Thymocytes self-renewal: a major hope or a major threat?

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Summary: Thymus transplants were never used to correct T-cell intrinsic deficiencies, as it is generally believed that thymocytes have short intrinsic lifespans. This notion is based on multiple thymus transplantation experiments, where it was shown that thymus-resident cells were rapidly replaced by progenitors migrating from the bone marrow (BM). This substitution occurs even when bone marrow precursors are unable to generate T cells, as in Rag1/2−/− or severe combined immunodeficiency (SCID)-deficient mice. In contrast, two groups reported that neonatal thymi transplanted into mice that cannot respond to IL-7 harbor populations with extensive capacity to self-renew, which maintain continuous thymocyte generation for several months after surgery. The consequences of this self-renewal capacity differed in these two laboratories. We found that these thymus transplants rapidly reconstitute the full diversity of peripheral T-cell repertoires 1 month after surgery, the earliest time point studied. Moreover, transplantation experiments performed across major histocompatibility barriers show that allogeneic-transplanted thymi are not rejected, and allogeneic cells do not induce graft-versus-host disease, both syngeneic and allogeneic transplants inducing rapid protection from infection. These results indicate a potential use of neonatal thymus transplants to correct T-cell intrinsic deficiencies. The other group observed that continuous thymocyte renewal from BM precursors was fundamental to prevent tumor development. In the absence of this input, thymocytes from the transplanted thymus generated tumors with all the characteristics of T-cell acute lymphoblastic leukemia (T-ALL). Moreover, they suggested that the absence of BM competition was responsible for the T-ALLs developing in X-linked severe combined immunodeficiency (SCID)-X1 patients, deficient in the expression of IL2-Rγc. These patients were treated with autologous CD34+ cells transfected with virus vectors expressing γc in the absence of myeloablation. We here review the potential therapeutic impact of thymus transplantation and compare the results of these two laboratories aiming to find an answer to the ‘Dr Jekill versus Mr. Hyde’ status of thymus transplantation experiments.

Keywords: T cells, thymus transplants, T cell deficiency, T cell ALL, T cell reconstitution, T cell generation

New therapeutic approaches are fundamental to treat congenic and acquired T-cell deficiencies

T lymphocytes are fundamental for the control of infections. In rare cases T-cell deficiencies are congenital, caused by
mutations preventing the expression of any of the genes required for T-cell generation or functional activity (1). However, in the majority of cases, T-cell deficiencies are induced either by infections such as the acquired immune deficiency syndrome, by aggressive anti-cancer therapies, or by aging. These situations are now frequent, rendering the reconstitution of the peripheral T-cell pool an important clinical goal. T-cell reconstitution may be achieved by the transplantation of a competent bone marrow (BM). However, BM transplants have two types of shortcomings: delayed T-cell generation and graft-versus-host disease (GVH).

After BM transplants, BM precursors must first transit through the thymus to generate T cells. Therefore, the peripheral T-cell reconstitution is delayed by many months during which patients are very susceptible to infections (2–5). Besides, BM transplantation cannot correct T-cell deficiencies once the thymus epithelium atrophies in adults.

The other major problem of BM transplants results from the difficulty of finding a BM donor fully histocompatible with the host. Partially allogeneic BM transplants have reduced engraftment capacity and induce GVH disease. It was reasoned that the elimination of T lymphocytes from the transplanted BM could prevent GVH, but the BM so depleted had a reduced engrafting capacity. Moreover, removal of antigen-experienced T cells from the transplant depletes the T cell-deficient patients of the sole source of functional T lymphocytes, increasing their susceptibility to infections and, in patients with cancer, accelerating their tumors growth.

Thymus transplants could constitute an advantageous alternative or complementary therapy to BM transplantation: these grafts could be a source of both a functional thymus epithelium and functional T cells and thus might correct T-cell deficiencies of both children and adults (6, 7). They would not necessarily require the conditioning of the patient and should export mature T cells immediately, overcoming the long lag-time required for T-cell generation after BM transplantation. It is therefore fundamental to clarify why in some cases these transplants are beneficial while in other cases a threat.

The transplantation of the thymus epithelium

Grafts of the thymus epithelium were successful in correcting deficiencies of the thymus epithelium, as found in the complete DiGeorge syndrome (8) or in FOXN1 gene mutations (7). In both cases, the thymus was first depleted of T cells in vitro, and slices of the thymus epithelium were transplanted into the leg muscle. Two aspects should be mentioned concerning these transplants. It is difficult to achieve their vascularization, requiring a precise technique that was only achieved by Markert et al. (7, 8) (A. Fischer & A. E. Sousa, personal communications). Surprisingly, the major histocompatibility complex (MHC) of the transplanted epithelium has no impact in the reconstitution of a functional peripheral T-cell pool. Hosts are able to mount adequate immune responses even when transplanted with fully allogeneic thymus epithelium.

