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Reconstitution of T-cell compartment after *in utero* stem cell transplantation: analysis of T-cell repertoire and thymic output

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A B S T R A C T

Background and Objectives. *In utero* transplantation of hematopoietic stem cells allows immune reconstitution of fetuses with severe combined immunodeficiency. The objective of this work was to study the quality of T-cell reconstitution following this procedure.

Design and Methods. We evaluated the kinetics and extent of T-cell reconstitution in five infants with severe combined immune deficiency (SCID), three with a B^+ and two with a B^- phenotype, who received haploidentical stem cell transplantation before birth. To this end, we measured the frequency of T-cell receptor excision circles (TREC) and the diversity of the T-cell repertoire.

Results. *In utero* transplantation led to engraftment of donor-derived T lymphocytes which attained normal numbers in four infants, who are in good health. In the three patients with a B^+ phenotype, generation of a heterogeneous T-cell repertoire was associated with development of TREC levels comparable to those of SCID patients treated by post-natal transplantation and of healthy babies. Of the two patients with a B^- phenotype, one developed mixed T-cell chimerism and a substantial number of circulating T cells, associated with a variable heterogeneity of the T-cell repertoire; TREC levels were normal soon after birth, but declined thereafter. The remaining B^- patient remained lymphopenic with a skewed T-cell repertoire and very low TREC levels. This patient eventually required transplantation from a matched unrelated donor at 5 years of age, but died of EBV-related lymphoproliferative disease.

Interpretation and Conclusions. These data indicate that *in utero* transplantation of fetuses with B^+ SCID allows generation of newly diversified T lymphocytes and ensures long-term reconstitution of cell-mediated immunity.

Key words: *in utero* stem cell transplantation, T-cell repertoire, TREC, severe combined immunodeficiency.

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Hematopoietic stem cell transplantation (HSCT) from HLA-identical siblings allows immunological reconstitution and long-term survival of nearly all children affected by severe combined immunodeficiency (SCID). Unfortunately this option is available only to a minority of patients. HSCT from haploidentical or from matched unrelated donors allows long-term survival in a lower but still considerable proportion of SCID infants.[1] When the graft is T-depleted, this type of transplantation is followed by profound immunodeficiency, which may last for several months and may contribute to the high incidence of infections.[2-5] Although the nature of these infections clearly points to a T-cell deficiency,[6-10] the reason why T-cell immunity is impaired after transplantation, in spite of the rapid recovery of normal numbers of T lymphocytes, has been elucidated only recently, and principally in transplanted adults. After T-cell-replete HLA-matched transplantation with marrow containing relatively large number of donor lymphocytes, early T-cell reconstitution results, at least in part, from the expansion of mature donor T cells that may provide protection against infections early after transplant. Because these donor-derived lymphocytes are the progeny of only a few precursors, their T-cell receptor (TCR) repertoire is of limited diversity.[11] In the case of haploidentical HSCT, the bone marrow is depleted *ex vivo* from mature T cells before transplantation in order to avoid severe graft-versus-host disease. Because of the low number of mature T cells co-transfused with the graft, early after transplantation some TCRBV (TCR variable β) families may consist of a single or a few clones that proliferate in response to antigens.[12] Conse-

quently, the first wave of T cells derives from a thymus-independent pathway of reconstitution,[13-15] and is often inadequate to protect against infections. The resulting mature T lymphocytes can show limited TCR diversity and can persist in the periphery for several years.[16,17] Both in HLA-identical and in haploidentical HSCT, the T-cell compartment of the recipients can be subsequently reconstituted through a second mechanism that involves selection of graft-derived precursor cells in the thymus[18-20] and/or, possibly, in the periphery.[21,22] This process of thymic T-cell selection accounts for a more durable reconstitution of the T-cell compartment and eventually creates a more diverse TCR repertoire. These two mechanisms of immune reconstitution have been reported not only in adults, but also in children: 3 months after HLA identical transplantation, leukemic patients receiving T-cell-depleted grafts and, to a lesser extent, also those receiving undepleted grafts, show a restricted diversity of TCR repertoire in the repopulating T cells, while one year after treatment, the complexity of the T-cell repertoire appears to be complete in almost all recipients.[23] The degree of T-cell heterogeneity, therefore, represents a useful marker of T-cell reconstitution after transplantation. More recently, a complementary assay has been developed to investigate the extent of immune reconstitution; this assay measures the frequency of TCR excision circles (TREC). TREC are by-products of TCR rearrangement; they are stable and do not replicate with cellular proliferation.[24,25] Consequently, TREC quantification after transplantation represents a sensitive measurement of the capacity to generate new peripheral blood T cells. In adults, TREC levels have been shown to remain low for 3 months after transplantation, to become higher by 6 months and to normalize, with respect to healthy age-matched controls, from 6 to 12 months after transplantation.[26] TREC are virtually absent in SCID babies; following HSCT in the neonatal period they peak earlier and to higher numbers than following HSCT carried out after 28 days of life.[27] Therefore, SCID infants who undergo HSCT in the neonatal period show higher and earlier level of thymic output than those receiving HSCT later in life, thus attaining reconstitution of a normal T-cell repertoire more rapidly. This difference may contribute to the higher survival rate following HSCT in the neonatal period. In spite of the progress in post-natal HSCT, this procedure is still associated with significant morbidity and mortality, as well as with an important economic and social burden. We and others have reported successful *in utero* HSCT (IUT) of fetuses affected with SCID.[28-30] The rationale for IUT in SCID fetuses is based on at least 4 assumptions. First, unlike the post-natal setting, the immature fetal immune system may be relatively more permissive to the transplantation of partial or non-HLA- matched donor cells that will ultimately be recognized as self by the fetus. Second, abundant hematopoietic niches or homing sites are supposedly generated during fetal development, facilitating engraftment and expansion of donor-derived stem cells. Third, IUT may avoid the need for prolonged hospitalization, both before and after transplantation, which makes conventional post-natal transplantation a risky and expensive treatment. Finally, if successfully treated by IUT, SCID newborns may develop functional T cells at birth or soon after birth, thus reducing the risk of post-natal opportunistic infections.[30] Successful allogeneic *in utero* transplantation has been reported in several animal models but, to date, it is unclear whether the failure of most cases of prenatal allogeneic hematopoietic stem cell transplantation in humans is related to fetal immune function, HLA mismatching, the lack of potential space for the donor cells, the types and numbers of transplanted cells, or to the inability of donor cells to home to the sites of engraftment. Furthermore, no information is available on the quality of T-cell reconstitution following IUT.

