Thymus autonomy as a prelude to leukemia

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Introduction

T lymphocytes develop in the thymus from bone marrow derived hematopoietic progenitors. This is a process that involves high cellular turnover and, in the mouse, essentially all thymocytes are progressively replaced every 4 weeks. This and other aspects of T lymphocyte development can be studied in thymus transplantation experiments using congenic markers to distinguish the origin of the cells [1]. Classical experiments in which wild-type thymi were transplanted into recipient mice with a cell-autonomous defect in T lymphocyte development have shown that the thymus graft generates and exports one wave of T lymphocytes but stops thereafter [2,3]. Indeed, those experiments established thymocytes as short-lived cells and paved the way to a long-lasting dogma in thymus biology: that the thymus lacks cells with self-renewal capacity [4]. However, performing thymus transplants, Rocha’s group and ours have shown that the thymus...
is capable of sustaining T lymphocyte differentiation and export independently of bone marrow contribution [5,6]. This unpredicted event always occurred when a wild-type thymus was grafted into a recipient that was deficient for Common Cytokine Receptor Gamma Chain (γc). These studies clearly indicate that the thymus holds cells that can self-renew under specific conditions. However, prolonging thymus autonomy leads to T cell acute lymphoblastic leukemia (T-ALL) identical to the human disease [7].

Our results resembled the emergence of T-ALL in patients treated by gene therapy for X-linked severe combined immunodeficiency (SCID-X1) [8,9]. SCID-X1 patients have loss-of-function mutations in the γc gene and require a transplant of healthy bone marrow to live. These patients receive healthy hematopoietic stem and progenitor cells and often are not preconditioned, which impacts on the reconstitution of the bone marrow compartment [10,11]. Specifically, no long-term engraftment of hematopoietic stem cells takes place and typically only the T lymphocyte defect is corrected. A total of 20 SCID-X1 patients lacking a suitable donor enrolled in a clinical trial for correction of the γc-deficiency in the early 2000’s, and none was preconditioned prior to infusion of the corrected cells. Upon therapy, the patients continuously produced T lymphocytes de novo for years, despite evidence that the bone marrow did not contribute to this process [12]. Although the results were promising, five of the 20 patients developed T cell leukemia as a consequence of the treatment [8,9]. In this context, two studies from independent laboratories have recently focused on the efficacy of transplant with healthy hematopoietic stem and progenitor cells for the reconstitution of the γc-deficient bone marrow [13,14]. Both were concordant in that lymphoid malignancies arose upon transplantation of a reduced proportion of healthy hematopoietic stem and progenitor cells. Here, we discuss the recent findings in T lymphocyte development in the steady state, including the conceptual framework provided by cell competition, and consider the possible malignant outcome that might result from manipulating the hematopoietic system.

Cell competition

Cell competition was originally described in 1975 in Drosophila [15] as a context-dependent process that contributes to tissue homogeneity and homeostasis. The concept of cell competition postulates that if different cell clones occur within a tissue, they are capable of comparing their relative levels of fitness, and the more fit cells (the winners) induce the elimination of the less fit cells (the losers), thereby ensuring optimal organ function [16–19] (Fig. 1A). Importantly, this is a noncell autonomous process, in which the elimination of the losers is dependent on the presence of the winners. Per se, losers are viable and capable of maintaining organ function. Cell competition regulates organ size during development, contributes to tissue repair, and ensures the removal of suboptimal or aberrant cells from tissues [20,21] (Fig. 1B). In mice, cell competition has been implicated in embryonic development [22,23], hematopoiesis [24], and cardiomyocyte homeostasis [25]. The sensing mechanism detecting differences in fitness remains elusive, and while there are some proposals for sensing molecules in Drosophila [26], they are still unknown in mammals [27]. In the thymus, cell competition favors replenishment of a population of early T lymphocyte precursors [7]. This ensures that there is a pool of pristine cells from which T lymphocyte development can constantly progress [7]. Specifically, using thymus transplantation experiments, we found that ‘young’ precursors, which seeded the thymus recently, led to the clearance of the ‘old’ precursors, with a longer time of thymus residency (Fig. 1C). If thymus colonization was experimentally blocked, then old precursors persisted, presumably self-renewing, and the organ could maintain T lymphocyte production and export – thymus autonomy [5,6] (Fig. 1D). In the steady state, cell competition occurs between precursor cells at the same developmental stage that are genetically identical [7]. We found that interleukin 7 (IL-7) plays an important role in the clearance of the old precursors, although the exact molecular mechanisms underlying this cellular interaction remain elusive [7]. The long-term consequence of impairing cell competition, thereby enabling thymus autonomy, is the development of T-ALL. Hence, in thymus biology, cell competition acts as a tumor suppressor, by clearing the old cells and promoting the constant replenishment of precursors [7]. Of note, T-ALL could develop from wild-type T lymphocyte precursors, without any genetic manipulation. This was the first study clearly linking cell competition to cancer in mammals and showing that cell competition is required for the maintenance of the healthy organism, while its impairment promotes leukemogenesis.