The MHC restriction of these T-cell populations was not determined. It is however surprising that T cells, positively selected in a thymus epithelium with a particular MHC are able to interact with antigen-presenting cells (APCs) or B cells from the host that have a different MHC. We will describe below that MHC restriction has also little impact in the transplants of neonatal thymus lobes into Ragγc-deficient mice.

Thymus grafts as a source of functional T cells

Thymus grafts were never used to correct intrinsic T-cell deficiencies, as it was generally accepted that the thymus does not harbor precursors with self-renewal capacities. Indeed, when a wildtype (WT) thymus is transplanted into WT mice, resident thymocytes generate a single wave of mature T cells, BM precursors from the host rapidly replacing the transplant resident cells (9). Moreover, this occurs even when the host cannot generate mature T cells. When WT thymi are transplanted into SCID- or Rag2-deficient hosts, the competent thymocyte populations from the graft are rapidly substituted by the incompetent precursors from the host BM. By 3 weeks after surgery, mature T-cell export fails (10, 11). Based on these data, it was believed that all thymocyte subpopulations were short-lived; their maintenance being dependent on a continuous input from BM derived progenitors.

By contrast, our laboratory (12) and Martins et al. (13) described that when neonatal thymus grafts were transplanted into host mice lacking BM precursors able to respond to IL-7 (as in IL-7Rc–, Rag2–γc–, or in Rag2–γc–, KitW/Wv host mice), the thymocytes from the graft persisted and maintained a normal CD4/CD8β profile (Fig. 1) up to 7 months after transplant (11), the latest time point studied. These results indicate that thymocytes are not necessarily short-lived. They show a capacity to self-renew in the absence of BM precursors that cannot respond to IL-7. However, the outcome of these experiments was very different in these two laboratories.
We found that after grafting a single neonate thymus lobe, the lobe increased in size up to 3 months after grafting, and underwent atrophy thereafter (12). As the graft would then be 3 months old, and the mouse 5 months old, the time course of this decline correlated with the age-related thymus atrophy found in WT mice. Therefore, we assumed that the reduced cellularity of the graft was age-related, mimicking the thymus atrophy found in older mice. However, it is also possible that the transplant-reduced cellularity was due to the exhaustion of the thymocyte precursors with self-renewal capacities (see below).

These transplants rapidly exported mature T cells, reconstituted a normal diverse peripheral T-cell repertoire and conferred the capacity to clear infections. Moreover, the peripheral T-cell reconstitution after thymus transplants is much more rapid than that found after BM injection. By 2 weeks after grafting, numerous peripheral T cells can be found in transplanted mice. By contrast at this time point BM-derived precursors had just reached the thymus and were yet at the TN1 (CD44+ CD25− TN thymocytes) differentiation stage. By 4 weeks after thymus grafting, 97% of peripheral T cells were from thymus transplant origin. By contrast, the progeny of the injected BM was mostly DP (CD4+ CD8β+ double-positive thymocytes) cells, generating the first CD4+ and CD8+ mature T cells which still resided in the thymus (12). Therefore thymus transplants export mature T cells directly, overcoming the long lag-time required for BM precursors to generate T cells.

Importantly, as found with transplants of the thymus epithelium, the transplants of thymus lobes functioned across histocompatibility barriers. Mice transplanted with syngeneic or semi-allogeneic thymi reconstituted the peripheral T-cell pools to equivalent levels. The peripheral reconstitution after fully allogeneic transplants was delayed (12) but equivalent numbers of T cells were reached later on. Syngeneic or semi-allogeneic transplants fully protected the hosts to Listeria monocytogenes (LM) infection, while protection in mice transplanted with fully allogeneic thymi was but slightly less efficient (Fig. 2). When testing the capacity of these thymus transplants to confer protection to infection, we wished to determine if such protection only occurred in conditions of thymocyte self-renewal, i.e. in IL-7R or Ragγc-deficient transplants.
The capacity of thymus transplants to confer protection to infection. CD45.2+ Rag2γ–c mice were grafted with a thymus lobe of neonatal wildtype mice. These graphs were syngeneic, semi-allogeneic (B6xBalb/c), or fully allogeneic (Balb/c). One month later, they were injected i.v. with 4 × 10^3 live *Listeria monocytogenes*. Results show bacterial loads evaluated as colony-forming unit/spleen at day 2 and 5 after infection (From Peaudecerf et al. J.Exp.Med. 209:1401–1408, 2012).

