Introduction

Severe combined immunodeficiency (SCID) encompasses a wide spectrum of disorders of both cell-mediated and humoral immunity. Defects in lymphoid differentiation may lead to the complete absence of T and B cells (T-B− phenotype) or, alternatively, may predominantly affect T lymphocyte development (T-B+ phenotype). In a few patients with SCID with a predominant T-cell defect, T cells are detectable in peripheral blood but are not functional because of abnormalities in the cell activation process rather than because of differentiation defects.

In recent years, the identification of several molecular alterations has led to a better understanding of the mechanisms underlying SCID. Alterations in at least 8 genes have been identified and associated with a clinical phenotype of SCID. Abnormalities of signaling molecules, such as the common γ element of several cytokine receptors, the α chain of IL-7 receptor, and the JAK-3 tyrosine kinase that associates with the γ chain, are responsible for the X-linked or the autosomal recessive forms of T-B− SCID. Mutations of RAG-1 and RAG-2 have been described in autosomal recessive forms of the disease. Mutations of elements of the T-cell receptor (TCR)/CD3 multimeric complex, such as CD3ε or CD3γ chains, or of the 70-kd kinase that associates with the ζ chain of the complex, also result in the less severe forms of activation defects. It is noteworthy that all the genes so far implicated in the pathogenesis of SCID encode for proteins selectively expressed in hematopoietic tissue. This implies that in most patients, the affected cell line can be replaced by stem cells from healthy donors. However, an appropriate, productive immune response also requires appropriate intercellular connections. A relation between the hematopoietic precursor and the thymic epithelial component plays a crucial role in cell ontogeny and in negative and positive selection.

We previously reported on 2 sisters affected by SCID because of a T-cell defect associated with congenital alopecia and nail dystrophy. This complex phenotype was considered the human homologue of the mouse Nude/SCID phenotype. Subsequently, the siblings were found to carry a nonsense mutation in the highly conserved winged–helix–nude (WHN) gene. Alteration of this gene is responsible for mouse Nude/SCID. WHN is a forkhead–winged helix transcription factor whose expression is restricted to the thymic epithelium, epidermis, and hair follicle. Human Nude/SCID is the first example of a human SCID caused by a gene not expressed in the hematopoietic cells. Six years ago, before the molecular alteration was identified, the younger of the 2 WHN−/− siblings underwent allogeneic human leukocyte antigen (HLA)-related bone marrow transplantation (BMT) because of her critical clinical and immunologic conditions. The current study was undertaken to evaluate, in this unique model, the role of the thymus in immunologic reconstitution and the long-term efficacy of BMT in the human Nude/SCID phenotype.

Patient, materials, and methods

Patient history

The patient was born of consanguineous Italian parents with a complete absence of hair and dystrophic nails, as seen in her older sister. Thereafter,
like her older sister, she was found to have severe functional T-cell immunodeficiency. No thymic shadow was evident in either child’s involvement of 100 U/mL. With age, 5 months, the second child underwent HLA-identical total bone marrow transplantation from her unaffected brother, after only 2 doses of antilymphocyte rabbit globulin at a dose of 2.5 mg/kg and no immunosuppressive therapy or myeloablation by irradiation. The engraftment of donor cells was established through the analysis of highly polymorphic DNA regions using a B-D flow cytometer was used. For the proliferation assays, peripheral blood mononuclear cells were isolated by density gradient centrifugation as described above. Magnetic beads coated with anti-CD8+ monoclonal antibody (Dynal Biotech, Oslo, Norway) were used for the positive and negative sorting of T cells. Total RNA was extracted from positively or negatively sorted lymphocytes by using Tryzol (Gibco-BRL, Bethesda, MD). Reverse transcription–polymerase chain reaction (RT-PCR) was performed by using the one-step RT-PCR kit (Gibco-BRL) according to the manufacturer’s instructions.