Thymus autonomy and leukemogenesis

T lymphocyte precursors, or thymocytes, go through several differentiation stages that are associated with proliferation or death as result of several selection
checkpoints in the thymus [28,29]. As a consequence, the majority of the cells generated in the thymus fails to fully develop into functional T lymphocytes. For those cells that complete T lymphocyte differentiation, transition through every given stage requires a certain time period that is characteristically short, in the range of days [30]. This is on the basis of the generalized notion that the short lifespan of thymocytes, usually not self-renewing, is a cell-intrinsic property. Our group and another have readdressed this aspect, and shown that thymocytes are, indeed, capable of self-renewing and autonomously sustaining T lymphocyte production under specific conditions [5,6]. Transplanting a wild-type thymus into recipients bearing a \( \text{c}c \)-deficiency, we have shown that T lymphocyte production can be maintained for several weeks. The repertoire generated was diverse and conferred protection to infection [6]. The physiological relevance of thymus autonomy remains elusive but it is plausible that it plays a role in securing thymus function in conditions of progenitor shortage. Infections can impact on hematopoiesis, affecting directly the activity and survival of hematopoietic stem and progenitor cells [31,32]. As a consequence, the production of certain hematopoietic lineages over others can be skewed in an pathogen-specific way [31,32]. Infections impacting on the production or homing of T lymphocyte progenitors could potentially lead to periods of progenitor shortage in the thymus, and it is tempting to speculate that thymus autonomy serves the purpose of sustaining T lymphocyte production in such cases. Nonetheless, thymus autonomy must be tightly regulated, as its prolongation leads to the emergence of T cell acute lymphoblastic leukemia (T-ALL) with onset at 16 weeks post-transplantation and affecting up to 75% of the animals [7]. The disease was identical to the human counterpart, including immunophenotype, genetic and genomic abnormalities, and transcriptomic profile [7]. Major genomic deletions and duplications were detected. Gain-of-function mutations in Notch1, similar to those described in over 50% of human T-ALL [33] were present in 80% of the leukemias. The transcription profile of the murine T-ALLs was compared and found to correlate with the transcriptome.

**Fig. 1.** Cell competition contributes to optimal organ function. (A) Cell competition was described and extensively studied in Drosophila, mostly in the imaginal discs. These are epithelial tissues in which the co-occurrence of clones with different levels of fitness leads to the elimination of those with lower fitness, the losers. Consequently, tissue composition tends toward homogeneity, in which winners prevail, thereby ensuring optimal function. (B) Tissues can be composed solely by losers, as they are viable. (C) In the thymus, cell competition occurs at a stage of differentiation (CD4-CD8-CD25\(^+\), triple negative 2–3, hereby DN2/3), in which young lead to the clearance of old precursors. (D) If cell competition is impaired, old precursors can self-renew and maintain T lymphocyte production independently of the bone marrow.
published from human T-ALL [34]. Nevertheless, T-ALL is a heterogeneous disease, and several subgroups can be defined based on their expression profile. The group of pediatric T-ALL overexpressing TAL/LMO corresponds to the largest subgroup of human T-ALL and is characterized by high levels of expression of LMO1/2 and/or TAL1/2 [34]. These can include, but are not restricted to, translocations involving these genes. Similar to this latter group, the T-ALL we described expresses both Lmo2 and Tal1. Specifically, we could find early expression of Lmo2 that was also retained in the leukemias. Expression of Tal1 was restricted to full-blown T-ALL, suggesting that Lmo2 expression is an earlier event that Tal1 [7]. A more recent study proposes some changes in the subgroups of human T-ALL and splits TAL1 from LMO2, grouping the latter with LYL1 [35]. This is more similar to the former description of the human T-ALL subgroups [36]. Taken together, we propose that T-ALL developing as a consequence of impaired cell competition in the thymus can now be used to gain new insights into the biology of neoplastic transformation from wild-type thymocytes.