The absence of competition from BM precursors is not sufficient to ensure thymocyte self-renewal

One of the surprising findings of our transplantation experiments is that T-cell committed precursors from the thymus transplant colonized the endogenous ‘empty’ thymus of the Rag2γ–c hosts (18). The endogenous thymus then increased in size and generated all thymocyte populations, including mature single-positive T cells. We aimed to identify the thymocyte population of graft origin responsible for the reconstitution of these endogenous thymi. Colonization should be mediated by TN thymocytes from the graft, as graft-derived thymocytes in the endogenous thymi showed a normal CD4/CD8 distribution by 2 weeks after transplantation (18). Indeed, only TN thymocytes are able to fully reconstitute the DP and SP compartments in 2 weeks. More immature BM precursors reconstitute the thymus more slowly, whereas more mature thymocytes cannot generate DP cells. The study of the lineage negative (Lin–) TN cells of graft origin in the blood of these transplanted mice showed the presence of cells with a peculiar TN phenotype. These Lin– cells of graft origin found in the blood were CD44+ cKitlow IL-7Rlow CD25int (19). These cells expressed Rag1 but not CD3ε (19) supporting they issued from thymocytes at the TN1 to TN2 (CD44+ CD25low TN thymocytes) transition (20). Moreover, TN1–TN2 transition populations (19) as well as a small subset of TN2 cells expressing the highest cKit levels (L. Peaudecerf and B. Rocha, unpublished observation) were the sole TN populations expressing the sphingosine 1-phosphate receptor 1 (SIP1) (19), a receptor required for the egress from the thymus (21).
If cellular competition from the BM was the sole mechanism involved in thymocyte persistence, the reconstituted endogenous thymi of these Rag2−γc− hosts should maintain a normal CD4/CD8 profile, comparable to that of the thymus graft. Surprisingly, this was not the case. The transplant-derived TN precursors generated a single wave of thymus reconstitution: by 3–4 weeks, the endogeneous thymi did not have immature TN or DP thymocytes of graft origin (18), while these cells were present in the thymus transplant up to 7 months (12).

These results show that the absence of competition from BM progenitors is not sufficient to explain the long-term thymocyte self-renewal in neonatal thymus transplants. They indicate that to ensure thymocyte self-renewal, the graft must also harbor precursors with self-renewal capacities. The TN1–TN2 transition migrants of graft origin that colonize the endogenous thymi do not have such precursors with self-renewal capacities, explaining why they only generate a single wave of thymocyte differentiation.

### The endogenous BM always colonizes the transplanted thymi

The notion of "BM competition" suggests that the endogenous BM does not colonize the thymus transplants in host mice that cannot respond to IL-7 (Rag2−γc− or IL-7R-deficient mice). By contrast, the analysis of our transplanted thymi showed that both BM precursors always colonized these transplants (Fig. 3A). In WT hosts, the BM-derived precursors substituted all the TN populations of the transplant generating all types (TN1–TN4) of TN precursors. In Rag2− hosts, the BM-derived precursors differentiated until the TN3 differentiation stage, as found in the Rag2− thymus. However, the DP compartment did not persist in these hosts indicating that the TN4 or DP populations do not harbor precursors with extensive self-renewal capacity. The BM-derived precursors from Rag2−γc− and IL-7R-deficient hosts also invaded the graft, arresting their differentiation at the TN2 differentiation stage, as it is characteristic of thymocyte differentiation in these host mice. However, in these hosts, the thymus transplants maintained a continuous thymocyte generation for many months.

Therefore, the thymocyte autonomous generation in the thymus transplanted into Rag2−γc− and IL-7R-deficient hosts is not due to a blockage of the input from the host BM. It rather depends on the capacity of the host BM to generate TN3 cells and substitute the TN3 populations of the thymus transplant. When TN3 populations from the WT thymus transplant are substituted (as found in WT and Rag2− hosts), DP and SP cells from the transplant do not persist. When TN3 cells from the WT thymus transplant remain (as found in Rag2−γc− or IL-7R− hosts), thymocyte self-renewal occurs.

