cells with lymphoid potential. Among the different factors, thrombopoietin (TPO), stem cell factor (SCF), and FLT3-ligand have been shown to allow the growth of human stem/progenitor cells [21]. In addition, the above-mentioned cytokines were successfully included in gene transfer protocols of $CD34^+$ cells [22–24] or $CD34^+/CD38^-$ cells [25,26]. However, in these studies maintenance and transduction of stem cells with lymphoid potential were not fully proven. The role of IL-3 during lymphoid development is controversial. The *in vitro* exposure of stem/progenitor cells to this cytokine has been described to be detrimental to the maintenance of murine B cell progenitors [27], T cell progenitors [28], and NK cell progenitors [15]. In contrast, IL-3 has been shown to enhance differentiation and proliferation of B cell progenitors from human primitive uncommitted cells obtained from distinct hematopoietic sources [29], although its effect can be very different when included in other culture conditions [30,31]. IL-7 is essential for T and B cell development in the mouse [32–34], in particular for the survival of T and B cell progenitors. In the human system, the *in vivo* relevance of this cytokine in the development of the T cell compartment has been highlighted by the observation that defective IL-7 receptor leads to an immunodeficiency syndrome [35]. However, the role of IL-7 in human B cell development is still controversial [19,36]. If either IL-3 or IL-7 represents a key factor for survival of human lymphoid progenitors, its use during *ex vivo* culture for gene transfer in BM cells from ADA-SCID patients may result in an increase in the number of transduced lymphoid progenitors. The reinfusion of a cell population enriched in lymphoid progenitors in addition to stem cells may favor immunoreconstitution and contribute to generation of a pool of long-term surviving lymphocytes.

ADA-deficient SCID is characterized by impaired development of T, B, and NK cells due to the accumulation of purine toxic metabolites. Little information is available on the effects of the purine metabolic defect on stem/progenitor cells, including lymphoid progenitors. Although ADA-SCID was the first disease to be treated with stem/progenitor cell gene therapy, no studies have attempted to characterize in detail the *in vitro* growth of $CD34^+$ ADA-SCID cells and their response to different growth factors, with specific regard to their lymphoid potential.

Here we studied the effects of different cytokines on maintenance and gene transfer of BM $CD34^+$ cells from ADA-SCID patients, compared to $CD34^+$ cells from healthy donors. Our data indicate that the addition of IL-3 or IL-7 to the TPO/FLT3-ligand/SCF (T/F/S) combination of growth factors resulted in substantial increase in the recovery of transduced $CD34^+$ cells and *in vitro* lymphoid progenitors from ADA-SCID patients, with preserved *in vivo* lymphoid potential of stem/progenitor cells. These findings thus allowed us to design a gene therapy protocol for the treatment of this disease.

RESULTS

Ex Vivo Culture Conditions Including IL-3 or IL-7 Increase the Yield of Transduced BM $CD34^+$ Cells from ADA-SCID Patients or Normal Donors

We first investigated whether the addition of IL-3 or IL-7 to the minimal culture conditions including T/F/S could increase gene transfer efficiency and proliferation of $CD34^+$ cells (Fig. 1A). We isolated purified BM $CD34^+$ cells from healthy donors and prestimulated them for 24 h with the cytokines T/F/S at constant concentrations, in the presence of increasing amounts of IL-3 or IL-7. We then subjected the prestimulated cells to three consecutive overnight cycles of transduction on fibronectin-coated plates and maintained them in the presence of the same growth factors utilized during the prestimulation. At day 4 we counted the cells and analyzed them by flow cytometry to assess the level of gene transfer in the $CD34^+$ cell population, evaluated by the expression of the ΔNGFR transgene in the cells still expressing the CD34 antigen. This allowed the evaluation of the final yield of transduced $CD34^+$ cells, measured as the proportion of $CD34^+$ cells expressing the transgene with respect to the input cell population. As shown in Fig. 1A, we observed a dose-dependent increase in the final yield of transduced $CD34^+$ cells in the presence of IL-3 and, to a lesser extent, IL-7, reaching a plateau at 60 and 20 ng/ml, respectively. The increase in transduced $CD34^+$ cells induced by these cytokines was not associated with a loss of phenotypically early progenitors. Transduced $CD34^+/CD38^-$ cells were increased in the presence of IL-3 and maintained with IL-7, compared to T/F/S alone (Fig. 1B).

We then studied the effect of IL-3 or IL-7 at optimal doses on stem/progenitor cells from ADA-SCID patients in comparison to healthy donors. We isolated $CD34^+$ cells from BM of 9 ADA-SCID patients and 6 healthy donors (including one child), as well as from 10 normal umbilical cord blood (UCB) samples, and transduced them as described above. The average percentages of $\Delta NGFR^+$ cells among CD34-expressing cells were 48 ± 9 and $44 \pm 14\%$ in ADA-SCID patient and in normal donor BM, respectively. The yields of transduced $CD34^+$ cells, shown as percentage of input, were 31 ± 35 and $24 \pm 13\%$ in ADA-SCID patient and in normal donor BM, respectively. The presence of IL-

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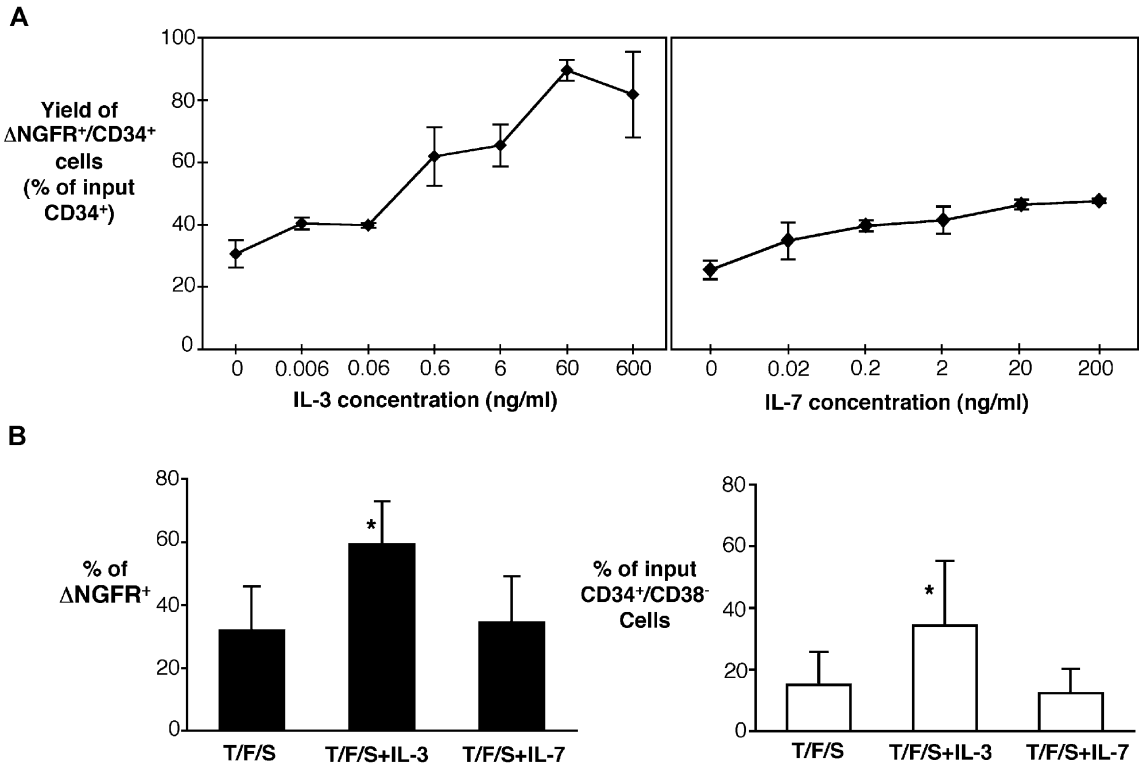