We have consistently observed T-ALL developing from wild-type thymocytes upon thymus transplantation into recipients of several different backgrounds (Rag2\(^{-/-}\)γc\(^{-/-}\)Kit\(^{w/w}\), Rag2\(^{-/-}\)γc\(^{-/-}\), γc\(^{-/-}\)), all of which were defective for γc. Since γc is part of the receptor for several cytokines (interleukins 2, 4, 7, 9, 15, and 21), we tested whether interleukin 7 receptor (IL-7r) could account for the aforementioned effect of γc in cell competition, and inhibition of thymus autonomy. Transplantation of wild-type thymus into IL-7r\(^{-/-}\) recipients (lacking the other chain of IL-7r) also led to development of T-ALL with a similar incidence to that observed from γc\(^{-/-}\) hosts, indicating that it is the ability of incoming precursors to respond to IL-7 that is key in inhibiting thymus autonomy [7].

We have been using consistently the experimental setup of thymus transplantation under the kidney capsule of recipients lacking either γc or IL-7rα for over 6 years [5,7]. The consistency of our results, tested in different backgrounds of recipients, of donors (e.g. T-ALL also developed from Rag-deficient thymus donors) [7], and at different Institutions (involving the establishment of new colonies from mice purchased from Taconic [7] and from The Jackson Laboratories (manuscript in preparation), indicates that leukemia is a frequent event following thymus autonomy. Nevertheless, Rocha’s group published a review article proposing thymus transplants as a possible therapy for patients with T lymphocyte deficiencies [37]. The group claims that no T-ALL emerged in their hands using an experimental setup similar to ours. While their previous study followed very few mice for long enough time to detect T-ALL [6], the review mentions a cohort of 19 animals that was followed up for at least 6 months and did not develop leukemia [37]. There is no obvious explanation that could account for the discrepancy of results, but the differences point at the existence of specific factors that might influence leukemogenesis and would be of interest to address. One important aspect could be the specific background of their mouse colony, that was kept for a very long time by interspecies at their animal facility and may have undergone genetic drift [37]. Nevertheless, additional factors including the environment in which animals are kept, their health status, diet or even their microbiota composition are potential candidates that could influence the results.

**Gene therapy for SCID**

Gene therapy is an attractive solution for the correction of monogenetic disorders, in particular those affecting the hematopoietic system. Hematopoietic stem and progenitor cells can be harvested, their genetic defect corrected ex vivo, and then infused back into the patients, with the aim of correcting the disease. Several primary immunodeficiencies fall into this category and SCID-X1 is one example, caused by loss-of-function mutations of the γc gene in boys [38]. Phenotypically, it is characterized by the absence of T and NK cells, and presence of dysfunctional B cells [38]. It is the most frequent form of SCID, accounting for 40% to 50% of the cases [39]. The standard treatment, applied since 1968, is hematopoietic stem cell (HSC) transplantation [10]. Transplantation of cells obtained from haploidentical twins confers a 3-year survival rate reaching 90% to 97%. However, it drops to 66–79% when cells are derived from an alternative donor [39,40], indicating an unmet need for the cure of SCID-X1. The first SCID-X1 patients treated by gene therapy date from the early 2000’s [41,42] and had full correction of their immunological defects, including production of T lymphocytes [43]. However, these early results were jeopardized by the emergence of T-ALL in 5 of the 20 patients [8,9]. The cause for malignant transformation was considered to be genotoxicity due to nonrandom integration of the retroviral vectors used for delivery of the correct form of γc [44,45]. Nevertheless, the T-ALLs had a plethora of additional genomic alterations, likely to have contributed to the malignant phenotype developed by the patients [8,9].