In our experiments thymocyte differentiation in these grafts appears to follow the normal course from the TN3 differentiation stage onwards. Within the TN populations of graft origin (Fig. 3B), TN1 and TN2 populations declined rapidly, likely substituted by the incompetent precursors from the host BM. The TN populations from the graft were progressively enriched in a CD44+ cKitlow IL-7Rlow CD25int TN1–TN2 intermediary cell set (12) which expressed Notch1, Gata3, Bc11b, and Rag1 (20). Only a very minor fraction of these cells divided. Up to 5 months after transplant, TN3 and TN4 populations were also present, their number declined with age, and eventually disappeared: they were not detected by 7 months after grafting (Fig. 3B). The analysis of the Tcra and Tcrb repertoires of the DP thymocytes from the graft showed that their repertoires were identical to those of DP from a WT thymus (12). These results indicate that from the TN3 populations onwards, these grafts support a normal thymocyte differentiation process.

We also observed that compensatory mechanisms were engaged in several thymocyte sets. Thus the progressive decline in the numbers of cells in each thymocyte set was associated with a progressive increase in its division rate. Thus, when the number of TN3 thymocytes of the graft declined with age, the TN3 cells in the S phase of the cell cycle increased from 34 to 53% from 2 weeks to 3 months after transplantation (Fig. 3B). Moreover, increased division rates were not restricted to the TN cell sets. When TN3 and TN4 precursors disappeared by 7 months after grafting, the DP division rates also increased from 10% to 50% (L. Peaudecerf and B. Rocha, unpublished observation). These results raise the possibility that homeostatic proliferation is not restricted to naive T lymphocytes that start dividing when transferred into lymphopenic hosts. Rather, increased division rates may be a general compensatory mechanism engaged by multiple cell types upon any reduction in their numbers.

### The capacity of different T-cell precursors to reconstitute the peripheral T-cell pools

Although thymus transplants can reconstitute the peripheral T-cell pools in both syngeneic and allogeneic conditions, several questions could be raised: (i) the reconstitution capacity was equivalent in both conditions?; (ii) The injection of BM...
precursors after myeloablation would be more efficient than thymus transplantation at later time points?; (iii) Besides thymus transplants, complementation with BM precursors would improve peripheral T-cell reconstitution?; (iv) Would the presence of allogeneic cells induce a late GVH reaction?

To address these questions we studied three groups of Rag-c/c mice (Fig. 4). The first group was first thymectomized (Tx) and 2 weeks later transplanted with a single neonatal thymus lobe (left columns). The thymectomy was performed to ensure that all peripheral T cells originated from the transplanted thymus, and not from the endogenous thymus (reconstituted with transplant-derived precursors). The second group was irradiated (600 rads) and reconstituted with BM LSKs (Lineage−/C0 Sca-1+/cKit+ BM precursors) (middle columns). The third group was first thymectomized and 3 weeks later irradiated (600 rads) and received simultaneously BM LSKs and a neonatal thymus transplant (right columns). We compared peripheral T-cell reconstitution in the spleen of these mice 5–6 months later. We found that BM injection alone was not more efficient than thymus transplants to reconstitute the peripheral T-cell pools. Actually, in syngeneic transplanted mice, the numbers of total T cells recovered in the periphery was lower in mice receiving only BM (P < 0.01) versus that found in both groups of thymus-transplanted mice (Fig. 4A). This difference could be due to the lag-time required for BM precursors to reach and expand in the periphery. The efficiency of thymus transplants to reconstitute the full peripheral T-cell pool recall early studies reporting that 10% of a normal thymus export is sufficient to reconstitute the peripheral T-cell pools (22). However, the association of BM to thymus transplantation could yet have advantages. The % of CD4+ CD44+ cells in BM-injected mice was reduced (P < 0.01) as compared to mice receiving only thymus transplants, indicating that the major advantage of the injection of BM precursors could be a significant increase in the representation of naïve cells in the peripheral T-cell pools (Fig. 4B). The major difference between allogeneic and syngeneic T-cell distribution was the CD4/CD8 ratio, which was higher in mice reconstituted with Balb/c allogeneic
cells (Fig. 4C). This difference likely reflects the CD4/CD8 ratio of these two different mouse strains. While in Balb/c mice the CD4/CD8 ratio is 2/1, our B6 strain has similar proportions of CD4+ and CD8+ T cells.

In mice reconstituted with allogeneic cells we did not find evidence of an ongoing GVH disease. Besides, we also did not find evidence that allogeneic T lymphocytes were more activated than syngeneic T cells. Within each group of mice, the percentage of CD4+ and CD8+ T cells expressing CD44+ cells was similar in syngeneic- and allogeneic-reconstituted mice. In all groups we also found rare cells expressing CD69 but the representation of CD69+ CD4+ or CD8+ T cells was similar in mice reconstituted with syngeneic and allogeneic cells (not shown). We conclude that thymus transplants are very efficient to induce the rapid reconstitution of the peripheral T-cell pools. However, supplementation with BM precursors can be beneficial, by increasing the representation of naive T cells and, in the case of T B SCIDS, by reconstituting the B-cell pool. Therefore, it is possible that difficult task of finding histocompatible BM for transplantation will no longer be required. The best therapeutic approach could be the association of thymus transplants with simultaneous injection of BM precursors from the same donor: the thymus transplant providing rapid T-cell reconstitution and the BM injection increasing naive cell output at later time points.