In this study, we have analyzed in detail the extent of T-cell reconstitution in the 5 SCID patients who underwent IUT in our institution.

Design and Methods

Study population

Between 1995 and 2001, 5 patients (IUT#1 through IUT#5) underwent IUT with purified haploidentical $CD34^+$ cells at Spedali Civili Hospital, Brescia (Italy), according to a clinical protocol approved by the Institutional Review Board. Molecular and immunological investigations revealed that two patients (IUT#1 and IUT#5) had T^- B^+ X-linked SCID (SCIDX1) due to defects of the common γ chain (γ_c), one patient (IUT#3) suffered from autosomal recessive SCID due to mutations of the interleukin-7 receptor α chain (*IL7RA*) gene, one patient (IUT#4) had a T^- B^- SCID due to compound heterozygosity for *RAG2/B*$^-$ gene mutations, and the remaining patient (IUT #2) had T^- B^- SCID of unknown genetic origin. Haploidentical purified $CD34^+$ cells were obtained and transplanted as previously reported[28] between the 21st and the 24th week of gestation. The haploidentical donor was the father in patients IUT#1, IUT#2, IUT#3 and IUT#5, and was the mother in patient IUT#4. Information on the type of donor, number and source of transplanted cells, the count of residual T cells that were present in the graft, the evaluation of maternal T-cell engraftment at birth, and a short description of post-natal clinical course are presented in Table 1, while a detailed report of the clini-

Table 1. Characteristics of the transplant and postnatal transplantation course.

Patients	*Donor*	*Type of cells*	*Number of cells ($\times10^6$/Kg)*	*Residual T cells ($\times10^4$/Kg)*	*Maternal engraftment at birth*	*Postnatal clinical course*
IUT #1	Father	BM CD34$^+$	19.4	6.7	no	Uneventful, no GvHD. Discharged at 24 days of life
IUT #2	Father	BM CD34$^+$	16.0	8.5	no	No evidence of GvHD. Persistent, severe lymphopenia. Boosting of paternal CD34$^+$ cells at 8 months and at 3.1 years. Growth arrest since 4 years of life. MUD transplant at 5 years. Deceased of EBV-LPD at day +102 after MUD-transplantation
IUT #3	Father	BM CD34$^+$	20.0	4.0	no	Uneventful, no GvHD. Discharged at 42 days.
IUT #4	Mother	PB CD34$^+$	21.0	5.5	ne*	Transient respiratory distress at birth. No GvHD. Discharged at 52 days of life. Granulomatous pneumonia at 3.4 years.
IUT #5	Father	BM CD34$^+$	16.5	2.6	no	Uneventful, no GvHD. Discharged at 14 days of life.

**ne: not evaluable because transplanted cells were of maternal origin: BM: bone marrow; PB: peripheral blood; MUD: matched unrelated donor; EBV-LPD: Epstein-Barr virus related lymphoproliferative disease.*

cal and laboratory follow-up of these patients, including the response to vaccines and the analysis of B-cell function, will be described elsewhere (*manuscript in preparation*). None of the patients presented clinical or biological signs of graft-versus-host disease (GvHD). Accordingly, none of them received GvHD prophylaxis or treatment.

Peripheral blood samples were obtained at multiple time points after IUT as part of the routine post-transplant clinical care, as indicated in the approved clinical protocol. To determine whether the peripheral blood T cells of IUT babies were of donor or recipient origin, chimerism analysis was performed at serial time points, using highly polymorphic DNA markers DQα and D1S80 and the DNA Typing kit (Applied Biosystems, Foster City, CA, USA).

Samples from SCID infants who were treated with post-natal haploidentical HSCT with purified CD34$^+$ cells and from healthy infants of the same ethnic origin were used as controls.

Analysis of T lymphocytes subsets and in vitro response to mitogens

The distribution of the major T-cell subsets at various time-points after IUT was analyzed by flow cytometry, using directly-conjugated monoclonal antibodies to CD3, CD4, CD8, and CD45RA (Becton-Dickinson, Mountain View, CA, USA). In order to measure *in vitro* proliferative response to mitogens, peripheral blood mononuclear cells (PBMC), immediately isolated from heparinized blood samples by Ficoll/Hypaque density gradient centrifugation, were cultured in triplicate (2×10^5/well) in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, and 50 mg/mL gentamycin, with or without phytohemagglutinin (PHA, 10 μg/mL, Difco Laboratories, Detroit, MI, USA). Cultures were incubated at 37°C, 5% CO_2 for 3 days, and then pulsed with 1 μCi per well of ^{3}H-thymidine (Amersham Biosciences, Cologno Monzese, Italy) during the last 6 hours of culture. Cells were then harvested, and incorporated radioactivity was determined with a scintillation β-counter.

Analysis of T-cell diversity

Total cytoplasmic RNA and cDNA for the analysis of TCRBV chain diversity were prepared from PBMC as previously described.[31] One to two micrograms of total RNA, prepared by the guanidium thiocyanate-phenol-chloroform method, were used to synthesize the first strand of the TCRB chain-specific cDNA using a primer specific for TCRBC1 and TCRBC2 genes (βcDNA: 5' GGG CTG CTC CTT GAG GGG CTG CGG 3'). For the analysis of TCRBV usage, PCR products, obtained with amplification performed with a TCRBV degenerate primer TCRBV (Vβd: 5' ACG TAG ATT CT(GT) T(ACT)(CT) TGG TA(CT) (AC)(AG)(AT)CA 3') and a TCRBC primer (βAI: 5' CCC ACT GTG CAC CTC CTT CC 3'), were hybridized with probes specific for each TCRBV segment. The hybridization event was revealed by a colorimetric method.[31] For heteroduplex analysis,[32] the TCRBV chains of interest were amplified by 35 cycles of PCR, using TCRBV-specific oligonucleotides (TCRBV1: 5' GCA CAA CAG TTC CCT GAC TTG CAC 3'; TCRBV2: 5' TCA TCA ACC ATG CAA GCC TGA CCT 3'; TCRBV3: 5' GAT ATG GAC CAT GAA AAT ATG TTC 3'; TCRBV4: 5' GCC CAA ACC TAA CAT TCT CAA CTC 3'; TCR-