FIG. 1. Effects of IL-3 or IL-7 on gene transfer into $CD34^+$ and $CD34^+/CD38^-$ cells. (A) BM $CD34^+$ cells from three different healthy donors were transduced with a retroviral vector, in the presence of T/F/S. Different concentrations of IL-3 (left) or IL-7 (right) were added to T/F/S. At day 4, the yield of transduced $CD34^+$ cells was calculated as follows: the number of $\Delta NGFR^+/CD34^+$ cells at the end of the culture was divided by the initial number of $CD34^+$ cells and multiplied by 100, to measure the proportion of $CD34^+$ cells that expressed the transgene with respect to the input cell population. Shown are results from two representative experiments performed in duplicate. (B) Percentage of gene transfer of $CD34^+/CD38^-$ cells at day 4 (left) and yield of transduced $CD34^+/CD38^-$ cells, measured as % of input $CD34^+/CD38^-$ cells (right). Gene transfer was performed in the presence of T/F/S, T/F/S + IL-3 (60 ng/ml), or T/F/S + IL-7 (20 ng/ml). Bars represent average values (±SD) from six different healthy donors. The difference between T/F/S + IL-3 and the other culture conditions is statistically significant, both for the percentage ($*P < 0.005$) and for the yield ($*P = 0.02$) of transduced $CD34^+/CD38^-$ cells.

3 increased significantly the proportion and the final yield of $CD34^+$ cells expressing the transgene, both in ADA-SCID patient and in normal donor BM (Fig. 2). We obtained similar results with UCB $CD34^+$ cells, although the increase in the proportion of transduced cells was minimal. In ADA-SCID patients, this increase was paralleled by a significant enhancement in the number of CFC obtained after culture (1.5-fold increase compared to T/F/S; $P < 0.04$; not shown). The effect of IL-3 was more pronounced on cell yield than on gene transfer rate, indicating that an increase in cell proliferation does not strictly correlate with an enhanced efficiency of gene transfer. In 6 of 9 ADA-SCID patients tested, IL-7 improved the percentages and yield of transduced $CD34^+$ cells, but overall the increase was not statistically significant.

The Addition of IL-3 or IL-7 to T/F/S Cytokines Allows the Maintenance of *in Vitro* Lymphoid Progenitors Derived from ADA-SCID Patients' BM

We initially studied if the T/F/S combination of cytokines was sufficient to maintain *in vitro* hematopoietic progenitor cells with lymphoid potential from ADA-SCID patients or healthy donors. We isolated $CD34^+$ cells from normal UCB, BM of healthy donors (adults or children), or from BM of ADA-SCID patients. We compared freshly isolated and cultured cells for their ability to differentiate *in vitro* into B and NK lymphocytes using a clonal assay that detects the presence of $CD19^+$ and $CD56^+$ cells after 3–4 weeks of culture. We seeded purified $CD34^+$ cells from each donor or patient directly in the clonal assay or prestimulated and transduced them in bulk in the presence of T/F/S, as previously described, and subsequently plated them in the clonal B/NK test. As shown in Figs. 3A and 3B, freshly isolated UCB $CD34^+$ cells displayed a higher content of B and NK cell progenitors compared to both normal BM, independent of donor's age ($P < 0.006$), and ADA-SCID BM $CD34^+$ cells (either from untreated patients or from patients under PEG-ADA enzymatic treatment, $P < 0.04$). Both B and NK cell progenitors from UCB were efficiently maintained after 4 days of culture in the presence of T/F/S. In contrast, we observed a dramatic loss of B cell progenitors (Fig. 3A) in

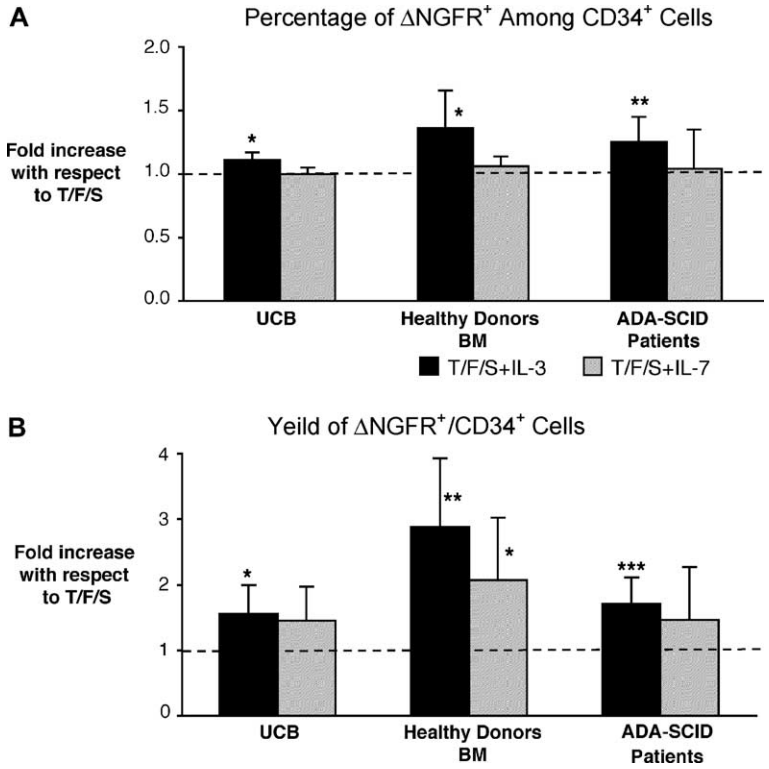

FIG. 2. Transduction efficiency in $CD34^+$ cells from different sources. $CD34^+$ cells from 10 different UCB samples, 9 ADA-SCID patients' BM, and 6 healthy donors' BM (5 adults, 1 child) were transduced with a retroviral vector, using different culture conditions (T/F/S, T/F/S + IL-3, T/F/S + IL-7). (A) Percentage of $\Delta NGFR^+$ cells was calculated among the cells still expressing the CD34 antigen at the end of culture. (B) The yield of transduced $CD34^+$ cells was calculated as described in the Fig. 1 legend. The ratios between the percentages or the yields obtained after transduction in the presence of either IL-3 or IL-7 and those after T/F/S treatment were calculated for each experiment. The values in A and B show the means ($\pm$SD) of these ratios, thus representing the fold increase with respect to T/F/S alone (dashed horizontal line) for each $CD34^+$ cell source. The fold increase in the control child's cells was 1.4 in A and 2.9 in B by adding IL-3, 1.1 in A and 2.1 in B by adding IL-7. The *P* values relate to the differences between the corresponding bars and T/F/S: $*P < 0.04$; $**P = 0.006$; $***P = 0.0006$.