Thymus transplants associated with the emergence of T-ALL

In contrast to our results, Martins et al. (14) described that thymocyte self-renewal leads to the development of T-ALL.

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Fig. 4. The impact of different therapeutic approaches in reconstitution of the peripheral T-cell pools of T cell-deficient mice. Six-week-old Rag2Δc−/− B6 mice were: left columns: thymectomized (TX) and 2 weeks later transplanted (TT) with one syngeneic or allogeneic neonatal thymus lobe. Middle columns: irradiated (600 rads) and injected with syngeneic- or allogeneic-sorted bone marrow (BM) LSKs. Left columns: irradiated and received in the same day: syngeneic-sorted BM LSKs and the transplant (TT) of a single syngeneic neonatal thymus lobe; or allogeneic-sorted BM LSKs and the transplant of a single allogeneic neonatal thymus lobe. All mice were studied 5–6 months after. Results are the mean ± SE of 3–5 mice per group. Black symbols correspond to syngeneic (Syng) transfers and grey symbols to allogeneic transfers (Alo). Results refer to T cells in the spleen. (A) Total T-cell numbers. The sole significant difference is the lower number of T cells in the syngeneic BM-injected mice (P < 0.01) with respect to all other groups. (B) The expression of CD44 in different populations. The sole significant difference in the increase in CD44 expression by CD4 T cells, in the first group with respect to all other groups (P < 0.01). (C) The number of CD4+ (left) and CD8+ (right) T cells. Mice with allogeneic graphs have significant increase in the number of CD4 T cells in groups receiving BM (P < 0.05) and decrease in CD8 T cells in group I and III (P < 0.01).
in a significant fraction of transplanted mice. The frequency of these tumors varied in different host mice from 64% in Rag2\(^{-/-}\) cKit\(^{W/Wv}\) to 50% in Rag2\(^{-/-}\) and 38% in \(\gamma_c\) mice. The comparison of thymocyte differentiation in both experiments could contribute to our understanding of these conflicting results. Regrettably, early thymocyte differentiation in these experiments was less characterized, the only data available being from CD4\(^+\)CD8\(^-\) double-negative (DN) thymocytes from Rag2\(^{-/-}\) cKit\(^{W/Wv}\) transplanted mice, 2.5 months after grafting (13).

In these mice, it is not shown if BM precursors from the host were present in the thymus grafts. This information is important, as the presence of such precursors may help to constitute a niche favoring the survival of the TN cells from the graft. However, it is known that Rag2\(^{-/-}\) cKit\(^{-/-}\) mice are devoid of TN thymocytes, and have major modifications of the thymus stroma with the disappearance of the cortical/medullary architecture (23). Therefore, it is likely that BM-derived precursors are quite rare in the thymus transplanted into Rag2\(^{-/-}\) cKit\(^{-/-}\) mice and that the niches for the maintenance of TN thymocytes are absent or deficient. Indeed, the CD44/CD25 profiles of DN thymocytes at 2.5 months after surgery clearly show that TN3 and TN4 populations are absent or deficient. Indeed, the absence of TN3 and TN4 in these transplanted thymi (13). However, in another manuscript, Martins et al. (14) refer the presence of TN3 precursors. Therefore, it is unclear if these TN3 were recovered from other hosts (Rag2\(^{-/-}\) c or IL-7R\(^-/-\)). Alternatively, TN3–TN4 precursors from grafts transplanted into Rag2\(^{-/-}\) cKit\(^{+/+}\) hosts may have short lifespans, and be no longer present at 2.5 months after grafting.

The absence of TN3 and TN4 precursors precludes that thymocyte differentiation in these transplants progresses normally from the TN3 to the DP compartment. As TN3 and TN4 populations are absent, the continuous division of DP cells likely maintains the thymocyte self-renewal. This notion is confirmed by studies of the Tcr\(\beta\) and Tcr\(\alpha\) repertoires in the DP cells from these transplanted thymi: holes in the TCR repertoires, reduced diversity, and evidence of clonal expansions were found. Overall these results indicate that the thymocyte differentiation process in the graft is abnormal. The absence of TN3 and TN3–TN4 populations indicate that thymocyte renewal in these grafts cannot progress normally from TN to the DP stage compromising the renewal of the DP compartment from more immature TN cells. The continuous division of the same DP cells, together with the repeated breaks induced by their successive Tcr\(\alpha\) rearrangements may be one of the reasons favoring transformation and T-ALL development.