BV6: 5' AGG CCT GAG GGA TCC GTC TC 3'; TCRBV8: 5' ATT TAC TTT AAC AAC AAC GTT CCG 3'; TCRBV13S1: 5' CAA GGA GAA GTC CCC AAT 3'; TCRBV13S2: 5' GGT GAG GGT ACA ACT GCC 3'; TCRBV14: 5' GTC TCT CGA AAA GAG AAG AGG AAT 3' and TCRBV17: 5' AGA TAT AGC TGA AGG GTA CAG CGT 3') and the TCRBC primer βAI. Amplification products were heated to 95°C for 5 minutes and then cooled to 50°C for 1 hour. The annealed samples, kept on ice until used, were run for 5-6 hours at 200 V, at room temperature, on a 12% non-denaturating polyacrylamide gel (PAGE; 29:1 acrylamide/bisacrylamide) performed in 1X TBE buffer (0.089 M Tris-borate and 0.002 M EDTA, pH 8.0). The gels were stained for 30 minutes, at room temperature, in the dark, in a solution containing 0.75 μg/mL ethidium bromide in 200 mL of 1X TBE and then photographed under UV light.

TCRBV1 and TCRBV13S2 amplified products were purified, cloned and sequenced as described elsewhere.[31,32] Sequences were compared with published data relative to TCRBV, TCRBD and TCRBJ segments.[33]

Real-time PCR detection and measure of TREC

TREC were determined at various times after IUT. Samples obtained from 30 healthy infants (age range: from 3 months to 2 years) were used as controls.

Genomic DNA was extracted from about 3×10^6 PBMC using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantification of the signal-joint TREC was performed by means of real-time PCR.[34] Each 50 μL reaction contained 200-400 μg of DNA; the final concentration of each component was as follows: 1X Universal PCR Master Mix (Applied Biosystems), 10 pmol/μL of probe and 45 pmol/μL of each primer. The primers (5' GGA TGG AAA ACA CAG TGT GAC ATG G 3' and 5' CTG TCA ACA AAG GTG ATG CCA CAT CC 3') amplified a 192 bp product. The probe (5' CCC TGT CTG CTC TTC ATT CAC CGT TCT CA 3'), containing FAM (6-carboxy-fluorescein) as fluorescent report dye covalently linked to the 5' end and quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) linked to 3' end, was included in the reaction mixture throughout PCR to serve as a real-time detector for the amplified product. PCR (45 cycles at 95°C for 15 seconds and 60°C for 90 seconds) was carried out in a spectrofluorimetric thermal cycler (ABI PRISM 5700; Applied Biosystems).

To avoid PCR contamination, DNA isolation and PCR analysis were performed in two different rooms with separate reagents and equipments. Aerosol-free tips were used throughout the PCR protocol. The primers and the probe were diluted with DNase-free water, aliquoted at 50 μL and frozen at -20°C until use.

Results

Development of T-cell immunity following IUT

The absolute number of circulating lymphocytes and the distribution of T-cell subsets are reported in Figure 1 (panels a to f). All patients with a B^+ SCID phenotype (i.e., patients IUT#1, IUT#3, and IUT#5) attained a normal lymphocyte count already by birth or early after birth (Figure 1, panel A). This was paralleled by a normal proportion of mature $CD3^+$ T cells (Figure 1, panel B). A normal distribution of $CD4^+$ and $CD8^+$ subsets was observed in patients IUT#3, and IUT#5, whereas patient IUT#1 developed an excess of $CD8^+$ T cells (Figure 1, panels C and D). In these patients, a high proportion of $CD4^+$ cells co-expressed the CD45RA antigen, suggesting efficient *de novo* thymopoiesis (Figure 1, panel E). Patients IUT#2 and IUT#4 (i.e., those with a B- SCID phenotype) remained lymphopenic (Figure 1, panel A); however, while patient IUT#4 developed a substantial number of $CD3^+$ and $CD4^+$ T cells, this was not the case for patient IUT#2 (Figure 1, panels B and C). Because of persistent T-cell lymphopenia, patient IUT#2 received HSCT from a matched unrelated donor at 5 years of age, after full conditioning with busulfan and cyclophosphamide, but died on day +102 of EBV-lymphoproliferative disease.

In all patients, molecular analysis at the highly polymorphic loci DQα and D1S80 showed the presence of donor-derived cells among $CD3^+$ cells that had been positively selected using CD3-coated magnetic microbeads. Donor-derived T cells represented the totality of $CD3^+$ T cells in patient IUT#5, and accounted for the vast majority of T cells in patients IUT#1 and IUT#3. A mixed chimerism, with a similar proportion of autologous and donor-derived T cells was observed in patient IUT#4. In patient IUT#2, the few circulating $CD3^+$ cells were mostly donor-derived.

In vitro response to PHA became normal early after birth in patients IUT#1, IUT#3, and IUT#5, and progressively attained low-normal levels in patient IUT#4, while it remained severely impaired in infant IUT#2 (Figure 1, panel F).

Analysis of TCRBV repertoire diversity

To guide the analysis of TCR repertoire in IUT patients, we first established which are the most frequently represented TCRBV chains in healthy infants. The percentage of TCRBV expression, studied in 12 healthy babies by PCR followed by analysis of the amplified products' specificity, demonstrated that, although there are differences in the absolute magnitude of T cells expressing a particular TCRBV chain, some TCRBV segments are preferentially detected in all babies (Figure 2A). The analysis of the diversity of the 10 most represented TCRBV segments, performed by