BM $CD34^+$ cells from ADA-SCID patients (6-fold decrease, $P = 0.002$ for untreated patients; 25-fold decrease, $P = 0.02$ for patients under enzymatic treatment). We observed a significant but less pronounced reduction in B cell progenitors from healthy adult donors (2.5-fold decrease, $P = 0.02$), with a trend toward a similar decrease in healthy children. We found no significant differences in the number of NK cell progenitors before and after the transduction procedure, either in patients or in healthy donors (Fig. 3B). We next studied if the addition of IL-3 or IL-7 to the T/F/S cytokines could prevent the loss of BM B cell progenitors from ADA-SCID patients or normal donors during *ex vivo* culture and allow transduction of lymphoid progenitors. We tested $CD34^+$ cells, freshly isolated or maintained using the three different culture conditions, for their content in total B cell progenitors in the clonal differentiation assay. Fig. 3C shows the absolute numbers of B cell progenitors obtained from BM $CD34^+$ cells of six ADA-SCID patients and of four normal adult donors. In ADA-SCID patients, the addition of IL-7 or IL-3 during the 4-day culture greatly improved the number of B cell progenitors compared to the T/F/S combination of cytokines, resulting in a 6-and 5-fold average increase, respectively (Fig. 3C, gray bars), and thus reaching values not significantly different from those of freshly isolated cells. We observed no significant differences with regard to the number of NK cell progenitors before or after culture (not shown). In BM from healthy donors, culture with IL-3 resulted in numbers of B cell progenitors comparable to those of fresh cells, although the effects of the different cytokine treatments were less pronounced, compared to BM from ADA-SCID patients.

Next, we determined the effects of different combinations of cytokines on transduction of lymphoid progenitors from four ADA-SCID patients and from an age-matched healthy donor. The presence of IL-3 or IL-7 resulted in higher yields of transduced B cell progenitors in 3/4 and 4/4 patients, respectively, and in the normal donor, compared to T/F/S alone (Fig. 4A), mainly as the consequence of an increase in total B cell progenitors obtained with these cytokines. The transduced B cells differentiated in culture expressed cytoplasmic IgM, indicating their ability to rearrange the Ig locus, and, after infection with Epstein–Barr virus, generated a stable B cell line that expressed CD20, CD22, and CD80 markers (data not shown), suggesting that the *ex vivo* culture and gene transfer did not interfere with the normal B cell development *in vitro*. We also observed a higher recovery of transduced NK cell progenitors in 3/4 and 4/4 patients, respectively, with both IL-3 and IL-7, compared to T/F/S alone (Fig. 4B), similar to what we observed with total NK cell progenitors. To study the function of the transduced NK cells ($CD56^+$) generated *in vitro,* we sorted cells for the expression of ΔNGFR and used them as effectors in a cytotoxic assay. The killing capacity toward K562 target cells of the $\Delta NGFR^+$ NK cells was comparable to that of NK cells from healthy donor

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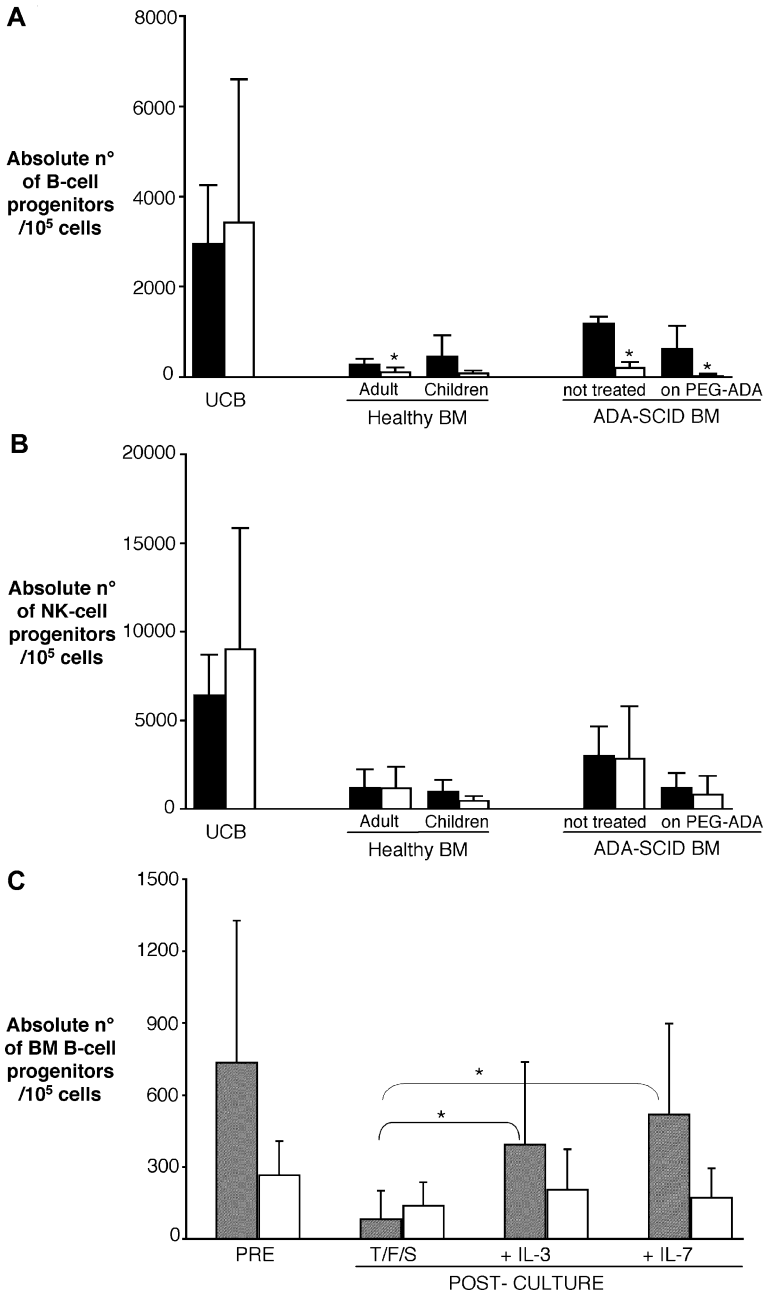

FIG. 3. Absolute numbers of freshly isolated or cultured lymphoid progenitors from various stem/progenitor cell sources. (A and B) $CD34^+$ cells were obtained from UCB donors ($n = 3$), healthy BM donors (adult, $n = 5$; children, $n = 3$), and ADA-SCID patients (three at disease onset and five on PEG-ADA therapy). Clonal B/NK assays (see Methods) were performed on freshly isolated cells or cells cultured for 4 days in the presence of T/F/S cytokines. Wells containing $CD19^+$ cells or $CD56^+$ cells after 3–4 weeks of culture were defined as positive for a B or NK cell progenitors, respectively. Absolute numbers of B cell progenitors (A) and NK cell progenitors (B), before culture (black bars) or after culture (white bars) with T/F/S, were compared. Bars shown are means (±SD) from independent experiments, one for each sample. The number of progenitors is normalized to 10^5 cells at the beginning of the culture and takes into account the total expansion of cells during the transduction protocol (see Methods). *Significant difference ($P < 0.03$) between cultured and freshly isolated cells, as assessed with two-tailed t test. Data obtained from children as controls were analyzed with a nonparametric (ANOVA), independent test (the data could not be paired in this case), which did not reveal significant differences due to the low number of samples. (C) BM $CD34^+$ cells from six ADA-SCID patients (gray bars) and from four healthy donors (white bars) were cultured for 4 days using T/F/S cytokines alone or with the addition of either IL-3 or IL-7 and then tested in a B/NK assay. The average absolute numbers (±SD) of B cell progenitors of 10^5 cultured BM $CD34^+$ cells were calculated as for A and B. *$P < 0.03$, T/F/S vs. +IL-3; *$P < 0.005$, T/F/S vs. +IL-7.

peripheral blood, suggesting that cells differentiated *in vitro* are functional (data not shown).