Leukemia cells are considered developmentally ‘frozen’, their characteristics reflecting their origin. The global gene expression profiles of these T-ALL compared to different thymocyte populations, reported a closer relationship with TN3/TN4/immature populations (14). However, these results are difficult to conciliate with the fully absence of TN3 and TN4 precursors in these grafts (13), and with the phenotype of these T-ALL, which co-express different levels of CD4 and CD8 (14). Moreover, a large proportion of the T-ALL clones express in-frame Tcr\(\beta\) chains, indicating that transformation occurred after pre-TCR selection (14).

To conclude, the analysis of thymocyte renewal in our experiments shows that immature TN progenitors in the graft are present until age-related thymus atrophy. Until then, the rate of division of the DP compartment and their Tcr\(\beta\) and Tcr\(\alpha\) repertoires are identical to those found in normal mice. These results support a virtually normal thymopoiesis in these grafts from the TN3 thymocyte differentiation stage onwards. By contrast, the sole data available in T-ALL-prone transplants show major alterations of thymopoiesis. These alterations may justify the development of T-ALL in Rag2\(^{-/-}\) cKit\(^{W/Wv}\)-transplanted mice. However, Martins et al. (14) also reported T-ALL development in thymus transplanted into Rag2\(^{-/-}\) and IL-7R\(^-/-\) hosts. It is not known if thymocyte differentiation in Rag2\(^{-/-}\) cKit\(^{-/-}\) and IL-7R\(^-/-\) hosts that developed T-ALL is equivalent to that found in our experiments. This information is fundamental for the understanding of the different outcomes of these thymus transplantation experiments.

With the information now available, it is yet unclear why our results differ. The different outcomes of these experiments likely rely on a different capacity of the different grafts to maintain the TN3 and TN3–4 cell thymocyte pool. Trivial reasons may be involved, as a reduced vascularization: we only transplanted a single neonatal thymus lobe while in other experiments a complete neonatal thymus was grafted. Adequate vascularization was found fundamental for the success of thymus epithelium transplants (7, 8). Differences in vascularization may compromise TN survival either directly, or by preventing the invasion of the graft by BM precursors from the host. These BM-derived precursors may modify thymus architecture and contribute to the generation of a niche allowing TN3–TN4 survival. Indeed, it was shown that cKit\(^+\) precursors are important to maintain the cortex/medulla stromal architecture, as when Rag2\(^{2\gamma_c-}\) mice are crossed to cKit-deficient mice, this architecture is lost (23).

Another trivial reason that could explain the different results was putative differences in the hosts or in the donors.
of the thymus graft. We fully backcrossed the Rag2γc− hosts we used to our B6 background, while it is not known if Martins et al. performed equivalent backcrosses. We used neonatal thymi from our B6 CD45.2+ mouse colony, brought from I. Weissman’s laboratory about 35 years ago. The other studies used mice from Charles River laboratory. These two colonies could differ in the expression of endogenous retrovirus, which in T cell-deficient conditions are known to be reactivated and promote transformation (24). Regretfully, this latter hypothesis cannot be verified since our B6 CD45.2 mouse colony is no longer available. The Central French breeding facilities where our mouse colony was kept, aware that the extensive intercrossing in our B6 colony could induce genetic deviation, decided to backcross our B6 mouse colony to Jackson’s B6 mice.

At this phase, the reason behind the different outcomes of thymus transplants experiments remains to be determined. However, one aspect must be immediately addressed, as it affects the choice of the therapeutic strategies for the reconstitution of the peripheral T-cell pools, and determines if thymus transplantation studies should be pursued. Martins et al. proposed that the disruption of the competition by incoming BM progenitor cells necessarily leads to intra-thymic leukemogenesis. Moreover, they suggested that this process would account for the occurrence of T-ALL in 5 of the 19 patients with SCIDX1, treated with autologous progenitor cells expressing IL-2Rγc (14). These patients did not undergo myeloablation, so BM engraftment of competent progenitors was not detected, preventing the competition between competent BM progenitors and resident thymocytes. By contrast, the clinicians involved in these clinical trials claimed that the T-ALL in these first-generation gene therapy trials was associated with the integration of the IL-2Rγc retroviral vector in the proximity of an oncogene (usually LMO2) and the transactivation of the latter (15–17). They proposed that removal of the enhancer of the retroviral vector would prevent the development of T-ALL. So, in the light of these two hypotheses, two different alternatives can actually be envisaged for the correction of T-cell deficiencies: performing extensive myeloablation in these T cell-deficient hosts or modifying the type of vector used for gene therapy. At this phase, further data on early thymocyte differentiation in Ragγc− and IL7R-deficient mice developing tumors are not available. Therefore, at present, the choice between myeloablation versus the modification of the virus vector used for gene therapy can only be made based on the results obtained in clinical trials.