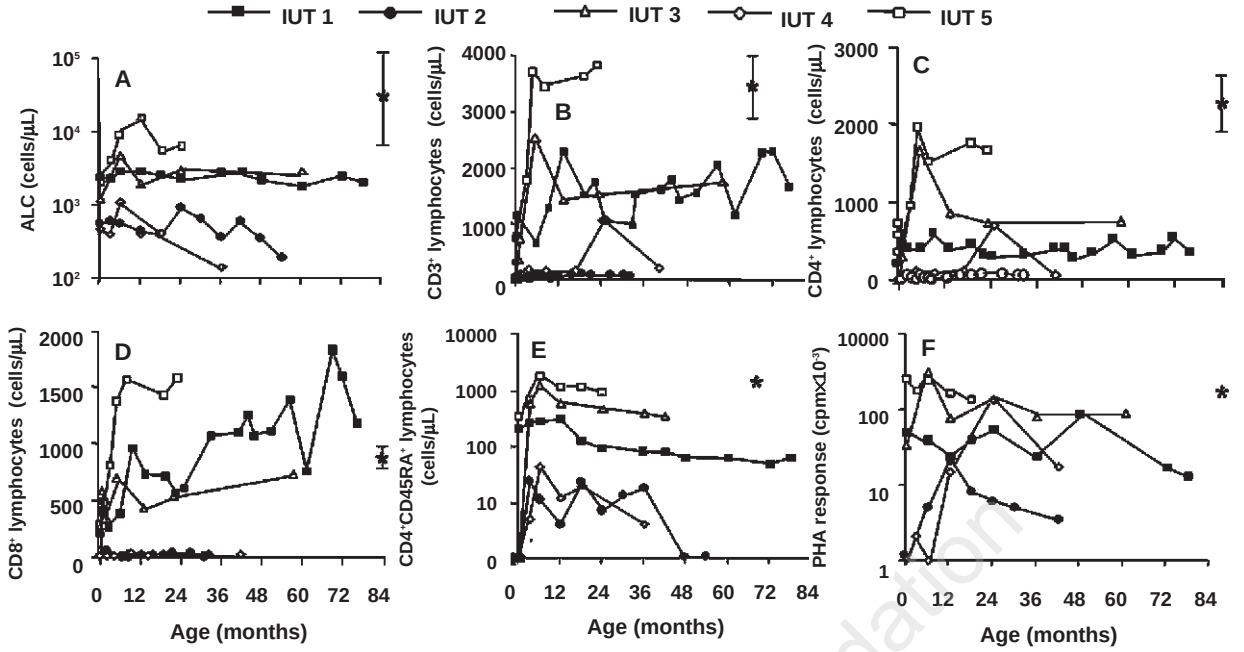

Figure 1. Immune reconstitution following IUT in 5 fetuses affected with SCID. *Panel A*: reconstitution of absolute lymphocyte counts (ALC); *panel B*: analysis of the absolute number of $CD3^+$ lymphocytes; *panel C*: absolute number of $CD4^+$ lymphocytes; panel D: absolute number of $CD8^+$ lymphocytes; panel E: absolute number of $CD4^+CD45RA^+$ lymphocytes; *panel f*: *in vitro* proliferative response to PHA. Age-specific values (*) are shown on the left side of each panel.

heteroduplex analysis in 4 of these babies, was consistent with our previous observations[31,35] showing a fully heterogeneous TCR repertoire profile, as indicated by the presence of smears across every TCRBV-specific PCR products (Figure 2B).

Based on these data, and due to the limited amount of available biological material obtained from patients at various time points after IUT, heteroduplex analysis was performed to assess the heterogeneity of the most represented TCRBV subfamilies. The results of such analysis for the 5 patients treated by IUT are shown in Figures 3 and 4. Patients with a B^+ SCID phenotype (IUT#1, IUT#3, and IUT#5) developed a more or less heterogeneous T-cell repertoire. In particular, in patient IUT#5 TCRBV-specific PCR products migrated in the polyacrylamide gels principally as smears at all time-points, suggesting the attainment of a truly polyclonal repertoire by birth. In patients IUT#1 and IUT#3, the simultaneous presence of homoduplex and heteroduplex bands, superimposed on smears, suggests oligoclonal expansions in the context of a polyclonal T cells (Figure 3). In contrast, in patient IUT#2, the initial evidence of broad T-cell diversity was followed by marked oligoclonality indicating a highly restricted T-cell repertoire. In patient IUT#4, a variable pattern of diversity of the TCR repertoire was observed during follow-up, with a final shift towards oligoclonality (Figure 4).

In order to confirm the results obtained by heteroduplex analysis, we decided to clone and sequence the TCRBV13S2 segment of patient IUT#4 (at various time-points, when, contrary to that observed for most of the other segments analyzed, heteroduplex analysis was suggestive of a shift from oligoclonal to polyclonal representation), and the TCRBV1 chain of patient IUT#1 which, at the time-points analyzed, was the most heterogeneous segment of this baby. As shown in Figure 5, the results of cDNA sequencing confirmed those obtained by heteroduplex analysis. In particular, in a first group of TCRBV13S2 sequences prepared from patient IUT #4 at one month of age, 10 out of 20 cDNA clones showed the same NDN region (Figure 5, panel A). In a second group of sequences, prepared 4 months after birth, there were only few clones with the same NDN regions (Figure 5, panel B). Finally, in the last group of sequences, from samples obtained at 8 months of age, all clones were different from each other (Figure 5, panel C). TCRBV1 clones generated from patient IUT#1 were free of predominant sequences in the 3 samples obtained at a one-year intervals from each other, thus confirming the heterogeneity of the cells expressing this TCRBV chain.

Analysis of TREC in peripheral blood T cells

As shown in Figure 6, patients with a B^+ SCID phenotype (IUT#1, IUT#3, and IUT#5) attained and main-