**CDR3 heterogeneity length analysis**

Conditions for CDR3 size distribution analysis are described elsewhere. Briefly, cDNA was amplified through PCR with specific primers to 24 different Vβ families and a fluorescent Cβ primer. One microliter amplified product was mixed with 1.5 μL 100% formamide and 0.5 μL size standard (Genescan-500 ROX, ABI 373; PerkinElmer, Urayasu, Japan), heated at 90°C for 3 minutes, and electrophoresed in a 6.75% denaturing polyacrylamide gel. The distribution of CDR3 size within the amplified product of each Vβ subfamily was evaluated by using an automatic sequencer (ABI 377; PerkinElmer) equipped with a computer program that allows the determination of the fluorescence intensity of each band. Results are depicted as peaks corresponding to the intensity of the fluorescence. CDR3 size patterns that did not have a bell-shaped distribution but had prominent peaks (less than 5) were judged to be abnormal.

**Results**

**Lymphocyte reconstitution in the WHN−/− patient after allogeneic BMT**

The patient’s immunophenotype before BMT is reported elsewhere. Briefly, the WHN−/− patient had CD3+ lymphocytes of reduced number and percentage (650 cells/μL and 25%, respectively), a low number of CD4+ cells, and a low-normal number of CD8+ cells. The T-cell subset and B-lymphocyte profiles during the 6-year post-BMT follow-up are shown in Figure 1. Consequent to the administration of antilymphocyte globulin, 12 days after BMT, CD3+, CD4+, and CD8+ cell levels were markedly reduced, whereas the CD19+ cell level was higher than normal (1400 cells/μL). The number of CD3+ cells started to increase progressively 3 months after BMT. Twelve months after BMT, T cells reached a level of 700/μL (27% of PBMCs). CD8+ cells increased faster than CD4+ lymphocytes. One year after BMT, chimerism analysis revealed that T cells were from the donor and B cells were from the host (data not shown). Four years after BMT, T cells remained of donor origin, and CD levels were as follows: CD3+ cells, 1100/μL; CD8+ cells, 500/μL; and CD4+ cells, 400/μL (18% of PBMCs). Therefore, CD4+, CD4+, and CD8+ levels progressively declined. The CD4+/CD8+ ratio persisted below 1.

**Regeneration of the CD45 RA “naive” phenotype within CD4 and CD8 compartments in the WHN−/− environment after BMT**

Despite the increase of CD3+ cells and, albeit to a lower extent, of mature CD3+CD4+ cells, nearly all these cells showed a memory CD45 RO phenotype, whereas only 1% to 3% of lymphocytes expressed the CD4+CD45 RA “naive” phenotype (Figure 2A). At 6 years of follow-up, no changes were observed in the CD4+CD45 RA→CD4+CD45 RO ratio. Because all T cells in the WHN−/− patient were of WHN−/− donor origin, we compared the memory and naive phenotype of CD4+ cells developed in vivo in the donor with those developed in the recipient environment. Six years after BMT, the CD4+CD45 RA compartment was significantly reduced only in the CD4+ cells obtained from the recipient WHN−/− environment (Figure 2B). This resulted in a very low RA:RO ratio compared with cells from the donor WHN−/− environment (0.06...
and 1.37, respectively). These findings indicate that a functional thymus is necessary to renew the naive CD4+ subset.

During the post-BMT period there was a marked increase in the percentage and number of CD3+CD8+ cells, which reached 27% and paralleled the increase of the whole CD3+ population (Figure 3A). This subset did not contain the CD3+CD8+ natural killer population, which represented 6% of PBMCs. In contrast to the CD4 compartment, the percentage of CD8+ CD45 RA cells was 12% and remained stable during follow-up. Six years after BMT, the CD45 RA–CD45 RO ratio was normal (1.30) (Figure 3B). This suggests that CD8+ CD45 RA lymphocytes are generated even in the absence of a functional thymus. Indeed, the CD8+ CD45 RA–CD45 RO ratio was also normal (1.66) also before BMT. CD8+ cells were also stained for the expression of CD28 and CD11b molecules. Fifty percent of CD8+ cells expressed CD28, whereas only 4% of these cells bore CD11b.