**Treatment of SCIDX1 patients by BM transplantation without gene therapy did not induce T-ALL**

Before the introduction of gene therapy, 556 patients with SCIDX1 (IL-2Rγc deficient) or other SCIDS, as well 512 patients with other congenital immune deficiencies, were treated by the transplantation of a competent histocompatible or non-histocompatible BM, and followed up for four decades from 1968 until 1999 (25). Many of these patients did not undergo myeloablation, as this previous conditioning was reported to have no effect, or to worsen the disease outcome (25, 26). If leukemia was the consequence of thymocyte renewal in the absence of competition from BM progenitors, the patients not undergoing myeloablation should be at high risk of leukemia. In fact, it was not reported a single case of T-ALL. The factors conditioning the disease outcome varied with the type of immunodeficiency, but as common features they were related to the age of the patient and the presence or absence of a protected environment. In particular, the presence of pulmonary infections before the BM transplantation had a major negative influence in the prognosis of the disease.

Martins et al. also argued that preconditioning, which allows hematopoietic stem cell engraftment and thus imposes BM competition, abrogates the risk of leukemia. Their hypothesis is also not supported by the observation that six of the nine patients with Wiskott–Aldrich syndrome, who received retrovirus-mediated gene therapy after preconditioning, developed T-ALL (27, 28).

Thus, patients without ‘BM competition’ and without gene therapy did not develop T-ALL, while a fraction of the patients with ‘BM competition’ and gene therapy developed T-ALL. Overall these clinical data suggest that the T-ALL developing in patients under gene therapy was a consequence of the insertion of the therapeutic retrovirus, rather than the absence of ‘BM competition’.

**The analysis of T-ALL in patients undergoing first-generation gene therapy**

The treatment of deficiencies in expression of the IL-2Rγc was initially performed using vectors derived from gammaretrovirus to allow the stable integration of the therapeutic gene in the patient DNA. These patients did not receive preconditioning and 5 of 19 developed T-ALL between 2–6 years after therapy (15–17).

The integration of these vectors is known to occur at the proximity of transcription initiation sites. This enables the viral long terminal repeat (LTR) enhancer to modify the
expression of the nearby cellular genes. The study of these T-ALLs showed that the virus integrated nearby or within proto-oncogenes in leukemia cells, frequently at the LMO2 (4 out of 5) and also at CCND2 and BMI1 sites, while these integrations were not detected in non-leukemia cells. The integration of the virus lead to the over-expression of these proto-oncogenes, as well as that of the adjacent genes, that was restricted to T-ALL cells. These results show that the virus integration promotes the over-expression of nearby genes.

However, the process of transformation involved further genetic modifications that varied in the different patients. These included mutations of the Notch1 expression, deletion of regulators of cell cycle progression as p16 (INK4A), and chromosome translocations. These results suggest an initiation event (over-expression of a proto-oncogene, usually LMO2) followed by further acquired somatic mutations. Indeed, it is known that over-expression of LMO2 in the mouse induces T-ALL, and spontaneous T-ALL in man are associated with upregulation of LMO2 (29–31).

Overall the absence of T-ALL in SCIDX1 patients undergoing BM transfers without myeloablation or gene therapy, in contrast to the development of T-ALL after similar BM transfers when associated with gene therapy, and the demonstration of virus integration near proto-oncogenes and their subsequent over-expression lead to the current notion shared by clinicians involved in these therapies that a powerful enhancer in the intact LTR region of the virus, could be responsible for the over-expression of the proto-oncogene, leading to transformation. Therefore, a recent therapeutic trial was performed using a modified virus, by generating self-inactivating (SIN) vectors (lacking the transactivating enhancer element of the viral LTR).