Prior to leukemia, the patients treated by gene therapy consistently produced T lymphocytes de novo for
several years. Furthermore, T lymphocyte reconstitution was faster in these patients as compared to others treated by haploidentical HSC transplantation [46]. However, myeloid cells and B lymphocytes originating from the autologous, corrected stem cells ceased, indicating that no long-term engraftment of HSC was achieved [12]. Generally, SCID patients do not receive preconditioning prior to bone marrow transplantation, as to avoid toxicity associated with chemotherapy, because the lack of T and NK cells renders them permissive to T lymphocyte reconstitution [10,11]. Nevertheless, there is some debate on whether preconditioning should be included as part of the therapy, and several centers include it in order to correct the deficiency in B and NK cells that would otherwise not be repaired [39,40]. In this context, a new gene therapy trial in the United States is using a new version of lentivirus to correct SCID-X1 in five patients with persistent disease who had undergone previous HSC transplantation [47]. In this study, patients were preconditioned prior to gene therapy and attained correction of the immune deficiency, including in the B and NK cell compartments [47]. Finally, a recent trial integrating teams in Paris, London and the United States is ongoing and has used a new generation of vectors for treating nine patients [48]. In seven, immune function was corrected, and vector integration sites seemed to differ from those in the first trial [48]. No adverse effects have been reported so far.

The importance of vector design is unquestionable for several aspects, namely the efficiency of transduction of the target cells, to obtain optimal levels of expression of the gene of interest, and safety for the patients. Nevertheless, it is likely that vector design was not the sole factor implicated in the emergence of T-ALL in earlier trials. Supportive of this view is the case of success of gene therapy for correction of ADA-SCID [49,50]. ADA-SCID is caused by deficiency in Adenosine deaminase and although the genetic and molecular defects differ between the two forms of SCID considered here, they both result in impaired lymphocyte development. While the first trials for correction of SCID-X1 were hampered by malignancy arising from the treatment, this was not the case for ADA-SCID. Of note, the vectors used in both trials were very similar [41,51]. The two major differences were the target gene itself, and the use of a mild non-myeloablative conditioning regimen prior to therapy in the case of ADA-SCID [51]. ADA-SCID can nowadays be treated successfully through gene therapy but always involves preconditioning, which enables effective engraftment of the corrected bone marrow, with consequent correction of the immune defects [49,50].