In contrast to the findings with BM, the addition of IL-3 or IL-7 to T/F/S did not further increase the number of total B and NK cell progenitors from UCB $CD34^+$ cells ($n = 3$, data not shown). Moreover, UCB lymphoid progenitors were efficiently transduced using all three cytokine combinations, including T/F/S, with no significant change in the final yield of transduced progenitors.

T/F/S + IL-3 or IL-7 Allows Gene Transfer into SCID-hu Repopulating Cells from ADA-SCID Patients

Since the *in vitro* studies may not be predictive of *in vivo* findings, we investigated engraftment and differentiation of $CD34^+$ cells transduced into B and T lymphocytes in the SCID-hu animal model. We transduced BM $CD34^+$ cells from four ADA-SCID patients *ex vivo* in the presence of T/F/S or T/F/S plus either IL-7 or IL-3 and then injected the same number of cells into irradiated BM/Thy/Liv SCID-hu mice. As controls, we

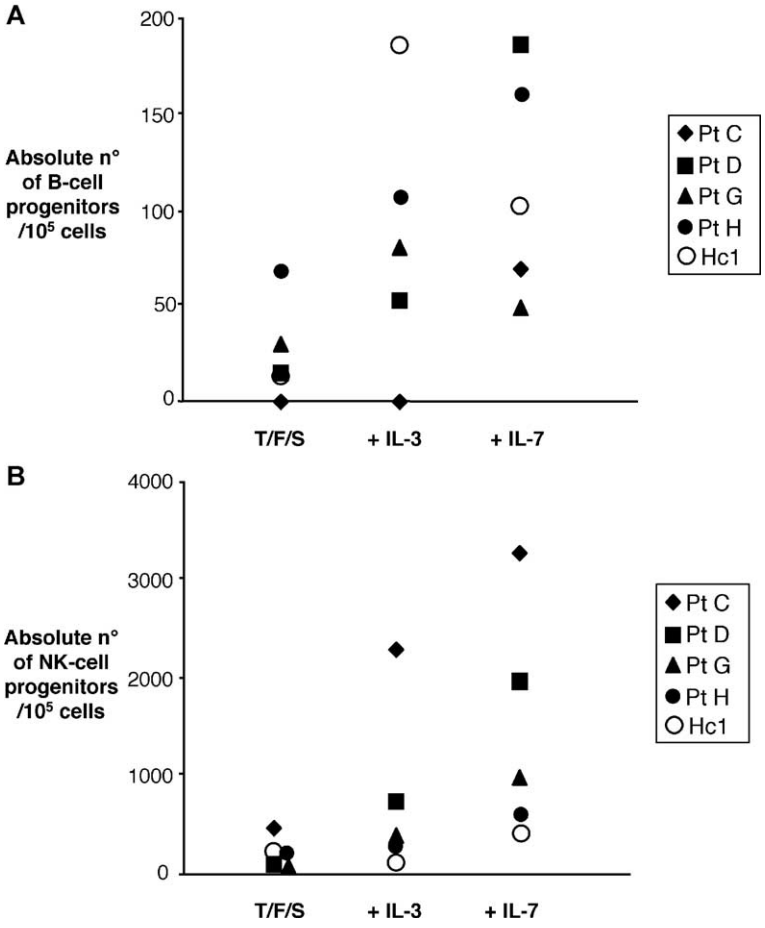

FIG. 4. Absolute numbers of transduced lymphoid progenitors from ADA-SCID BM CD34$^+$ cells. BM CD34$^+$ cells from four ADA-SCID patients and one healthy child (Hc1) were transduced using T/F/S alone or with the addition of either IL-3 or IL-7, and at day 4 cells from the bulk cultures were plated in the clonal B/NK assay, without prior purification of gene-modified cells. After 3–4 weeks, cells from each well were labeled with mAbs against CD19, CD56, and ΔNGFR and analyzed by FACS. The values represent mean absolute numbers of ΔNGFR-expressing (A) B cell progenitors and (B) NK cell progenitors of 10^5 cultured CD34$^+$ cells for each patient (calculated as for Fig. 3 and as described under Methods), with all the experiments performed in duplicate. The percentages of transduction into initial CD34$^+$ cells seeded in the B/NK assay in these four patients were on average 46 ± 9, 60 ± 5, and 45 ± 18% in the presence of T/F/S, T/F/S + IL-3, and T/F/S + IL-7, respectively.

transplanted unmanipulated CD34$^+$ cells into a set of animals. We analyzed the levels of engraftment and transgene expression after 8–9 weeks. A representative analysis of lymphoid reconstitution in SCID-hu mice transplanted with *ex vivo*-transduced CD34$^+$ cells is shown in Fig. 5. Among the human (CD45$^+$) cell population, donor cells were distinguished from the recipient human fetal tissue by HLA mismatch, and we analyzed the proportion of T and B lymphocytes, as well as of ΔNGFR-expressing cells (Figs. 5 and 6).

The overall levels of engraftment of human donor cells in the BM/Thy/Liv mice were 80 ± 22% in 31 mice analyzed, with no significant differences between animals transplanted with fresh CD34$^+$ cells or transduced cells (not shown). The high chimerism observed may indicate that plateau levels of engraftment have been reached, thus not allowing us to detect differences between the repopulating potential of fresh and cultured cells. On the other hand, it is noteworthy that, despite the transduced cells containing on average 1/3 of CD34$^+$ cells compared to fresh cells, due to the expected differentiation occurring in culture, we observed similar levels of engraftment. T and B lymphoid differentiation of CD34$^+$ cells from ADA-SCID patients was achieved with all three culture protocols (Fig. 6A), including the IL-3-containing cytokine combination. We observed similar levels of engraftment and differentiation capacity with BM CD34$^+$ cells from healthy donors (n = 3, data not shown). As previously observed in the SCID-hu model [37], the ratio between donor SP4 and SP8 cells was close to 1. The proportion of transduced cells among the engrafted donor cell population was higher in mice transplanted with cells cultured in the presence of IL-3 or IL-7 (Fig. 6B), with the exception of a single mouse injected with patient A CD34$^+$ cells. We found transduced donor lymphocytes in all thymic subsets analyzed, as well as in B lymphocytes (Fig. 6C and not shown). We observed similar levels of lymphoid differentiation when gating on total donor or ΔNGFR$^+$ cells, with all the expected thymic subpopulations present at the appropriate ratio (Fig. 6 and data not shown). Overall, we found a higher proportion of transduced thymocytes in mice injected with CD34$^+$ cells cultured with IL-3 or IL-7 compared with T/F/S only. The differences did not reach statistical significance, as the number of mice injected with patients' cells was limited to sample availability. It is