The trial aiming to correct γc deficiencies involved several centers in Europe and in USA. Eight SCIDX1 patients were recruited. Patients in the USA were only enrolled if they had life-threatening infections. From these patients, one died before therapy. The other seven patients survived and cleared the infections. The kinetics of the reconstitution of the peripheral T-cell pools was comparable to that found in first-generation SCIDX1 trials, using complete gammaretrovirus vectors. The virus insertions were also similar. In particular, insertions at the proximity of the LMO2 gene were also present. Importantly, these insertions were not associated with over-expression of the LMO2 or the nearby genes. These patients were followed up for 3.8 years and none developed T-ALL (32).

These data support the critical role of the enhancer of the retroviral vector rather than the lack of 'BM competition’ in the leukaemogenesis process observed in first-generation gene therapy trials. They suggest that using SIN vectors for therapy may by-pass the transactivation of oncogenes mediated by the viral LTR and circumvent leukaemogenesis. It must be noted that these results are encouraging, but yet require longer follow-up. In first-generation therapeutic trials, T-ALL emerged in average by 3 years after therapy (15–17). Although this ‘average point’ is by-passed in this SIN vector trial, the risk of T-ALL is yet not formally excluded. In first-generation trials, rare patients only developed T-ALL 6 years after gene therapy.

**Thymus transplants as a source of T lymphocytes – a major hope, and a long way to go**

At this phase, thymus transplantation may become an important therapeutic tool for the reconstitution of T-cell deficiencies in man. Besides, the differences in the ontogeny of T-cell generation in the mouse and man favor the feasibility of thymus transplantation in man. While in mice the generation of mature T cells and their egress from the thymus only starts after birth, in man full thymocyte differentiation is achieved by 3 months of intrauterine life. Therefore, very small-sized human grafts able to export T cells immediately will be available from aborted fetus at legally accepted gestations periods for abortion. However, a method adequate to vascularize such grafts must be determined, as preservation of the three-dimensional structure of the graft is likely required for an adequate T-cell output. It is possible that, as found in mice, vascularization will be obtained after grafting under the kidney capsule.

Before arriving to that stage, several experiments can be conceived in the mouse. To be able to use such grafts for the treatment of the acquired immune deficiencies in the adult/or in old patients, it will be important to determine if the transplants will survive and grow in old hosts. In the mouse, we found that these grafts undergo age-dependent atrophy. If the hormonal environment mediates this atrophy, thymus transplantation will be useless in adults, where it is most frequently needed. However, it is possible that atrophy is mediated by the exhaustion of the thymocytes able to self-renew from the graft. In such case, fetal grafts should survive and export T cells for long time periods in aged hosts.

Several other questions must be addressed. The first concerns the surprising behavior of allogeneic immature
precursors reconstituting the peripheral pools with T cells that apparently do not obey the strict rules of MHC restriction. These findings were obtained both in the mouse (12) and in man (7, 8). In the Rag\textsuperscript{γc} mice, allogeneic cells did not mediate GVH, and were able to exert control of LM infection. It will be important to establish if mice receiving allogeneic thymus transplants will be able to respond to vaccination and to clear other infections, as it is the case in patients receiving allogeneic thymus epithelium (7, 8). As NK cells could recognize and eliminate allogeneic T cells, it will be also important to establish if allogeneic grafts yet survive in other types of T cell-deficient mice that, in contrast to Rag\textsuperscript{γc} mice, have an intact NK compartment. It was reported that allogeneic neonatal cells are resistant to NK activity (33), and we induced NK tolerance in the adult mouse by a single injection of anti-NK1.1 (B. Rocha, unpublished observations). Therefore, the presence of NK cells in the host may not be a major factor preventing allogeneic fetal transplants in man, but the potential impact of NK cells must be addressed.

The apparent indifference of T lymphocytes to MHC differences in lymphopenic hosts also raises a fundamental question. Can MHC restriction imposed in the thymus be modified in the peripheral pools? It is clear that antigen-experienced memory T cells cannot change their MHC restriction. The adult BM is a reservoir of CD8\textsuperscript{+} memory T cells. The injection of histoincompatible adult BM into lymphopenic patients induces GVH. However, in the experiments of thymus transplantation, the peripheral T-cell pool is repopulated by naïve and not by memory cells. As TCR repertoires are extensively cross-reactive, it is possible that homeostatic proliferation, by selecting cross-reactive T cells which are able to interact with the host APCs modify/enlarge the restriction of the peripheral T-cell pool. Therefore, at this phase, the use of thymus transplants for the correction of T-cell deficiencies is but a dream. However, the routes to achieve this dream are worth pursuing, as they may provide the possibility to overcome the major shadows of MHC histoincompatibility and GVH disease, and allow T-cell reconstitution in many conditions of T-cell deficiency. Besides, they should provide important information for the holistic understanding of the immune system.

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