### Proliferative responses in the WHN−/− patient after allogeneic BMT

To assess the functional reconstitution of peripheral lymphocytes after BMT, we analyzed at regular intervals during the 6-year follow-up the proliferative response of PBMC from the WHN−/− patient to CD3 cross-linking. From the third month after BMT there was a progressive improvement in the proliferative response to CD3 cross-linking (Figure 4A). The response was completely normal 2 years after BMT. Proliferative capability began to decline 48 months after BMT. Again, because all T cells in the recipient were of donor origin, we compared the proliferative response to common mitogens of PBMC obtained from the WHN−/− recipient with the genotypically identical donor cells developed in vivo in the WHN−/− environment. Six years after BMT a remarkable deficiency of cells from the WHN−/− recipient was evident (Figure 4B). Even though exogenous IL-2 induced an approximately 3-fold increase of the response to CD3 cross-linking, the response never reached normal levels.

### Humoral immunity studies

To evaluate whether immunologic reconstitution, as revealed by cell number and proliferative response to mitogens, was associated with normal capability to generate specific antibody production, an immunization program was scheduled in the post-BMT period. To discriminate between a response due to committed donor cells and that due to newly in vivo–primed lymphocytes, the program included immunizations both to known antigens to the donor at the time of BMT and to neoantigens such as HBsAg. Table 1 illustrates the comparison of humoral immunity in the pre- and post-BMT (36 months) periods and, in particular, antibody responses in the WHN−/− (recipient) versus the WHN−/− (donor) environment. A full immunologic reconstitution was achieved 2 years after BMT as revealed by the rise in anti-tetanus toxoid antibodies. Notably, even in the WHN−/− environment, B cells were able to generate a specific antibody response to HBsAg.

### Table 1. Humoral immunity studies

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<thead>
<tr>
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<th>WHN−/− recipient</th>
<th>WHN−/− donor</th>
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<tr>
<td>Serum IgG (g/L)</td>
<td>4.94</td>
<td>6.55</td>
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<tr>
<td>Serum IgA (g/L)</td>
<td>0.49</td>
<td>0.77</td>
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<tr>
<td>Serum IgM (g/L)</td>
<td>0.80</td>
<td>0.74</td>
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<td>Autoantibodies</td>
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<td>Allantibodyglutaminins</td>
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<tr>
<td>Pre-BMT</td>
<td>abs</td>
<td>anti-A/B (1:128)</td>
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<tr>
<td>Post-BMT</td>
<td>anti-A (1:8)</td>
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<tr>
<td>Tetanus toxoid</td>
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<tr>
<td>Pre-BMT</td>
<td>abs</td>
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<tr>
<td>Post-BMT</td>
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<td>HBsAg</td>
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<tr>
<td>Pre-BMT</td>
<td>abs</td>
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<td>Post-BMT</td>
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abs, absent; pos, positive.
specific antibody response toward antigens unknown at the time of the transplantation. In fact, 2 boosters of HBsAg led to the appearance of specific antibodies, which demonstrates that efficient priming also occurs in the WHN\(^{-/-}\) environment.

Maintenance of T-cell receptor diversity in the WHN\(^{-/-}\) environment

The biologic mechanisms ensuring maintenance of T-cell repertoire diversity in mature lymphocytes are not completely known and involve central and peripheral lymphoid tissue. In this context, the analysis of TCR diversity in our WHN\(^{-/-}\) patient after BMT provides a unique opportunity to analyze the role of peripheral factors in maintaining T-lymphocyte diversity. We evaluated the TCR repertoire by analyzing V\(\beta\) CDR3 heterogeneity length (spectratyping) 6 years after BMT in genotypically identical T cells obtained from the WHN\(^{-/-}\) recipient and from the WHN\(^{+/+}\) donor. Figure 5A shows the CDR3 length profiles of 18 V\(\beta\) families in the CD4\(^+\) cells. In cells from the WHN\(^{-/-}\) recipient there was a marked alteration in the CDR3 length profile in only 3 of the 18 V\(\beta\) families, 8,12,15 Notably, in 2 of them (families 8 and 15), the altered profile overlapped in donor and recipient cells. Spectratyping analysis of CD8\(^+\) lymphocytes (Figure 5B) revealed that most of the V\(\beta\) families displayed an altered profile (11 of the 18 families). Most of them (families 5,3,8,9,12,14,17) exhibited oligoclonal expansion. The complexity of the TCR repertoire was reduced in the remaining families. Even though all the families were altered in both WHN\(^{-/-}\) and WHN\(^{+/+}\) cells, within individual families the distribution pattern differed between recipient and donor, unlike what occurred in CD4\(^+\) cells. Spectratyping analysis confirmed the different behavior of CD4\(^+\) and CD8\(^+\) cells in the absence of a functional thymus.