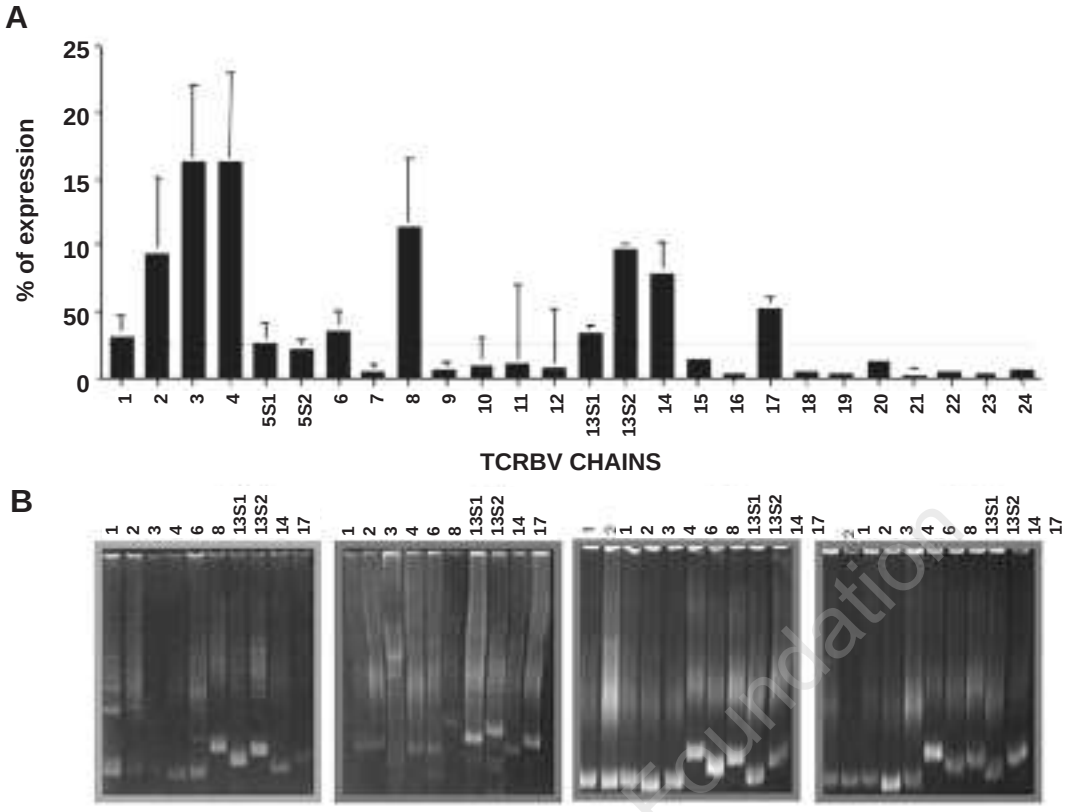

Figure 2. Analysis of T-cell repertoire of lymphocytes of healthy babies. (A) Percentage of TCRBV usage. The data are expressed as mean ± standard deviation of values obtained with lymphocytes of 12 babies whose age ranged from 3 months to 2 years. TCRBV segments with an expression > 2.5% (dots line) are considered as preferentially represented (B) Hetereroduplex analysis performed on the most expressed TCRBV chains of 4 representative babies. The number TCRBV segments loaded in each line is shown at the top of each gel.

tained TREC levels that were comparable to those of SCID patients treated by post-natal HSCT and only slightly lower than those of age-matched healthy infants. In patient IUT#4, after an initial evidence of effective thymopoiesis (as demonstrated by detectable number of TREC), the levels of TREC progressively declined. With the exception of very low TREC at 6 months of age, no evidence of active thymopoiesis was obtained for patient IUT#2.

Discussion

Although HSCT is a life-saving procedure in infants with SCID, it is often associated with prolonged immune deficiency when performed with T-cell depleted, haploidentical stem cells. Early after transplantation, circulating T lymphocytes often carry activation markers, and are functionally impaired in their proliferative response to mitogens and antigens. This first wave of T-cell reconstitution results most often from the peripheral expansion of mature T cells contained in the graft,[1,36-38] and is therefore associated with a restricted T-cell repertoire.

The recovery of normal T-cell function after HSCT depends on the generation of large numbers of T cells with a heterogeneous TCR repertoire, a process that, in adults, usually requires several months to be completed, probably also because of the physiologic involution of the thymus occurring after childhood.[26] Indeed, most of the T-cell pool is produced around birth, and it is not known whether the adult thymus retains the capacity to sustain generation of a completely new repertoire.[39,40] T-cell reconstitution appears to occur more rapidly in children[23,41,42] and SCID infants who receive HSCT in the neonatal period have a better thymic output, and show a higher survival rate and lower morbidity than do those treated later in life.[27] These data suggest that thymic function plays an important role in the reconstitution of T-cell immunity following HSCT, that even the hypotrophic thymus of SCID is able to support T-cell development from donor stem cells and, more importantly, that the success of HSCT may also depend on the precocity of the intervention. The ability to diagnose SCID in the first trimester of pregnancy led us and others to