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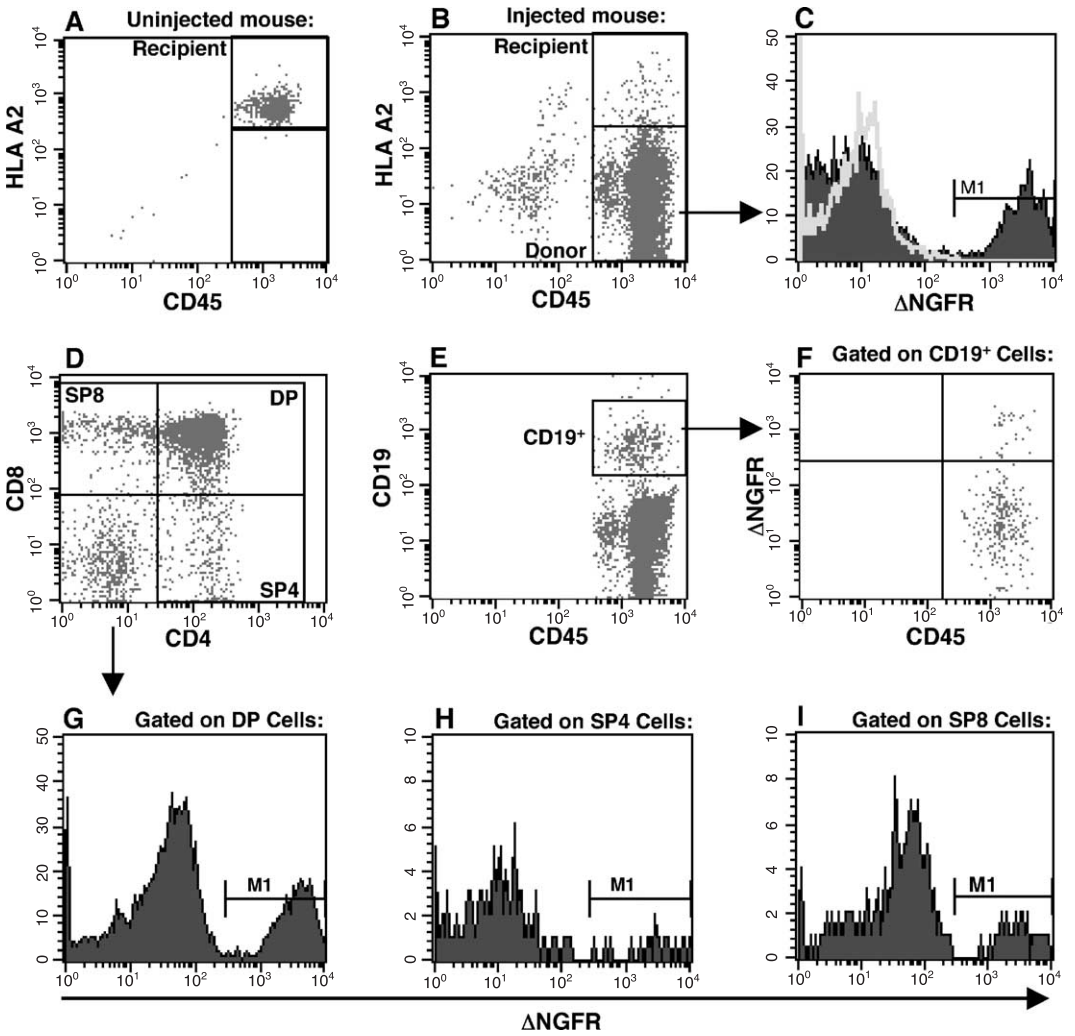

FIG. 5. *In vivo* T and B lymphoid differentiation capacity of transduced CD34$^+$ cells. BM CD34$^+$ cells from healthy donors or patients were subjected to retroviral-mediated gene transfer using T/F/S combination of cytokines with the addition of either IL-3 or IL-7. After the transduction procedure, cells were injected into the human grafts of BM/Thy/Liv SCID-hu mice. Eight to nine weeks later the grafts were analyzed by FACS for the presence of the injected cells. Results obtained from (A) one uninjected mouse and (B–I) one representative mouse transplanted with transduced cells from an ADA-SCID patient are shown. (B) CD45 and HLA staining, gated on lymphoid cells; (C) ΔNGFR staining, gated on donor cells; (D and E) staining for B and T cells, gated on CD45$^+$ and donor cells; (F–I) ΔNGFR staining, gated on B or T cells.

noteworthy that, despite the fact that cells cultured with IL-3 gave rise to two-to threefold more transduced CD34$^+$ cells than T/F/S, they showed similar engraftment and differentiation capacity compared to fresh CD34$^+$ cells. Collectively, these data indicate that the expansion observed in transduced BM CD34$^+$ cells did not occur at the expense of transplantable early progenitors with lymphoid potential.

Repopulation Capacity of BM CD34$^+$ Cells from an ADA-SCID Patient Treated with Gene Therapy Combined with Nonmyeloablative Conditioning

Based on the above results, we designed a new clinical gene transfer protocol for ADA-SCID patients, associated with nonmyeloablative conditioning (busulfan 4 mg/kg) [6]. We chose the protocol with the T/F/S + IL-3 combination based on: (1) the reproducibly higher gene transfer rates and yields of transduced CD34$^+$ cells, which would allow us to transplant almost twofold more transduced progenitor cells into the patients; (2) the maintenance of stem/progenitor cells with lymphoid potential as assessed by *in vitro* experiments; and (3) the demonstration that the treatment with IL-3 did not alter the *in vivo* lymphoid reconstitution capacity of ADA-SCID patient CD34$^+$ cells. We transduced BM CD34$^+$ cells from two ADA-SCID patients with a retroviral vector expressing the ADA therapeutic gene and the neo^R marker gene and reinfused them into the patients