Discussion

We used a unique model, the human counterpart of the Nude/SCID phenotype recently described by our group,\(^1\) to explore the role of the thymus in post-BMT immunologic reconstitution. Nude/SCID is the first form of SCID whose immunodeficiency is substantially and exclusively related to an intrinsic abnormality of the thymus.\(^19,20\) The gene involved in this disease is the WHN gene, which encodes a forkhead–winged helix transcription factor and is associated with both the murine and human Nude/SCID phenotypes.\(^12-14\) Because WHN is selectively expressed in the thymic epithelia and not in hematopoietic cells, this rare and novel condition in humans is a reliable model with which to investigate the role of intrathymic intercellular connections, and namely the connections between thymic epithelia and hematopoietic progenitors, in the regeneration and maintenance of T-cell responses after allogeneic BMT. The analysis of immunologic reconstitution during the 6-year follow-up after BMT in the patient described herein revealed that the lack of a functional thymus did not prevent the repopulation kinetics of CD3\(^+\), CD4\(^+\), and CD8\(^+\) cells.

In the absence of a functional thymus, in the WHN\(^{-/-}\) environment no CD4\(^+\) CD45\(^{RO}\) RA naive lymphocytes were detected in the periphery. Moreover, the long-term follow-up revealed that the defect in the generation of the naive CD4 population was not restored by BMT. Conversely, the WHN\(^{-/-}\) patient was able to regenerate and maintain a normal amount of CD8\(^+\) CD45 RA T lymphocytes. Our findings are in keeping with the recent observation in cancer patients who underwent thymectomy, that after BMT the reconstitution of the CD4\(^+\) CD45 RA subset relies on a functional host thymus.\(^21\) As did our patient, patients who undergo thymectomy retain the capacity to regenerate the naive CD8\(^+\) subset, suggesting the presence of a thymus-independent pathway for the generation of this subset.\(^21\) Indeed, recovery of the CD4\(^+\) CD45 RO memory subset after BMT in thymus-deficient patients presumably results from expansion of the donor’s mature lymphocytes. Taken together, these findings also argue against the possibility that in humans CD4\(^+\) CD45 RO cells can convert to the naive CD45 RA phenotype.\(^22\) The prompt recovery of the CD8\(^+\) CD45 RA naive cells may indicate the involvement of extrathymic lymphopoiesis. These cells could also originate from conversion of the CD45 RO phenotype.\(^23\) However, it should be noted that the CD8\(^+\) CD45 RA compartment is heterogeneous in that it contains unprimed and primed cells.\(^24\) Unlike unprimed naive cells, primed cells express low levels of CD27 and CD28 and high levels of the CD11b adhesion molecule. Of note, a large fraction of CD3\(^+\) CD8\(^+\) cells in our patient expressed CD28 and only a small percentage expressed CD11b, which suggests that they were unprimed naive CD8\(^+\) T cells. In patients with DiGeorge syndrome, BMT can lead...
to donor T-cell engraftment, \(^\text{25}\) though it is unclear whether reconstitution is stable over time and whether donor T cells represent an expansion of mature T lymphocytes differentiated before transplantation. \(^\text{26}\) However, in DiGeorge syndrome, there is residual thymic function as testified by the post-BMT increase in CD4\(^{+}\) cells grown in the host environment. Feasibly, in the absence of the thymus, T-lymphocyte survival and homeostasis depend principally on continuous stimulation by lymphoid tissue accessory cells. \(^\text{27-28}\) Because all T cells were of donor origin, this progressive functional impairment could reflect accelerated accumulation of aged lymphocytes that have a low proliferative capability, somewhat similar to what occurs in the elderly. \(^\text{30,31}\)