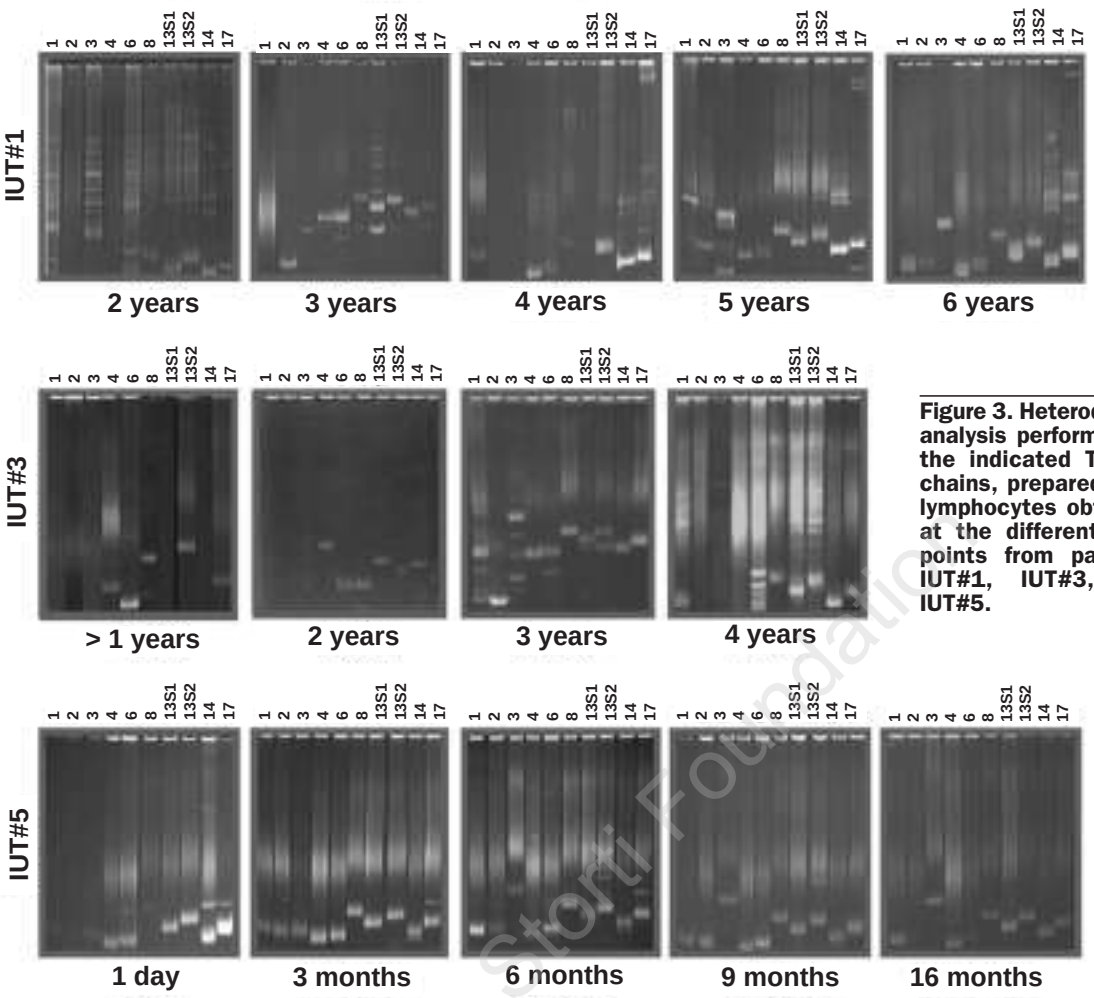

Figure 3. Heteroduplex analysis performed on the indicated TCRBV chains, prepared from lymphocytes obtained at the different time points from patients IUT#1, IUT#3, and IUT#5.

attempt prenatal HSCT, with the hope that the infant would be born with a fully reconstituted immune system or that the immune system would be rapidly reconstituted after birth, thus decreasing the risk of infections and avoiding the need for prolonged hospitalization in a protected environment. Several reports have illustrated the successful use of IUT in the treatment of SCID.[28-30,43-45] These studies indicate that lymphoid reconstitution can be achieved with prenatal HSCT, but they do not comment on the kinetics and quality of T-cell reconstitution.

We have confirmed that prenatal transplantation of parental, haploidentical hematopoietic stem cells into SCID fetuses can be safely performed, and is associated with engraftment without use of chemotherapy and/or immune suppression. We also found that, in most cases, IUT may allow appearance of donor-derived T cells that have undergone maturation in the recipient's thymus, thus confirming that the thymus of SCID fetuses is able to support active thymopoiesis. Since our data indicated that it is unlikely that the quality and efficiency of T-cell reconstitution following IUT for SCID can be related to the number of transplanted cells, the count of residual T cells present in the graft, or the presence of maternal T-cell engraftment, we hypothesize that the different outcome of IUT in SCID may depend on the nature of the genetic defect, which in fact is different in different genetic forms of SCID.

In particular, IUT of fetuses affected with B^+ SCID (patients IUT#1, IUT#3, and IUT#5) resulted in normalization of the number and function of circulating T cells with more or less polyclonal repertoires. This recovery of T-cell immunity was paralleled by the appearance of high numbers of TREC, whose values were only slightly lower than those of healthy age-matched infants.

In contrast, results of IUT were less satisfactory in infants with B^- SCID (patients IUT#2 and IUT#4). In particular, in spite of engraftment of donor-derived T cells, infant IUT#2 remained profoundly lymphopenic, and his T-cell repertoire rapidly became highly restricted. Moreover, no evidence for active thymopoiesis was ever recorded in this infant, as indicated by the negli-

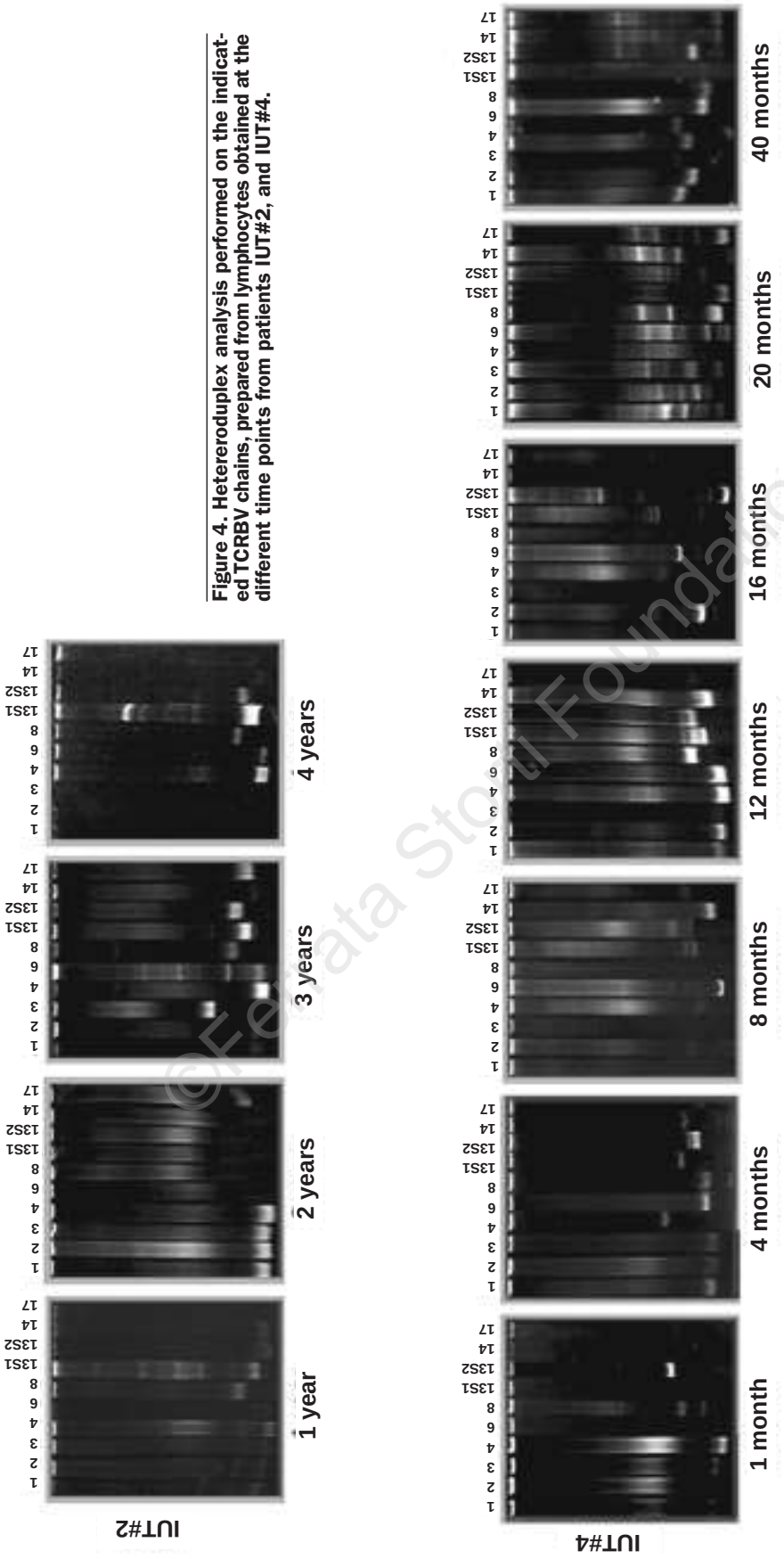

Figure 4. Hetereroduplex analysis performed on the indicated TCRBV chains, prepared from lymphocytes obtained at the different time points from patients IUT#2, and IUT#4.