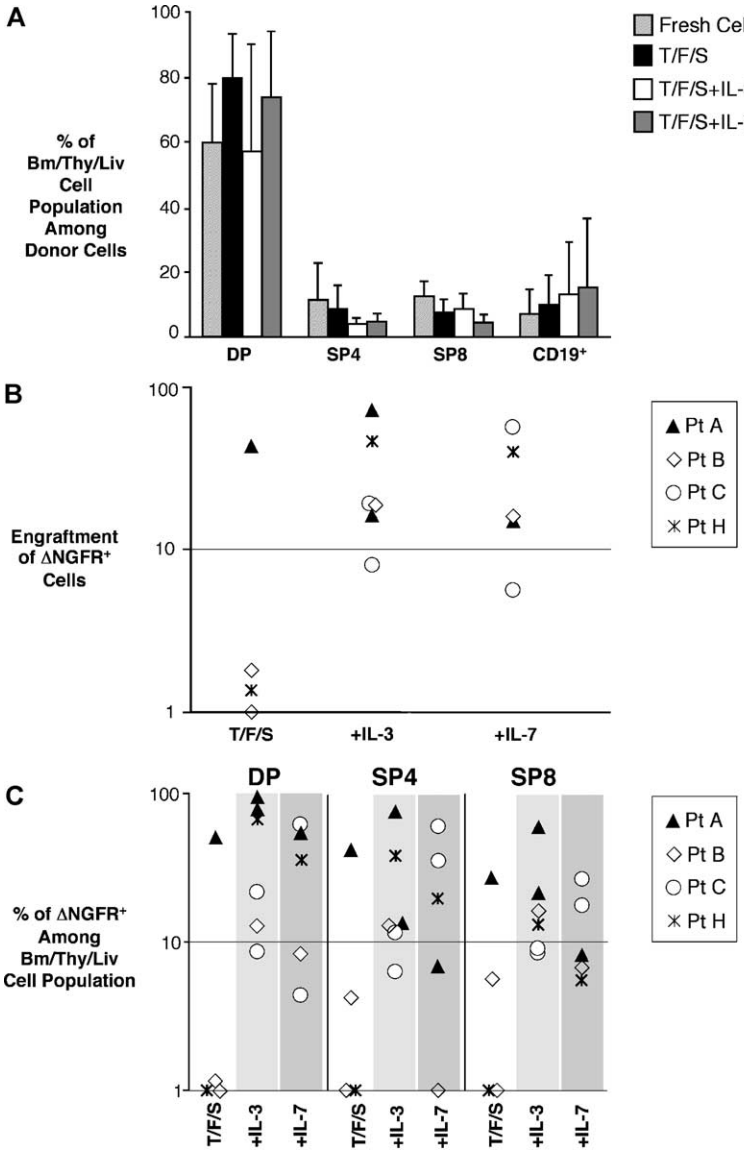

FIG. 6. Lymphoid differentiation capacity of transduced CD34$^+$ cells. (A) Fresh BM CD34$^+$ cells from ADA-SCID patients were injected into the human grafts of seven BM/Thy/Liv SCID-hu mice or subjected to retroviral-mediated gene transfer using three different combinations of cytokines. At day 4 equal numbers of total transduced cells were injected into SCID-hu mice (3 to 5 mice/condition), and after 8–9 weeks cells from the BM/Thy/Liv grafts were analyzed by FACS for chimerism (see text). The values represent mean (±SD) percentages of CD4/CD8 double-positive cells (DP), CD4 single-positive cells (SP4), CD8 single-positive cells (SP8), or CD19$^+$ cells among the donor gate, for fresh or cultured cells. (B) Engraftment of transduced cells. The symbols represent percentages of ΔNGFR$^+$ cells among donor cells in each mouse. Mice injected with cells from the same patients are represented with the same symbol. For patient C (Pt C), only mice injected with cells transduced with T/F/S + IL-3 or T/F/S + IL-7 were included in this graph, since no engraftment was achieved with cells transduced in the presence of T/F/S. (C) Presence of transduced cells among the donor thymic subpopulation. The values represent percentages of ΔNGFR$^+$ cells in individual mice. In injected cells cultured with T/F/S, T/F/S + IL-3, or T/F/S + IL-7, the proportions of transduced CD34$^+$ cells were 53 ± 8, 62 ± 10, and 56 ± 8%.

after 4 days of culture [6]. We purified BM CD34$^+$ cells from the first patient 4 and 11 months after gene therapy, injected them into SCID-hu mice to study their repopulation capacity, and analyzed them 8 weeks after. Engraftment levels of donor cells, obtained 4 and 11 months after gene therapy, ranged from 85 to 98%, as assessed by HLA typing (Table 1). CD34$^+$ cells from the ADA-SCID patient were able to give rise to a T and B lymphoid progeny in SCID-hu mice (Table 1 and Fig. 7), with all the thymic subpopulation present in the appropriate ratio (Fig. 7 and data not shown). Furthermore, we isolated T cells of donor origin from the SCID-hu graft 8 weeks after injection of CD34$^+$ cells, purified them based on the absence of the HLA-A2 marker, and expanded them *in vitro* in the presence of phytohemagglutinin (PHA) and IL-2 to generate T cell lines. We analyzed purified B and T lymphocytes as well as T cell lines of donor origin by quantitative PCR (Q-PCR) for the presence of transduced cells (Table 1). We found vector-containing cells within B cells (range 0.3–15.2%), T cells (range 0.14–31.2%), and T cell lines (range 4.2–41.7%). We detected no significant increase in the proportion of transduced cells compared to the initial frequency in the input CD34$^+$ cell population, most likely due to the lack of a selective advantage in the normal, detoxified microenvironment of the SCID-hu mouse. However, the frequency of transduced T cells increased 4 weeks after culture, suggesting an *in vitro* proliferative advantage of gene-corrected over untransduced T cells.

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TABLE 1: Long-term *in vivo* persistence of transduced stem/progenitor cells evaluated in SCID-hu secondary transplants

			BM/Thy/Liv tissue		% neo$^+$ in donor cells		
Time after GT[a]	SCID-hu No.	% Donor	% B cells in donor cells	% T cells in donor cells	B cells	T cells	T cell line
4 months	1	85.7	57.1	42.8	10	n.d.	n.d.
	2	97.3	11.1	77.1	13	n.d.	6.5
11 months	3	95.3	6.8	89.3	3.2	0.14	4.2
	4	98.1	1.0	95.8	0.3	2.6	6.5
	5	96.7	4.8	90.7	15.2	31.2	41.7

CD34$^+$ cells were purified from BM aspirates of an ADA-SCID patient 4 and 11 months after GT and injected into two and three SCID-hu mice, respectively. The percentage of neo$^+$ CD34$^+$ cells by Q-PCR (prior to SCID-hu injection) was 11.6 in the 4-month and 11.9 in the 11-month samples. The proportion of donor cells was measured as percentage of HLA-A2-negative cells (donor cells) among the CD45$^+$ cells. B and T cells were calculated among donor cells. B cells were measured by the expression of CD19 antigen. T cells were measured by the expression of CD3 antigen in the 4-month experiment, while in the 11-month the T cell group comprised CD4$^+$/CD8$^+$, CD4$^+$/CD8$^-$, and CD4$^-$/CD8$^+$ cells. In the 4-month SCID-hu, the T cell line (see Methods) was set up from the CD19-negative fraction. Cells were cultured for 2 weeks in the presence of PHA and IL-2 and then subjected to a second round of stimulation in the presence of feeders, PHA, and IL-2. In the 11-month SCID-hu, purified T cells were subjected to two rounds of stimulation, both in the presence of feeders, PHA, and IL-2. For all T cell lines, percentage neo$^+$ was calculated at the end of the second stimulation. Q-PCR was also run at the end of the first stimulation for mouse 2 (2.2% of neo$^+$ cells).
[a] GT, gene therapy.

We also tested a fraction of the BM CD34$^+$ cells obtained from the ADA-SCID patient 11 months after gene therapy for their capacity to differentiate into B and NK cells *in vitro*. Q-PCR on CD19$^+$ cells and CD56$^+$ cells purified from the B/NK assay showed that 4 and 9%, respectively, of these cells were transduced. These data further demonstrate that long-term engraftment of transduced hematopoietic stem/progenitor cells with preserved lymphoid potential (T, B, NK) can be achieved in ADA-SCID patients treated with gene therapy combined with nonmyeloablative conditioning.

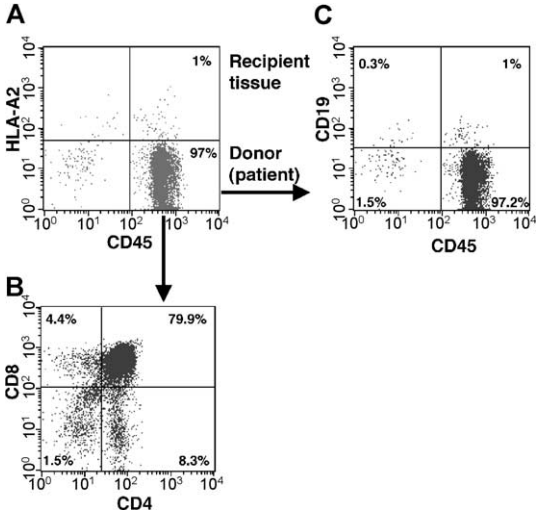

FIG. 7. Secondary repopulation capacity of BM CD34$^+$ cells from an ADA-SCID patient treated with gene therapy. BM was collected from an ADA-SCID patient 11 months after transplant of autologous CD34$^+$ cells engineered with GIADAI vector [6]. CD34$^+$ cells were purified and injected into the human BM/Thy/Liv grafts of three SCID-hu mice. Mice were sacrificed after 8 weeks and the implants analyzed by FACS. A flow cytometric analysis of one representative mouse is shown. (A) Engraftment of patient's cells (HLA-A2 negative). (B and C) Thymocytes and B lymphocytes gated on cells of donor origin.