**Relevance of bone marrow reconstitution for correction of SCID-X1**

Even though SCID-X1 patients are often transplanted without preconditioning [11,52], thymus function can be maintained for years. However, only a minority achieves restoration of the B lymphocyte compartment [52]. Likewise, the first SCID-X1 gene therapy trials also did not employ preconditioning to ensure hematopoietic stem and progenitor engraftment [41,42]. Infusion of γc-corrected HSC to SCID-X1 patients without preconditioning is likely to have led to thymus reconstitution, without reconstitution of the bone marrow, consistent with the authors’ report [12]. If this occurred, thymus autonomy could have created the conditions for leukemogenesis. Therefore, it remained plausible that the low-level to no correction of the bone marrow contributed to T-ALL onset in the SCID-X1 patients enrolled in the gene therapy trial. When we proposed this as a possible scenario [7], there was some debate both from a group working in developmental biology [53] and from clinicians working on gene therapy for SCID [54]. While the first described our findings as an example of Darwinian tumor-suppressor [53], the latter claimed our proposal was unsupported by the experience in the clinics of hematopoietic reconstitution of immunodeficient patients [54]. Nevertheless, and in line with our view, two independent studies in mice have recently shown that the relative number of healthy hematopoietic cells engrafting into the γc-deficient bone marrow is central for efficient reconstitution of the immunological function [13,14]. If the bone marrow is efficiently corrected, hematopoietic progenitors colonize the thymus continuously, and maintain the characteristic turnover associated with normal T lymphocyte differentiation. These data go beyond the original thought that only vector design was responsible for the malignancies arising in the SCID-X1 gene therapy trial. In fact, they are in concordance with the proposal we originally put forward, that progenitor deprivation in the thymus is permissive to thymus autonomy, and thus causes leukemia in the long run [7]. Using the same γc-deficient mouse model that we have used previously [5,7], one study detailed that even if preconditioning is applied (irradiation) to open up the niches in the bone marrow, thus enabling long-term engraftment, lymphoid malignancies can still develop [13]. Specifically, the relative proportion of engrafted wild-type stem cells determined whether leukemia develops or not, and correlates inversely with the incidence of leukemia [13]. Hence, preconditioning leading to effective long-term
Fig. 2. Thymus autonomy enables T-ALL. Manipulation of the hematopoietic system in a γc-deficient host/patient by intravenous injection (or infusion, in the case of patients) of healthy hematopoietic stem cells (HSC) can lead to full (A), partial (B) or no (C) reconstitution of the HSC compartment, which impacts on thymus function and in the correction of the immune system. Reconstitution of the HSC compartment requires preconditioning prior to injection to open the bone marrow niches (A) and (B). (A) If all injected HSC are healthy, which is the case for cells from a healthy donor, or for gene therapy-corrected cells (with at least 10% correction of all HSC [14]) and the niches were previously opened, then continuous thymus colonization takes place, and turnover is attained. Furthermore, a functional immune system is established. (B) If only a small percentage of injected HSC are healthy and the bone marrow niches were previously opened, then the HSC compartment is only partially rescued, and the majority of HSC remain γc-deficient. The differentiation of progenitors capable of thymus seeding at any given time point, involves only a fraction of active HSC. This leads to periods of low to no representation of γc-proficient progenitors in the pool of cells that seed the thymus. Although all those progenitors colonize the thymus, the healthy, γc-proficient progenitors will have an advantage over γc-deficient progenitors, i.e. cell competition will be impaired, and consequently thymus autonomy will take place. (C) Lack of preconditioning will not open the niches for HSC engraftment in the bone marrow, but progenitors (and HSC) will be capable of colonizing the thymus directly. Since no further colonization with healthy, γc-proficient progenitors will follow, cell competition will be compromised and thymus autonomy is predicted to occur. Although experimental design differs, the same cellular setting can be established by thymus transplantations of wild-type thymi into hosts that are γc-deficient. The conditions that enable thymus autonomy (B) and (C) are permissive to T-ALL.

Table 1. Manipulation of the hematopoietic system in γc-deficient hosts. Different studies involving hematopoietic stem and progenitor cell injection, or thymus transplants into γc-deficient hosts are summarized, as well as the experimental outcomes of every study. Hematopoietic stem cells (HSC), intravenously (i.v.), n/a – not applicable or not reported.

| Study | Experimental setup | Healthy donor (%) | Engraftment (%) | Incidence (%) | Onset of T-ALL (wk) |
|-------|-------------------|------------------|----------------|--------------|-------------------|
| [7]   | Thymus transplant |<br> (Rag2<sup>−/−</sup>γc<sup>−/−</sup>Kit<sup>W/Wv</sup>, Rag2<sup>−/−</sup>γc<sup>−/−</sup>γ<sup>−/−</sup>)<br>n/a<br>0<br>up to 75 | n/a | 16 |
| [55]  | HSC and progenitors i.v. |<br> (Rag2<sup>−/−</sup>γc<sup>−/−</sup>c5<sup>−/−</sup>)<br>21.8<br>n/a<br>28.5<br>21 | n/a | 21 |
|       | Pre-conditioning   |<br>100<br>64.9 ± 3.0<br>n/a<br>0<br>n/a | n/a | n/a |
| [13]  | HSC and progenitors i.v. |<br> (Rag2<sup>−/−</sup>γc<sup>−/−</sup>)<br>10<br>n/a<br>20<br>43 | n/a | 43 |
|       | Pre-conditioning   |<br>1<br>3.4 ± 0.3<br>n/a<br>40<br>30 | n/a | n/a |
| [14]  | HSC and progenitors i.v. |<br>(γc-deficient)<br>100<br>10<br><1<br>45<br>22 | n/a | n/a |
|       | Pre-conditioning   |<br>10<br><1<br>65<br>20 | n/a | n/a |

*aHosts were all γc-deficient, and the specific genotype is indicated in parenthesis.

bPercentage of healthy (wild type or corrected by gene therapy) donor hematopoietic stem and progenitor cells injected.