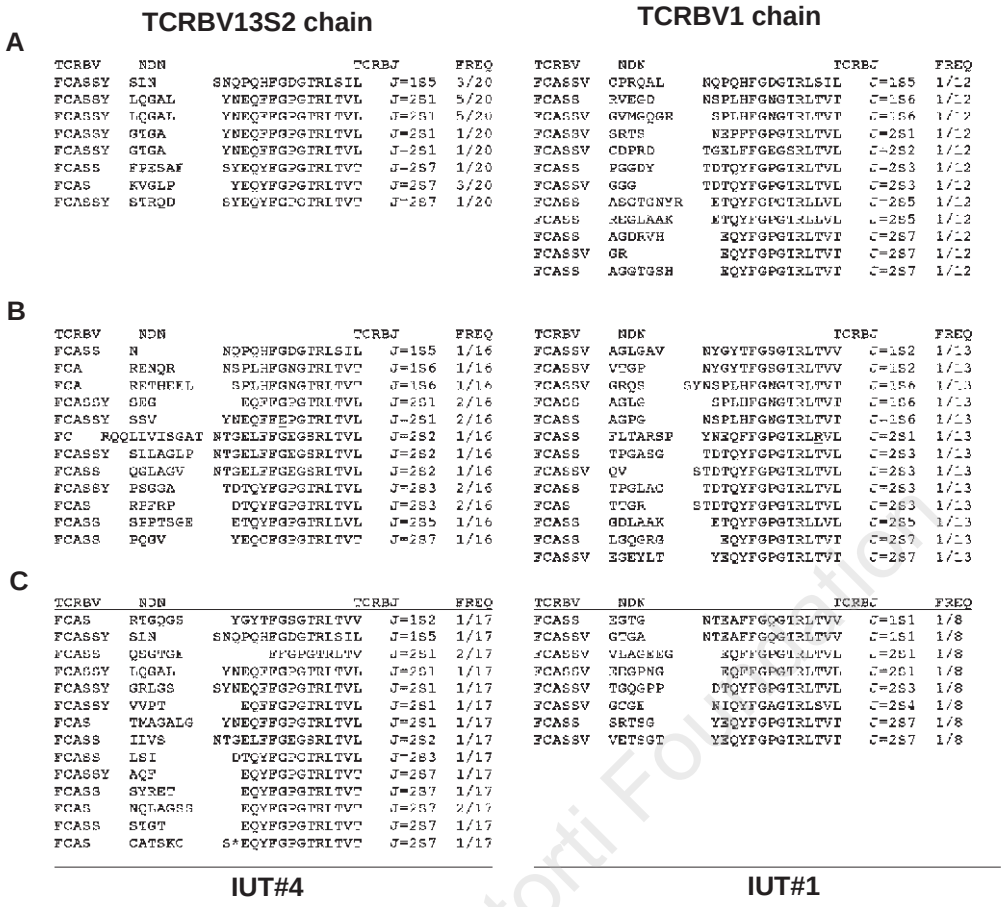

Figure 5. Sequence analysis. TCRBV13S2 cDNA clones were prepared from lymphocytes of patient IUT#4, obtained one (A), 6 (B) and 8 (C) months after birth. TCRBV1 cDNA clones were prepared from lymphocytes of patient IUT#1, obtained one (A), 2 (B) and 3 (C) years of life. Only the last amino acids of TCRBV segments are reported. FREQ: Number of clones with the same NDN region within the total number of clones sequenced for each groups. Mutated amino acids in the J regions are underlined. *indicates a stop codon.

gible number of TREC consistently documented after birth. The outcome of IUT in this infant was therefore considered a failure. Results were more difficult to assess in infant IUT#4. In spite of the progressive increase in the number of circulating T cells, and of low-normal T-cell function, as assessed by *in vitro* response to mitogens and ability to cope with post-natal infections, this patient failed to show active thymopoiesis, as indicated by the low number of both $CD4^+CD45RA^+$ T lymphocytes and TREC. Moreover, his T-cell repertoire progressively became oligoclonal.

Experience with post-natal HSCT from HLA haploidentical family donors supports the notion that this procedure is associated with a better outcome in B^+ vs. B^- SCID.[46] Among the hypotheses that have been put forward to account for this difference, B^-SCID, which includes cases with abnormal radiation sensitivity due to *Artemis* gene defects,[47] have been suggested to display high toxicity in response to radiomimetic drugs used for conditioning prior to HSCT. However, this interpretation cannot account for the results observed in our study because IUT was carried out without previous conditioning or any pharmacologic treatment whatsoever. One alternative explanation that may explain the better outcome of HSCT in B^+ vs. B^- SCID is based on the different selective advantage of donor-derived early T-cell precursors transplanted into SCID recipients pertaining to these two groups of SCID. In our series, B^+ SCID were due to defects of γ_c, shared by several cytokine receptors (as in patients IUT#1 and IUT#5), or of the *IL7RA* gene (as in patient IUT#3). In these genetic conditions, autologous early T-cell precursors are completely prevented from receiving any differentiative/proliferative signal through the IL-7R. The thymus is therefore empty and may be easily and rapidly repopulated from donor-derived precursors. Such a strong selective advantage of genetically normal cells has also been demonstrated in infants with X-linked SCID treat-