DISCUSSION

Efficient correction and engraftment of hematopoietic stem/progenitor cells with lymphoid potential is the main goal of gene therapy for primary immunodeficiencies, including ADA-SCID. In the present study we show that BM CD34$^+$ cells from ADA-SCID patients can differentiate *in vitro* into B and NK cells, but B cell progenitors have a poor survival when cultured *ex vivo* in the presence of T/F/S. The addition of IL-3, and to a lesser extent IL-7, favored both gene transfer efficiency and final yield of transduced CD34$^+$ cells and allowed efficient transduction of T, B, and NK cell progenitors. Furthermore, the frequency of B and NK cell progenitors was not reduced in ADA-SCID compared to healthy BM. Thus, the peripheral NK and B cell defect present in this disease is not due to a reduced content of early lymphoid progenitors in the BM. Little is known about the effect of ADA deficiency on the growth and differentiation of B cell progenitors. It can be hypothesized that, similar to T lymphocytes [12,38], toxic metabolites may interfere with later stages of B cell differentiation in the BM or may alter the survival of immature B cells in peripheral lymphoid organs. This is also suggested by the finding that in patients treated with gene therapy, the selective advantage for gene-corrected cells is stronger in circulating B lymphocytes compared to BM B cells ([6] and unpublished observations).

Results from our study show that B cell progenitors are significantly less frequent in BM compared to UCB. This difference was not due to a different seeding efficiency, which was comparable between BM and UCB (data not shown). In addition, this finding is consistent with previous observations showing a substantial reduction

in the number of B cell progenitors [39], compared to the UCB. The most striking difference between BM and UCB B cell progenitors is that the former are greatly reduced in culture in the absence of IL-3 or IL-7. These data indicate that results obtained with UCB cannot be generally extended to BM progenitor cells, which can have different cytokine requirements.

The consistent and marked increase in B cell progenitors from ADA-SCID $CD34^+$ cells cultured with T/F/S + IL-3 or IL-7 resulted in an improved transduction of B cell progenitors, suggesting that both cytokines act on patients' B cell progenitors as survival and proliferation factors. Indeed, IL-3 and IL-7 induce Stat5 phosphorylation, through Jak-2 and Jak-3 activation, respectively [40,41]. Interestingly, it has been shown that in organ cultures the antiapoptotic protein Bcl-2, which is downstream of both IL-3 and IL-7 signaling [41], protects thymocytes from the toxic metabolites produced in the ADA deficiency [38]. Furthermore, IL-3 overexpression can rescue the immunodeficiency in Jak-3 deficient mice [42], overcoming the impaired IL-7 signaling. These data point to a redundant role for IL-3 and IL-7 on human B cell progenitor differentiation and expansion, as also suggested by the observation that a defect in the IL-7Rα does not result in a B lymphocyte deficiency [35].

NK cell progenitors from all sources could be effectively maintained and transduced under all the conditions tested, including the T/F/S cytokine combination, indicating that IL-3 or IL-7 is not strictly required for NK cell progenitor growth and differentiation. These data emphasize that analyses of one cell type alone may not be predictive for all lymphoid progenitors.

Importantly, we demonstrate that conditions including IL-3 or IL-7 allow maintenance and efficient transduction of ADA-SCID T and B cell progenitors analyzed *in vivo,* as assessed in BM/Thy/Liv SCID-hu mice. SCID-hu mice have been extensively used as an *in vivo* assay for transplantable human hematopoietic stem cells [43–45]. Although the *in vivo* reconstitution and developmental potential by stem cells contained in the $CD34^+$ cell population was not formally proven in the current study, it is unlikely that the lymphoid engraftment shown in the SCID-hu mice was contributed by committed cells, since $CD34^+/CD38^+$or $CD34^+/Thy1^-$ cells do engraft in this model [44,46]. On the other hand, even transduction of "short-term" progenitor cells would be clinically relevant. First, gene-corrected progenitors may be crucial in the early phases of immune reconstitution to speed and enhance the immune recovery. Second, they may contribute to generating a pool of long-term surviving mature T lymphocytes, which are known to persist for several years in ADA-SCID patients [47,48]. Here we demonstrate that stem/progenitor cells from ADA-deficient patients are able to be engrafted, expand, and differentiate into B and T lymphocytes after *ex vivo* culture, displaying a normal T cell maturation pattern. Both genetically corrected and wild-type cells can survive and differentiate in the SCID-hu mice, which provide an exogenous source of ADA in the local human microenvironment and systemically. Importantly, the IL-3 or IL-7 *ex vivo* treatment does not compromise the SCID-hu reconstitution potential of transduced $CD34^+$ cells, demonstrating that engraftment of transduced stem/progenitor cells with lymphoid potential can be achieved under these conditions. The significantly higher yields of transduced $CD34^+$ cells obtained following 4 days of culture with T/F/S + IL-3 compared to those obtained with the other culture conditions would allow reinfusion of a higher number of transplantable hematopoietic $CD34^+$ cells with lymphoid potential. This is highly relevant, since we found a strong correlation between the number of infused $CD34^+$ cells and the levels of reconstitution ([6] and unpublished observation in our clinical trial).

A previous *in vitro* study showed that the use of the T/F/S + IL-3 combination of cytokines resulted in efficient transduction of UCB $CD34^+$ cells with maintenance of the *in vitro* lymphoid differentiation potential [49]. However, in that study only healthy UCB was used as the source of stem/progenitor cells, no clonal assays for lymphoid progenitors were performed, and the *in vivo* T cell differentiation was not studied. In addition, no direct information on the role of IL-3 was provided. Early clinical trials [3,50–52] used IL-3 but lacked one or more of the other critical growth factors used in our experiments (SCF, TPO, FLT3-ligand), which have been shown to improve gene transfer greatly. In the clinical trial for SCID-X1 [4], the *ex vivo* use of T/F/S + IL-3 for transfer of the common γ chain into BM $CD34^+$ cells in the absence of conditioning resulted in engraftment of transduced T and NK cells, but with low long-term reconstitution of B and myeloid cells. In contrast, in the gene therapy trial for ADA-SCID combined with low-intensity myeloablation, long-term engraftment of transduced cells belonging to all the lineages was demonstrated [6]. Our present findings further confirm that long-term engraftment of gene-corrected stem/progenitor cells was achieved in ADA-SCID patients following reinfusion of $CD34^+$ cells transduced with T/F/S + IL-3. This is demonstrated by the presence, almost 1 year after gene therapy, of transduced BM $CD34^+$ cells, which were able to differentiate into B and NK cells *in vitro* and retained the capacity to engraft and to develop into mature B and T lymphocytes in a secondary SCID-hu host. In addition to the distinct gene and immune defect, the different outcome of the two studies was likely due to the low-intensity myeloablation of ADA-SCID patients prior to stem/progenitor cell infusion, conferring additional selective and proliferative advantage to transplanted $CD34^+$ cells.

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In conclusion, we demonstrate that the addition of IL-3 or IL-7 to T/F/S during *ex vivo* gene transfer not only increases the final yield of transduced ADA-SCID patient BM CD34$^+$ cells and of lymphoid progenitors assayed *in vitro,* but also preserves the engraftment and lymphoid reconstitution capacity *in vivo,* at levels comparable to fresh cells. Under these conditions efficient transduction of B, T, and NK cell progenitors, as well as long-term engraftment of gene-corrected stem/progenitor cells in ADA-SCID patients, can be achieved.

METHODS

Purification of human CD34$^+$ cells. UCB was obtained from discarded umbilical tissues at the Ospedale San Raffaele (Milan, Italy). Human BM was obtained as residual material of BM harvest from the iliac crest of healthy adults or children, under informed consent, or obtained from ADA-SCID patients for diagnostic or therapeutic purposes, under informed consent. After mononuclear cells were isolated by density gradient (Lymphoprep; Axis-Shield, Norway), CD34$^+$ cells were isolated by magnetic selection (Miltenyi Biotec, Bologna, Italy).