The thymus plays a major role in T-cell ontogeny. \(^\text{32,33}\) In this organ, T cell precursors undergo a complex mechanism of positive and negative selection to shape the TCR repertoire of mature lymphocytes. Mature cells become capable of recognizing exogenous peptides in the context of self-major histocompatibility complex molecules. Cortical epithelial cells of the thymus are unique in their ability to mediate positive selection efficiently, \(^\text{10}\) whereas bone marrow–derived dendritic cells are efficient mediators of negative selection; they are not involved at all in the positive selection process. \(^\text{34}\) Patients with DiGeorge syndrome have a marked reduction in lymphopoiesis and severe alterations in the lymphocyte repertoire. \(^\text{35}\) In this context, our analysis of T-cell repertoire complexity provides insights into the mechanisms involved in the maintenance and regeneration of T-cell repertoire diversity. The WHN\(^{+/−}\) environment did not influence the long-term maintenance of TCR repertoire diversity in the CD4\(^{+}\) population, which consisted mostly of memory cells. Furthermore, the altered V\(\beta\) families had a similar profile in host and donor lymphocytes, suggesting that the donor’s repertoire is maintained in the host environment. Unlike the CD4 compartment, the complexity of the TCR CDR3 profile was reduced in most CD8\(^{+}\) cells, in a few cases indicating oligoclonal expansion. In addition, within individual V\(\beta\) families and unlike what was observed in the CD4 compartment, recipient and donor cells had different CDR3 profiles. Whether extrathymic lymphopoiesis or peripheral mechanisms may have contributed to the CD8\(^{+}\) reconstitution is an intriguing question. \(^\text{36,37}\)

It is noteworthy that 6 years after BMT, and despite the progressive immunodeficiency status and the absence of anti-infectious prophylaxis, the patient remains free of infection, probably because of a residual immune response estimated to be 20% of donor cell proliferative potential. In addition, Nude/SCID mice can clear some viral infections. This is presumably because of a T-cell–independent antibody response and mechanisms of innate immunity. \(^\text{38,39}\) Clearance of the polyoma virus in T-cell–deficient mice results from T-cell–independent antibody-mediated responses. \(^\text{39}\) In our patient HBsAg immunization led to specific antibody production. The generation of specific antibody responses toward a neoantigen unknown to the donor cells at the time of BMT may indicate that donor-derived CD4\(^{+}\) cells can provide help for B cells. However, it should be noted that we observed specific antibody responses during the phase of optimal immune response; we did not test the response when immunity declined. Alternatively, the generation of a B-cell response against HBsAg may be obtained in a thymus-independent fashion.

In conclusion, our findings on T-cell reconstitution in the WHN\(^{+/−}\) environment indicate that a functional thymus is essential to regenerate the CD4\(^{+}\) naive subset. The long-term maintenance of TCR diversity of the memory–effector CD4\(^{+}\) lymphocytes may be ensured by a peripheral mechanism that does not depend mainly on antigen-driven clonal expansion. \(^\text{28,29}\) Conversely, the regeneration of CD8\(^{+}\) naive lymphocytes after BMT is less thymus dependent and also occurs in the WHN\(^{+/−}\) environment. Lastly, despite the favorable clinical course after BMT, our data indicate that this therapeutic procedure is ineffective in the long-term cure of this form of SCID.

References


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Human equivalent of the mouse Nude/SCID phenotype: long-term evaluation of immunologic reconstitution after bone marrow transplantation

Claudio Pignata, Lucia Gaetaniello, Anna Maria Masci, Jorge Frank, Angela Christiano, Eliana Matreccano and Luigi Racioppi