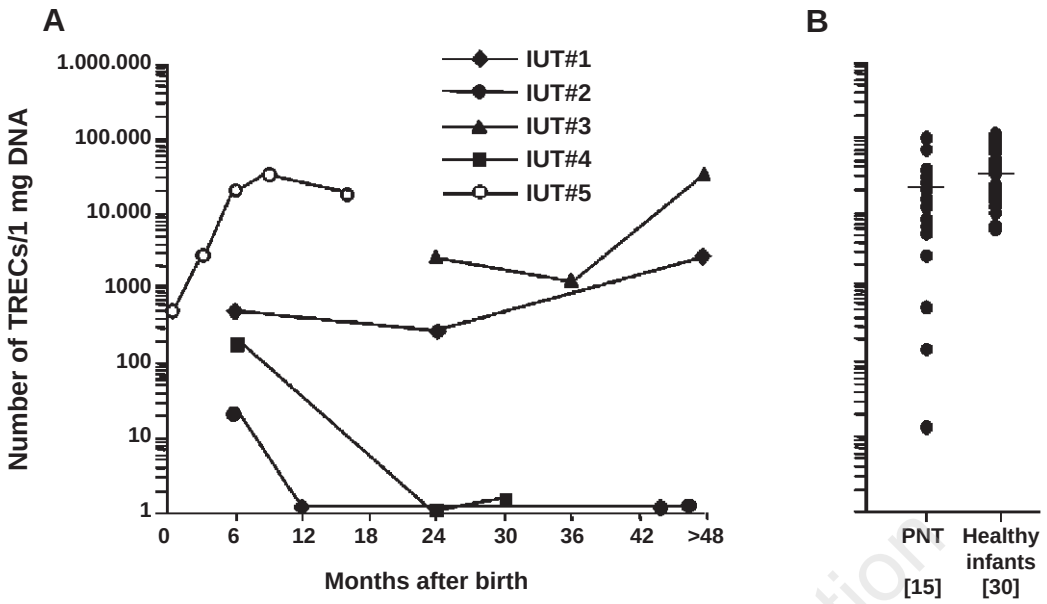

Figure 6. Evaluation of TREC. (A) Number of TREC in samples obtained at different time points from IUT patients. (B) Number of TREC in babies undergoing post-natal transplantation (PNT) and in healthy infants (age ranging from 3 months to 2 years after birth).

ed by gene therapy.[48-49]

In contrast, the block in thymopoiesis observed in B- SCID due to *RAG* or *Artemis* gene defects occurs later in T-cell development, and allows some thymic colonization by early lymphoid progenitors. We were unable to find the nature of the genetic defect in patient IUT#2, whereas compound heterozygosity for two *RAG2* gene mutations was documented in patient IUT#4. Importantly, a previous sibling in the latter family suffered from Omenn syndrome, and developed a substantial number of autologous, poorly functioning T cells. Patient IUT#4 is a compound heterozygote for a nonsense and a missense mutation. The latter results in a single amino acid change (F206C) which is likely to preserve some function of the RAG2 protein and hence to allow for residual autologous T-cell development, as observed also in other patients with Omenn syndrome.[50] Consequently, donor-derived lymphoid progenitors transplanted into this fetus may well have competed with autologous precursors for intrathymic differentiation. It is well known that conditioning is strictly required for postnatal HLA haploidentical transplantation to succeed in Omenn syndrome.[51] The prolonged survival, with substantial T-cell function in patient IUT#4, may further illustrate the potential of IUT vs. post-natal HSCT.

With the exception of patient IUT#5 (whose T-cell repertoire was polyclonal since birth), in all other patients discrete bands were demonstrated at the heteroduplex analysis, indicating the presence of some dominant clonotypes. These predominant TCR clonotypes appeared to vary from time to time in each patient, because homoduplex and heteroduplex bands were differently distributed in gels loaded with samples obtained at the different time points. Since all grafts may contain some residual T cells in spite of the T-cell purification, TCR clonotypes observed at birth most likely represent mature graft-derived T cells that have undergone expansion in the recipient. This may reflect proliferation in response to alloantigens or to exogenous antigens, as also suggested by the evidence of a highly restricted repertoire in SCID patients with maternal T-cell engraftment.[52,53] Finally, it is possible that post-natal factors may also play a role in shaping T-cell repertoire these patients.

However, other explanations for the observed oligoclonal expansions of T-cell repertoire must also be considered. Although heteroduplex analysis allows detection of subtle changes in the T-cell repertoire,[32] this method cannot determine the origin of the T cells examined. This is an important point because persistence of recipient hematopoiesis has been shown to affect the reconstitution of a normal TCR repertoire after transplantation.[54] Indeed, chimerism analysis allowed detection of recipient T cells in all of our patients, with the exception of IUT#5. In keeping with the nature of the genetic defect, autologous T cells were particularly abundant in patient IUT#4, accounting for approximately 50% of circulating T cells. The persistence of recipient T lymphocytes, expressing

alloantigens, can cause expansion or deletion of alloreactive T cells, and thus exert a profound effect on the development of the T-cell repertoire.

Overall, we found that IUT may support generation of a diverse T-cell repertoire in SCID fetuses, as shown by heteroduplex analysis and by evaluation of NDN diversity. Importantly, our finding of a high number of TREC in the periphery of B^+ SCID infants treated by IUT are in keeping with the recent demonstrations of active thymopoiesis following early post-natal HSCT in patients with SCID,[27] and indicate that, even if hypotrophic, the thymus of SCID infants is functionally capable of supporting T-cell development thus providing normal immune function for several years.[41] In evaluating these data, however, it must be considered that TREC levels can also be influenced by other variables, such as an elevated cell division rate or the induction of apoptosis.[55,56] Whether the impact of these variables is different in IUT and post-natal HSCT remains to be established.

In conclusion, the results of this study confirm that prenatal HSCT of fetuses with SCID allows survival and reconstitution of T-cell function. Moreover, our data suggest that quality of T-cell reconstitution following IUT is better for B^+ than for B- SCID. A long-term analysis of a larger series of patients is needed to confirm these results and to compare the quality of immune reconstitution following IUT with that obtained with post-natal transplantation in the neonatal period or later in life.

SP, FM and SG performed all the laboratory work. EM and FP were the physicians responsible for the patients' care, provided the material from the patients and collected clinical data; together with AL, SZ and SP carried out the IUT protocol. AA is the head of the division where the analyses were carried out. AGU and LDN developed the protocol of IUT transplantation and together with LI, designed, coordinated and analyzed data throught-out this project. All authors approved the final version of this article. The authors reported no potential conflicts of interest.

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