cDetermined by flow cytometry in bone marrow lineage-negative Sca1<sup>+</sup> Kit<sup>+</sup>.
reconstitution of the bone marrow HSC compartment can reduce the risk of leukemogenesis in gene therapy for SCID-X1. Of note, these data explain previous results from the same group in which leukemogenesis occurred from γc-deficient, lentivirus-treated, HSC, where genotoxicity could not account for the malignancy [55]. In that study, although the cells that originated the malignancy were corrected (transduced) for the γc-deficiency, the leukemic cells had a transcription pattern identical to normal wild-type, nontransduced T lymphocytes, suggestive that genotoxicity did not occur [55]. The work from Naldini’s laboratory elegantly explored several additional aspects that must be taken into account when attempting to correct the γc genetic defect by gene therapy [14]. This group generated their own mouse model of SCID-X1, mimicking a null mutation from patients. Their data show that it is important to create the niches in the bone marrow to enable long-term engraftment of healthy HSC. Furthermore, the relative proportion of corrected (or wild-type) cells is fundamental to guarantee that immune reconstitution is complete and no lymphoid malignancy emerges as a side effect. Finally, promising data are presented that shows that a targeted correction of the genetic defect is an attainable goal in the future of gene therapy [14].

General considerations

Normal thymus function relies on the constant seeding of the thymus by bone marrow derived hematopoietic progenitors. In the thymus, they commit to the T lymphocyte lineage [56,57] and further differentiate. T lymphocyte development is associated with migration of thymocytes in a characteristic pattern that reflects the existence of specific niches. These provide thymocytes with the necessary cues in a sequential way. We found that cell competition regulates thymus turnover [7], and that impairing it (for instance, under conditions of progenitor deprivation), is permissive to thymus autonomy [5,6]. Although competition for Notch1 ligands [58] and cytokines [59] have been reported during early T lymphocyte development, it is unclear to what extent, if at all, they are involved in the process of cell competition that we described [7].

The number of hematopoietic progenitors seeding the thymus at any given time point is very low [60] and derive ultimately from few active HSC [61]. If the HSC compartment is fully corrected, progenitors colonize the thymus continuously, and guarantee cell competition and turnover (Fig. 2A). However, in a bone marrow chimera in which one cell type is very much underrepresented, simple stochastic activation of a subset of HSC will predictably lead to differences in the levels of chimerism detected in the bone marrow or in the thymus (Fig. 2B). In addition, when reconstituting a γc-deficient organism, there are two additional aspects to consider: 1) the γc-deficient thymus can be directly colonized by healthy progenitors injected in the blood stream [59], and 2) γc-proficient cells are the only ones that respond to γc-cytokines [62]. The first aspect implies that thymus function is established from the first cohort of progenitors upon injection. The latter aspect means that the γc-proficient cells will be in advantage upon thymus seeding, and γc-deficient progenitors colonizing the thymus later will not be capable of inhibiting thymus autonomy. In other words, bone marrow chimeraism, consisting of low percentage of γc-proficient cells, is likely to enable sporadic events of successful thymus function (Fig. 2B). If the time period between waves of successful thymus seeding lasts long enough, i.e. if there is a period of (γc-proficient) progenitor deprivation, it is likely that thymus autonomy is switched on, regardless of whether cells were γc-corrected [55] or wild-type [13] (Fig. 2B). This highlights that the efficacy of transduction of the cells in the setting of gene therapy, and their capacity to engraft the bone marrow, are important to prevent thymus autonomy. In the same line, reconstitution of γc-deficient mice without preconditioning led to lymphoid malignancies, and incidence was highest when the percentage of injected wild-type cells was low [14]. This is in line with the outcome of the first trials for gene therapy of SCID-X1, which were consistent with a scenario in which the ratio of γc-corrected to noncorrected cells was very low [8,9] (Fig. 2C). Finally, thymus autonomy in mice leads to T-ALL [7] and although there are differences between clinical settings and the experimental designs employed, most studies that are consistent with thymus autonomy were followed by malignant transformation (Table 1). This highlights the need to limit thymus autonomy, particularly in the context of correction of immunodeficiencies, in order to prevent the risk of T-ALL development.

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Conflict of interest

The authors have no conflicts of interests.

Author Contribution

RAP and CVR wrote the manuscript, with stronger contributions in the sections referring to thymus autonomy and cell competition, respectively. VCM outlined and wrote the manuscript. All authors edited and contributed to the final version of the manuscript.

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