Gene transfer into CD34$^+$ cells. CD34$^+$ cells were prestimulated for 24 h in X-VIVO 10 medium (Biowhittaker, Verviers, Belgium) supplemented with 4% FCS (Hyclone), L-glutamine (Gibco BRL, Paisley, UK), penicillin/streptomycin, and the cytokines TPO (100 ng/ml), Flt3-ligand (300 ng/ml; both from PeproTech, London, UK), and SCF (300 ng/ml; R&D Systems, Minneapolis, MN, USA), plus/minus IL-3 (60 ng/ml; R&D) or IL-7 (20 ng/ml; PeproTech). Cells were then subjected to three overnight transductions on Retronectin-coated plates (Takara Biomedicals, Shiga, Japan), with retroviral supernatant supplemented with the same cytokines utilized during prestimulation, the first transduction being performed with the preloading method [53]. At day 4 cells were counted, washed, and plated in the B/NK differentiation assay and/or injected *in vivo* into BM/Thy/Liv grafts of SCID-hu mice; an aliquot was analyzed by FACS for the expression of ΔNGFR and CD34.

Flow cytometric analysis. The mouse anti-human monoclonal antibodies used were CD34, CD38, CD56, CD19, CD4, CD8 (Becton–Dickinson, San Jose, CA, USA); CD45, IgM (Caltag, Burlingame, CA, USA); and ΔNGFR (clone 20.4), HLA-A2 (clone CRII531), and HLA-A3 (clone HB122) produced at HSR. Antibodies were conjugated with phycoerythrin, fluorescein isothiocyanate, TC, or biotin. Streptavidin–FITC (Vector, Burlingame, CA, USA), –phycoerythrin, or –TC (Caltag) revealed biotinylated antibodies. Flow cytometric analyses were performed on a FACScan using the CellQuest software (Becton–Dickinson).

Retroviral vectors and supernatant production. The BML-1 vector contains the ΔNGFR gene under the control of MoMLV [53]. In the GIADAl vector, the ADA gene is under the control of the MoMLV LTR, and the neo^R gene is driven by the SV40 promoter [6,47]. Packaging cell lines were seeded at 5×10^4 cells/cm^2 and subjected to two replacements of supernatant with fresh medium in 2 days, before retroviral production. Supernatants were produced in IMDM (Biowhittaker) containing 10% FCS (Mascia Brunelli, Milan, Italy) and L-glutamine for 12 h, filtered, and stored at −80°C until further use.

In vitro assay for B/NK cell progenitors. Fresh or transduced CD34$^+$ cells were plated at 10 cells/well on flat-bottom 96-well plates preseeded with a semiconfluent layer of the MS5 stromal cell line. Culture medium was the same for all the CD34$^+$ cells plated, independent of the cytokines used during transduction, and consisted of RPMI (Euroclone, UK) supplemented with 10% human serum from healthy blood donors, 5% FCS, L-glutamine, penicillin/streptomycin, and the recombinant human cytokines TPO, Flt3-ligand, SCF, IL-7, and IL-15 (R&D) (20 ng/ml) and 250 IU/ml IL-2 (Roche, Milan, Italy) ([54, modified). Half the medium was replaced weekly. At weeks 3–4, wells with visible cell proliferation were selected and cells collected to be analyzed by FACS for the presence of CD19 B cell marker and CD56 NK cell marker. When cultures were established from transduced cells, additional staining to detect ΔNGFR$^+$ cells was included. The CD34$^+$ cell concentration chosen for the assay ensures clonality for lymphoid progenitors, as assessed after limiting dilution experiments in normal or ADA-SCID BM as well as in UCB CD34$^+$ cells (data not shown). Based on these data, to evaluate the absolute numbers of lymphoid progenitors it was assumed that each well containing B or NK cells represented a clone initiated by a single progenitor of a B or NK lymphocyte. Transduction experiments were normalized for 10^5 cells/culture condition to test. The formula used was

$$\text{Absolute number} = n \times (10^5/\text{SC}) \times \text{FI},$$

where n is the number of wells positive for B or NK cells, SC (seeded cells) is the total number of cells seeded in the assay (for example, for one 96-well plate, 10×96), and FI (fold increase) is the ratio between the number of cells at the end of the transduction experiment (day 4) and the number of cells plated at day 0. When referring to the absolute numbers of transduced lymphoid progenitors, n is the number of wells containing cells coexpressing ΔNGFR and CD19 (transduced B cell progenitors) or cells coexpressing ΔNGFR and CD56 (transduced NK cell progenitors).

In vivo assay for T/B cell progenitors. C.B-17 *scid/scid* (SCID) mice or *scid/beige* mice transplanted with human fetal thymus, bone, and liver (BM/Thy/Liv SCID-hu mice) were prepared as described [55], in accordance with the guidelines set forth by the local ethical committee. Before surgery, animals were anesthetized with tribromoethanol (240 mg/kg) administered intraperitoneally. Pieces of human tissue were placed subcutaneously and allowed to vascularize for 10 weeks before reconstitution with CD34$^+$ cells. Mice received 2.3 Gy whole-body γ-irradiation, followed by the direct injection into the grafts of the same number of cells for each patient or healthy donor ($0.5–2.5 \times 10^5$), either fresh or cultured. Engraftment was analyzed 8–9 weeks postinjection. Mice were sacrificed and grafts reduced to cellular suspensions for phenotypic analysis. Cells derived from secondary transplant mice injected with ADA-SCID patient's cells were incubated with anti-human CD19 microbeads (Miltenyi Biotec) to isolate B lymphocytes using the miniMACS system. T lymphocytes were purified from the CD19-negative fraction after simultaneous incubation with anti-human CD4 and CD8 microbeads. T cell lines were established from the T cell fraction by stimulating the cells with irradiated primary allogeneic peripheral blood mononuclear cells and JY cell line feeder mixture, in the presence of PHA and IL-2, as described elsewhere [56].

Quantitative PCR. Real-time PCR analysis was performed on sorted T and B lymphocytes collected from secondary transplant SCID-hu mice 8 weeks after ADA patient BM CD34$^+$ cells were injected to detect transduced (neo) cells. The glyceraldehyde-3-phosphate dehydrogenase gene was used to standardize for DNA content. DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA, USA) or after proteinase K digestion for fewer than 10^5 cells. Amplification reactions and analyses were carried out as described elsewhere [6,47]. The frequency of transduced cells was calculated using a standard curve (from 100 to 0.01%) and expressed as the proportion of cells containing the neo^R gene.

Statistical analysis. Statistical analyses were performed using two-tailed Student t tests, after the Shapiro–Wilk test for normality, unless otherwise stated. The paired t test was chosen to compare: (a) percentage and yield of transduced CD34$^+$/CD38$^-$ cells under the different culture conditions (Fig. 1B) and (b) lymphoid progenitor numbers before and after culture of CD34$^+$ cells (Fig. 3). The one-sample, two-tailed t test was chosen in Fig. 2 to assess the average fold increase of percentages (Fig. 2A) or yields (Fig. 2B) of transduced CD34$^+$ cells when IL-3 or IL-7 was added to T/F/S, against the null hypothesis $\mu_0 = 1$ (representing T/F/S alone). The independent t test was applied to compare the different CD34$^+$ cell sources for their content in lymphoid progenitors (Fig. 3 and

Results) and data from SCID-hu mice injected with $CD34^+$ cells (Fig. 6). Differences in the data were considered significant when $P < 0.05$.

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